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Scientific Research

Evidence for a Non-β_2-Adrenoceptor Binding Site in Human Lung Tissue for a Subset of β_2-Adrenoceptor Agonists

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ABSTRACT

The aim of this study was to compare the binding profile of a range of β_2-adrenoceptor (β_2-AR) agonists and antagonists in human lung tissue. Radioligand saturation and competition binding experiments were performed by filtration with a β_2-AR antagonist ([^{3}H]propranolol) or agonist ([^{3}H]vilanterol) radioligand and membrane fragments generated from lung parenchyma in the presence of 100 µM guanosine 5'-[β,γ-imido]triphosphate (Gpp(NH)p). In membranes prepared from human lung parenchyma, carmoterol, formoterol, ICI118551, propranolol and salbutamol resulted in inhibition of [^{3}H]vilanterol binding to levels that were significantly different from indacaterol, salmeterol and vilanterol (ANOVA, Bonferroni post-test, $P < 0.001$ except formoterol vs indacaterol where $P < 0.01$). Indacaterol and salmeterol resulted in inhibition of [^{3}H]vilanterol binding to levels that were not significantly different from vilanterol (ANOVA, Bonferroni post-test, $P > 0.05$). Indacaterol, salmeterol and vilanterol resulted in full inhibition of [^{3}H]propranolol binding to levels not significantly different from ICI118551 (ANOVA, Bonferroni post-test, $P > 0.05$). Indacaterol, salmeterol and vilanterol bind to an additional site in human lung parenchyma membranes that is distinct from the β_2-AR.

KEYWORDS

β_2-Adrenoceptor; Radioligand Binding; Human Lung; Tissue Binding Site

1. Introduction

Inhaled β_2-adrenoceptor (β_2-AR) agonists are used in the treatment of both asthma [1] and chronic obstructive pulmonary disease (COPD) [2] by causing relaxation of the airways and increased airflow into the lungs. Early drug discovery efforts in this area yielded short acting β_2-AR agonists like salbutamol in the late 1960s [3] and during subsequent decades a number of pharmaceutical companies focussed on developing molecules with longer duration of action for better control of symptoms and lung function. The fruits of this labour included formoterol [4] and salmeterol [5] that provided relief of symptoms for at least 12 hours. These drugs were branded long acting β_2-AR agonists (LABAs) and are still used routinely as twice daily bronchodilators in both asthma and COPD, in combination with a corticosteroid. The next generation of LABAs, that include vilanterol and indacaterol, were developed to be fast acting and have 24-hour duration of action as this is predicted to improve patient convenience, and therefore compliance, within these patient populations [6].

The mechanism by which both the established (formoterol and salmeterol) and recently developed (indacaterol, olodaterol and vilanterol) LABAs achieve their long duration of action has never been fully elucidated despite almost 20 years of literature dedicated to it. A number of hypotheses have been put forward that include the "microkinetic" theory [7], "exosite" theory [8] and slow dissocation kinetics from the high affinity agonist receptor state [9]. The "microkinetic" theory or model describes a highly lipophilic molecule partitioning into

cell membrane and forming depots of drug maintaining active concentrations of drug in the tissue for longer. The "exosite" theory puts forward the hypothesis of a second, distinct binding site on the β_2-AR itself that interacts with the long hydrophobic chains of salmeterol and vilanterol, trapping the agonist in the vicinity of the orthosterically active binding site. Recently it was also suggested for the LABA olodaterol that the duration of action observed in tissue studies was due to a slow dissociation rate from the β_2-AR high affinity agonist receptor state. However, the validity of this study remains debatable due to the non-physiological temperature (room temperature) the experiments were completed at, as much faster kinetics would be predicted at 37˚C [10]. As proposed in a recent review of these hypothesises, framed in the context of explaining salmeterol's duration of action [11], further in-depth studies in sub-cellular systems are required to aid in the determination of the exact mechanism/s of action that account for the duration of action of LABAs. One potential method to further investigate the interaction at the sub-cellular level is radioligand binding studies in membranes prepared from human lung tissue. Aside from the saturation binding studies completed on a radiolabelled form of formoterol in human lungs [12] these types of study have not been routinely completed with the LABAs.

As part of the vilanterol drug development programme a radiolabelled form of this LABA was generated to determine its β_2-AR binding characteristics in recombinant systems [13]. In this study [^{3}H]vilanterol has been used as a tool radioligand, in parallel with [^{3}H]propranolol, to investigate the binding characteristics of a range of β_2-AR agonists and antagonists in membranes generated from human lung tissue in an effort to provide further evidence to explain the duration of action of the LABAs.

2. Materials and Methods

2.1. Materials

Indacaterol, salbutamol, salmeterol and vilanterol (**Figure 1**) were synthesised by the Respiratory TAU Medicinal Chemistry department at GlaxoSmithKline Medicines Research Centre (Stevenage, UK). The chemical synthesis of vilanterol is detailed in Procopiou *et al.* [14]. Carmoterol was purchased from Creative Dynamics Inc. (New York, NY, USA). CGP20712, formoterol, ICI118551, propranolol, and all other chemicals were purchased from Sigma-Aldrich Co. Ltd. (Gillingham, UK) unless otherwise stated. [^{3}H]propranolol (specific activity 23 Ci/mmol) was purchased from PerkinElmer LAS UK Ltd. (Beaconsfield, UK). [^{3}H]vilanterol (specific activity 92 Ci/mmol) was synthesised by Quotient Bioresearch (Radiochemicals) Ltd. (Cardiff, UK). All studies were completed with a final dimethyl sulphoxide (DMSO) concentration of 1%.

2.2. Human Lung Parenchyma Membrane (HLPM) Preparation

Non-diseased human lungs from organ donors were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA, USA) in accordance with local human biological sample management procedures. The human biological samples were sourced ethically and

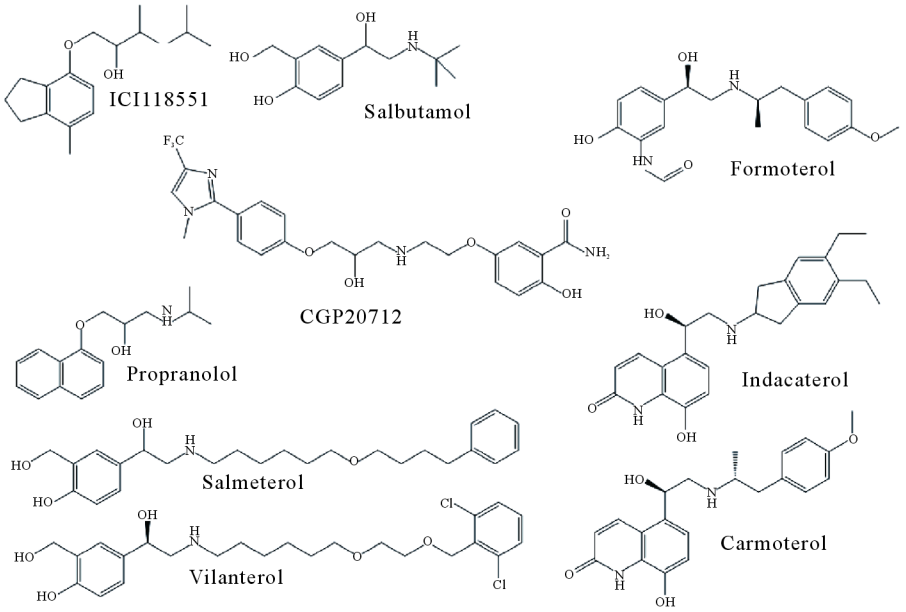

Figure 1. Chemical structures of carmoterol, formoterol, indacaterol, salbutamol, salmeterol, vilanterol (β_2-AR agonists), ICI118551, propranolol (β_2-AR antagonists) and CGP20712 (β_1-AR antagonist).

their research use was in accord with the terms of the informed consents. 5 - 10 g samples of human lung parenchyma tissue obtained from 2 donors were dissected and cleaned of adherent connective and fatty tissue. Tissue samples were suspended in ice-cold assay buffer (50 mM Tris, 154 mM NaCl, 10 mM $MgCl_2$ and 2 mM EDTA, pH 7.4 (5M HCl)) and homogenised with an Ultra-Turrax homogeniser (IKA, Staufen, Germany) for 20 s followed by 4 × 4 strokes in a glass-teflon homogeniser. Homogenised tissue was washed in assay buffer and centrifuged at 500 g for 10 min at 4˚C. The supernatant was then harvested and centrifuged at 40,000 g for 15 min at 4˚C with the resulting pellet resuspended in assay buffer and centrifuged a second time at 40,000 g for 15 min at 4˚C. Membrane pellets were then passed 10 × through a 0.22 mm needle, resuspended in assay buffer and protein concentration determined using the bicinchoninic acid method [15] using bovine serum albumin as a standard. The membrane suspensions were frozen in aliquots at −80˚C until required.

2.3. Radioligand Binding Assays

All radioligand binding experiments were performed in 96-deep well plates at 37˚C. Binding buffer consisted of RPMI1640 containing 100 µM Gpp(NH)p (pH 7.4). Radioligands ([^{3}H]propranolol or [^{3}H]vilanterol) were incubated with 50 µg/well membranes and either vehicle (1% DMSO to give total radioligand binding) or unlabelled β_2-AR agonists/antagonists (10 µM). Non-specific binding (NSB) values were determined by either 10 µM ICI118551 or salmeterol and were used to calculate specific binding. [^{3}H]propranolol saturation binding curve studies were completed in the presence of 0.1 µM of CGP20712 (selective β_1-adrenoceptor (β_1-AR) antagonist [16]). Plates were incubated with gentle agitation for 1 h and binding terminated by rapid vacuum filtration through a 48-well Brandel harvester (Brandel Inc. Gaithersburg, MD, USA) onto GF/B filter papers pre-soaked in 0.3% v/v poly-ethylenimine. Samples were washed rapidly three times with ice cold binding buffer and filters transferred into liquid scintillation (LS) vials containing 4 ml LS fluid (Ultima-Flo™ M, PerkinElmer LAS UK Ltd., Beaconsfield, UK). The amount of radioligand bound to receptor was measured by LS spectroscopy using a TriCarb 2900 TR LS counter (PerkinElmer LAS UK Ltd., Beaconsfield, UK). To ensure binding parameters were determined at the low affinity agonist state of the β_2-AR receptor *i.e.* G-protein uncoupled form of the receptor, radioligand experiments were completed in the presence of 100 µM Gpp(NH)p, a non-hydrolysable analogue of the nucleotide guanosine triphosphate. Saturation binding studies were performed with [^{3}H]propranolol and [^{3}H]vilanterol to determine β_2-AR binding parameters in HLPMs (equilibrium dissociation constant (K_D) and total number of receptors (B_{max}) were calculated as described under *Data Analysis*) using 10 µM ICI118551, a selective β_2-adrenoceptor (β_2-AR) antagonist, to determine NSB. For saturation binding, membranes were incubated with increasing concentrations of [^{3}H]propranolol (~0.04 to 6.0 nM) or [^{3}H]vilanterol (~0.03 - 5.3 nM) for 1 h prior to filtration. Single shot competition displacement studies were also completed where membranes were incubated with a fixed concentration of either [^{3}H]propranolol (~1.7 nM) or [^{3}H] vilanterol (~0.3 nM) and 10 µM of β_2-AR unlabelled agonist/antagonist.

2.4. Data Analysis

Analysis of all radioligand binding experiments was completed using Prism 5.0 (GraphPad Software, San Diego, CA, USA). Specific binding data from saturation experiments were fitted to a one affinity site model to determine K_D and B_{max} values. Unless otherwise indicated, data shown graphically are mean ± standard error of the mean (SEM). For comparison of model fitting the extra sum-of-squares F test was used with a threshold $P < 0.05$.

All statistical analyses were completed using SAS® (SAS Institute Inc., NC, USA) and differences of $P < 0.05$ were considered to be statistically significant. Statistical significance between two data sets was tested using a Student's unpaired *t*-test. One-way analysis of variance (ANOVA) was used for comparison of more than two datasets to highlight specific inter-group P-values, with Holm's method [17] used to adjust P-values for multiple comparisons and so lessen the occurrence of false positive results.

3. Results

3.1. Radioligand Saturation Binding HLPMs

[^{3}H] vilanterol and [^{3}H] propranolol saturation binding studies were carried out to determine binding affinity and compare the receptor populations labelled in HLPMs. Specific binding data from saturation experiments for both radioligands were best fitted to a one affinity site model (**Figure 2**).

This analysis resulted in a pK_D for [^{3}H]vilanterol of 8.80 ± 0.30 and 8.98 ± 0.14 for [^{3}H]propranolol (n = 6). The average Hill slope coefficient for [^{3}H]vilanterol was 1.17 (0.94, 1.41) and for [^{3}H] propranolol was 0.80 (0.67, 0.93) (n = 6 (2 donors, n = 3/donor), 95% confidence limits shown in parentheses). The B_{max} values for [^{3}H] vilanterol and [^{3}H]propranolol saturation binding were 0.37 ± 0.07 and 0.51 ± 0.11 pmol/mg (n = 6 (2 donors, n = 3/donor)) respectively, with no significant difference

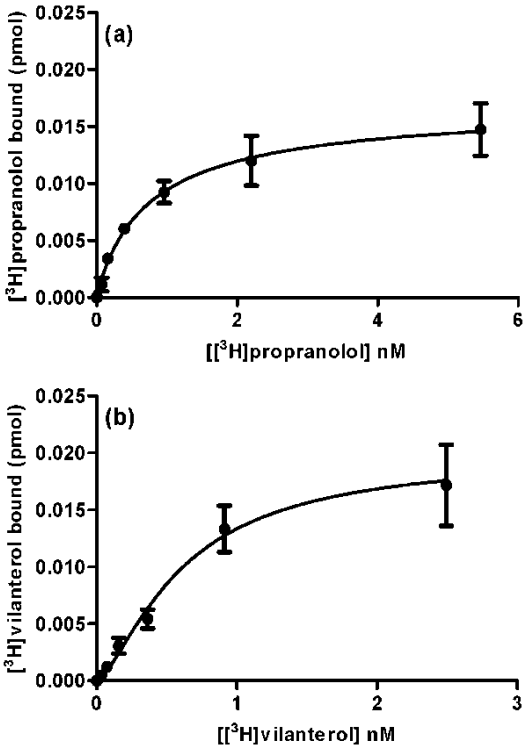

Figure 2. Saturation binding of (a) [^{3}H]propranolol and (b) [^{3}H]vilanterol with the β_2-AR in HLPMs. Specific saturation binding data for (a) [^{3}H]propranolol and (b) [^{3}H] vilanterol were best fitted to a one-site affinity model (extra-sum-of-square F test, P < 0.05). Data shown are the mean ± SD of duplicate points and are representative of 6 individual experiments with similar results (2 donors, n = 3/donor).

observed between radioligands (Student's *t*-test, P > 0.05). This suggested that both radioligands were labeling the same population of receptors.

3.2. Effect of β_2-AR Agonists and Antagonists on [^{3}H]vilanterol Binding in HLPMs

Competition binding with unlabelled β_2-AR agonist and antagonists was determined against [^{3}H]vilanterol at single concentrations (10 µM) in HLPMs following a 1 h incubation period at 37˚C at a concentration of radioligand that ensured measurement of β_2-AR binding only (*i.e.* approximately >400-fold lower concentrations shown to engage other endogenous receptors (screened against panel of 7TM receptors and transporters using radioligand binding assays by Eurofins Panlabs Inc. (Bothell, WA, USA), data not shown) including $\beta_{1/3}$-adrenoceptors [13]). Carmoterol, formoterol, ICI118551, propranolol and salbutamol resulted in inhibition of [^{3}H]vilanterol binding to levels that were significantly different from indacaterol, salmeterol and vilanterol (ANOVA, Bonferroni post-test, P < 0.01). Indacaterol and salmeterol resulted in inhibition of [^{3}H]vilanterol binding to levels that were not significantly different from vilanterol (ANOVA, Bonferroni post-test, P > 0.05) although indacaterol binding inhibition of [^{3}H]vilanterol was shown to be significantly different to salmeterol (ANOVA, Bonferroni post-test, P < 0.01) (**Figure 3**). CGP20712 did not inhibit the control binding of [^{3}H]vilanterol confirming that this radioligand was not binding to β_1-ARs in the HLPMs (**Figure 3**).

3.3. Effect of β_2-AR Agonists and Antagonists on [^{3}H]propranolol Binding in HLPMs

Single concentrations (10 µM) of indacaterol, salmeterol and vilanterol resulted in inhibition of [^{3}H]propranolol binding to levels not significantly different from ICI118551 and propranolol (ANOVA, Bonferroni post-test, P > 0.05) (**Figure 4**). CGP20712 partially inhibited the binding of [^{3}H]propranolol, compared to control binding, to a significant level (ANOVA, Bonferroni post-test, P < 0.001) confirming that this radioligand was binding to a population of β_1-ARs in the HLPMs (**Figure 4**).

4. Discussion

LABAs have been used for the last 20 years in the treatment of asthma and COPD to provide continuous relief of symptoms via sustained relaxation of the airways and increased airflow into the lungs. The mechanism accounting for the observed extended duration of airway relaxation following LABA inhalation has never fully been elucidated and although a number of hypotheses

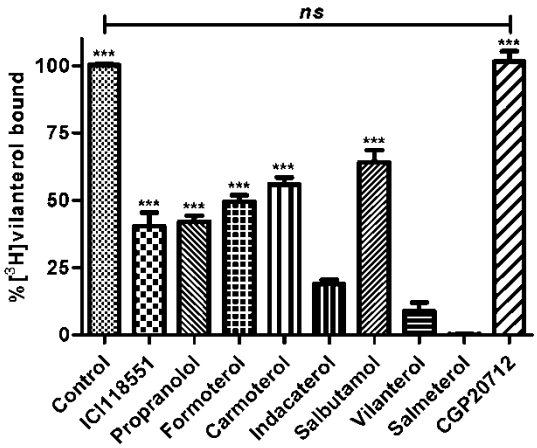

Figure 3. Competition binding of a range of β_2-AR agonists and antagonists against [^{3}H]vilanterol in HLPM. Percentage [^{3}H]vilanterol bound was calculated from control (1% DMSO) and NSB (10 µM salmeterol). Inhibition of [^{3}H] vilanterol (~0.3 nM) binding to levels that were significantly different from salmeterol and vilanterol shown as * (ANOVA, Bonferroni post-test, P < 0.001 with ns = not significant P > 0.05). Data shown are the mean ± SEM, n = 5 - 16 (2 donors, n ≥ 2/donor).**

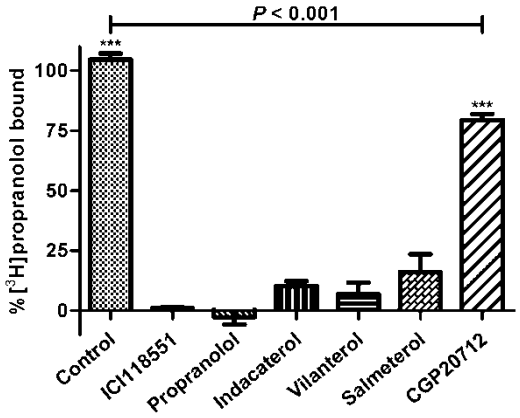

Figure 4. Competition binding of a range of β_2-AR agonists and antagonists against [^{3}H]propranolol in HLPMs. Percentage [^{3}H]propranolol bound was calculated from control (1% DMSO) and NSB (10 µM ICI118551). Inhibition of [^{3}H]propranolol (~1.7 nM) binding to levels that were significantly different from ICI118551 shown as *(ANOVA, Bonferroni post-test, P < 0.001). [^{3}H]propranolol competition binding was carried out in the presence of 0.1 µM CGP20712. Data shown are the mean ± SEM, n = 4 - 9 (2 donors, n ≥ 2/donor).**

have been put forward [7-9] none of these have been categorically proven. In this study [^{3}H]vilanterol has been used as a tool radioligand in unison with [^{3}H]propranolol to investigate the binding of a range of β_2-AR agonists (both short and long acting) and antagonists in sub-cellular membrane preparations generated from human lung tissue. The aim being to provide further evidence to either add weight to or rule out the current theories that have been put forward for the duration of action displayed by LABAs.

Historical studies have been completed using membrane preparations from CHO cells recombinantly expressing the human β_2-AR allowing characterisation of the affinity and maximal inhibition of binding of unlabelled ligands using [^{3}H]vilanterol [13]. This allowed the characterisation of binding at the β_2-AR in the absence of any human lung tissue architecture *i.e.* measure receptor based binding interactions only. All the agonist and antagonists tested were shown to inhibit binding of [^{3}H]vilanterol to NSB levels defined by ICI118551 in this system suggesting they were all binding to a common binding site on the β_2-AR [13] *i.e.* the orthosteric binding site. In order to investigate the binding characteristics of [^{3}H]vilanterol in a more physiological relevant system, radioligand binding was measured in membranes prepared from human lung parenchyma tissue.

Membranes generated from human lung parenchyma contain a range of tissue architecture including membranes from cells making up alveoli, blood vessels and small airways. Therefore, it is worth noting that human lung membranes used in this study will contain a range of receptors in addition to the β_2-AR, including the β_1-AR subtype. To aid in the dissection of β_2-AR versus β_1-AR subtype binding, a tritiated version of the non-selective β_2-AR antagonist propranolol [16] was investigated in addition to [^{3}H]vilanterol. When competition binding against both these radioligands was completed in the presence of the β_1-AR selective antagonist CGP20712, a significant inhibition of [^{3}H]propranolol was observed compared with no inhibition of [^{3}H]vilanterol (**Figures 3** and **4**). This confirmed that there was indeed a population of β_1-ARs in the human lung membrane preparations and highlighted the requirement to complete all subsequent [^{3}H]propranolol binding studies in the presence of CGP20712 to ensure measurement of β_2-AR binding only. It was also a further confirmation of the lack of [^{3}H]vilanterol β_1-AR binding at the concentrations of this radioligand tested in competition studies (~0.3 nM).

The saturation binding data for [^{3}H]vilanterol and [^{3}H]propranolol showed that both radioligands were labelling the same number of β_2-AR binding sites in human lung membranes, that would be predicted to be the β_2-AR orthosteric binding site in its low affinity state due to the presence of Gpp(NH)p. Subsequent single concentration competition binding for a range of β_2-AR agonists and antagonists against [^{3}H]vilanterol showed a subset of LABAs (indacaterol, salmeterol and vilanterol) inhibiting binding to a significantly greater level than other test agents (**Figure 3**). In contrast, when tested against [^{3}H] propranolol, indacaterol, salmeterol and vilanterol all inhibited binding to the same level as all other test agents. This showed that [^{3}H]vilanterol/vilanterol was binding to a secondary binding site in human lung membranes distinct from the β_2-AR, that it shared with indacaterol and salmeterol but not the other β_2-AR agonists and antagonists tested. With the binding to other endogenous receptors ruled out and this observation not repeated in the recombinant β_2-AR CHO membranes where binding to the β_2-AR in isolation is measured [13], this would suggest that the secondary binding site is only present in membranes generated from human lung tissue.

Due to the structural similarities between salmeterol and vilanterol *i.e.* saligenin head with a long hydrophobic tail **(Figure 1)**, it may not be a surprise that they share this tissue binding site and it could be hypothesised that the long hydrophobic tail could be a contributing factor. The data showing that indacaterol also shares this tissue binding site is a much more interesting observation due to its structural differences with vilanterol and salmeterol. As carmoterol in this study displayed no interaction with the tissue binding site and as it shares a carbostyril head with indacaterol (**Figure 1**), this suggests it is indacaterol's more hydrophobic tail that is interacting with the tissue binding site. In addition, comparing the cLogP data for the LABAs tested (**Table 1**) with the inhibition

Table 1. Calculated Log P (cLogP) values calculated for the β_2-AR agonists and antagonists tested in this study.

β_2-AR Agonist/Antagonist	cLogP[a]
Salbutamol	0.06
Formoterol	1.26
Carmoterol	1.31
Propranolol	2.75
Indacaterol	2.97
Salmeterol	3.06
Vilanterol	3.19
ICI118551	3.40

[a]cLogP values calculated by Daylight (Daylight Chemical Information Systems Inc., Laguna Niguel, CA, USA).

level of [^{3}H]vilanterol binding observed in human lung membranes there is a trend observed that the increased lipophilicity of this structural region (hydrophobic tail) contributes to engagement with the tissue binding site. High lipophilicity *per se* does not result in an interaction with this secondary site as propranolol and ICI118551 did not bind to the tissue site and have either a comparable or greater cLogP than indacaterol, salmeterol and vilanterol (Table 1).

The novel evidence for a non-β_2-AR human lung tissue site that has been generated in this study does not provide any further evidence for confirmation nor invalidation of either the "microkinetic" [7] or "exosite" [8] theories put forward for the duration of action of LABAs in the early 1990s. Further research into the characterisation of this human lung tissue site could be extremely beneficial, especially if a structure-activity relationship could be identified by the profiling of an increased number of chemical entities. This could result in the development of inhaled drugs acting in the lungs that target this tissue binding site, in addition to their primary target, as a means of increasing their duration of action.

5. Conclusion

In summary, it has been shown using β_2-AR agonist and antagonist tool radioligands that a tissue binding site distinct from the β_2-AR is present in parenchyma membranes prepared from human lung tissue. This non-β_2-AR binding site appears to be exclusive to a select number of LABAs (indacaterol, salmeterol and vilanterol), with potentially a link between their lipophilicity and the ability to interact with the site. This may provide a further hypothesis for the duration of action exhibited by these drugs, in addition or as an alternative to the "microkinetic" and "exosite" theories, where binding to a tissue site holds these LABAs in the lung for a longer period of time manifesting in a prolonged activation of the β_2-AR and subsequent relief of the symptoms of asthma and COPD.

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REFERENCES

[1] E. D. Bateman, S. S. Hurd, P. J. Barnes, J. Bousquet, J. M. Drazen, *et al.*, "Global Strategy for Asthma Management and Prevention: GINA Executive Summary," *European Respiratory Journal*, Vol. 31, No. 1, 2008, pp. 143-178. http://dx.doi.org/10.1183/09031936.00138707

[2] M. Cazzola and M. Molimard, "The Scientific Rationale for Combining Long-Acting β_2-Agonists and Muscarinic Antagonists in COPD," *Pulmonary Pharmacology & Therapeutics*, Vol. 23, No. 4, 2010, pp. 257-267. http://dx.doi.org/10.1016/j.pupt.2010.03.003

[3] V. A. Cullum, J. B. Farmer, D. Jack and G. P. Levy, "Salbutamol: A New, Selective β-Adrenoceptive Receptor Stimulant," *British Journal of Pharmacology*, Vol. 35, No. 1, 1969, pp. 141-151. http://dx.doi.org/10.1111/j.1476-5381.1969.tb07975.x

[4] G. P. Anderson, "Formoterol: Pharmacology, Molecular Basis of Agonism, and Mechanism of Long Duration of a Highly Potent and Selective β_2-Adrenoceptor Agonist Bronchodilator," *Life Sciences*, Vol. 52, No. 26, 1993, pp. 2145-2160. http://dx.doi.org/10.1016/0024-3205(93)90729-M

[5] M. Johnson, P. R. Butchers, R. A. Coleman, A. T. Nials, P. Strong, *et al.*, "The Pharmacology of Salmeterol," *Life Sciences*, Vol. 52, No. 26, 1993, pp. 2131-2143. http://dx.doi.org/10.1016/0024-3205(93)90728-L

[6] M. Cazzola, L. Calzetta and M. G. Matera, "β_2-Adrenoceptor Agonists: Current and Future Direction," *British Journal of Pharmacology*, Vol. 163, No. 1, 2011, pp. 4-17. http://dx.doi.org/10.1111/j.1476-5381.2011.01216.x

[7] G. P. Anderson, A. Lindén and K. F. Rabe, "Why Are Long-Acting Beta-Adrenoceptor Agonists Long-Acting?" *European Respiratory Journal*, Vol. 7, No. 3, 1994, pp. 569-578. http://dx.doi.org/10.1183/09031936.94.07030569

[8] R. A. Coleman, M. Johnson, A. T. Nials and C. J. Vardey, "Exosites: Their Current Status, and Their Relevance to the Duration of Action of Long-Acting β_2-Adrenoceptor Agonists," *Trends in Pharmacological Sciences*, Vol. 17,

No. 9, 1996, pp. 324-330.
http://dx.doi.org/10.1016/0165-6147(96)10040-7

[9] P. Casarosa, I. Kollak, T. Kiechle, A. Ostermann, A. Schnapp, *et al.*, "Functional and Biochemical Rationales for the 24-Hour-Long Duration of Action of Olodaterol," *Journal of Pharmacology and Experimental Therapeutics*, Vol. 337, No. 3, 2011, pp. 600-609.
http://dx.doi.org/10.1124/jpet.111.179259

[10] R. M. Wallace and J. M. Young, "Temperature Dependence of the Binding of [^{3}H]Mepyramine and Related Compounds to the Histamine H_1 Receptor," *Molecular Pharmacology*, Vol. 23, No. 1, 1983, pp. 60-66.

[11] A. Szczuka, M. Wennerberg, A. Packeu and G. Vauquelin, "Molecular Mechanisms for the Persistent Bronchodilatory Effect of the β_2-Adrenoceptor Agonist Salmeterol," *British Journal of Pharmacology*, Vol. 158, No. 1, 2009, pp. 183-194.
http://dx.doi.org/10.1111/j.1476-5381.2009.00296.x

[12] J. C. W. Mak, B. Grandordy and P. J. Barnes, "High Affinity [^{3}H]Formoterol Binding Sites in Lung: Characterization and Autoradiographic," *European Journal of Pharmacology*: *Molecular Pharmacology*, Vol. 269, No. 1, 1994, pp. 35-41.
http://dx.doi.org/10.1016/0922-4106(94)90023-X

[13] R. J. Slack, V. J. Barrett, V. S. Morrison, R. G. Sturton, A. J. Emmons, *et al.*, "*In Vitro* Pharmacological Characterization of Vilanterol, a Novel Long-Acting β_2 Adrenoceptor Agonist with 24-Hour Duration of Action," *Journal of Pharmacology and Experimental Therapeutics*, Vol. 344, No. 1, 2013, pp. 218-230.
http://dx.doi.org/10.1124/jpet.112.198481

[14] P. A. Procopiou, V. J. Barrett, N. J. Bevan, K. Biggadike, P. C. Box, *et al.*, "Synthesis and Structure-Activity Relationships of Long-Acting β_2 Adrenergic Receptor Agonists Incorporating Metabolic Inactivation: An Antedrug Approach," *Journal of Medicinal Chemistry*, Vol. 53, No. 11, 2010, pp. 4522-4530.
http://dx.doi.org/10.1021/jm100326d

[15] P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, *et al.*, "Measurement of Protein Using Bicinchoninic Acid," *Analytical Biochemistry*, Vol. 150, No. 1, 1985, pp. 76-85.
http://dx.doi.org/10.1016/0003-2697(85)90442-7

[16] J. G. Baker, "The Selectivity of β-Adrenoceptor Antagonists at the Human β_1, β_2 and β_3 Adrenoceptors," *British Journal of Pharmacology*, Vol. 144, No. 3, 2005, pp. 317-322. http://dx.doi.org/10.1038/sj.bjp.0706048

[17] S. A. Holm, "A Simple Sequentially Rejective Multiple test Procedure," *Scandinavian Journal of Statistics*, Vol. 6, No. 2, 1979, pp. 65-70.