Scientific Research Publishing

World Journal of Cardiovascular Surgery, 2022, 12, 293-303
https://www.scirp.org/journal/wjcs
ISSN Online: 2164-3210
ISSN Print: 2164-3202

The *in Vitro* Characterisation of the Effect of Selexipag on Small Human Pulmonary Arteries

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How to cite this paper: Arif, S., Abdi, M., Qaddoura, B., Elshafi, G., Qadri, S., Chaudhry, M., Loubani, M. and Hussain, A. (2022) The *in Vitro* Characterisation of the Effect of Selexipag on Small Human Pulmonary Arteries. *World Journal of Cardiovascular Surgery*, **12**, 293-303.
https://doi.org/10.4236/wjcs.2022.1212026

Received: December 1, 2022
Accepted: December 28, 2022
Published: December 31, 2022

Abstract

Objective: There is a lack of data on the direct effects of Selexipag, a novel prostacyclin Inositol phosphate-3 receptor (IP3) agonist with a long half-life, on human pulmonary arteries. This study aims to establish the efficacy and potency of Selexipag on human pulmonary arteries. **Methods:** Patient consent was obtained prior to lung resection for primary lung cancer. The pulmonary artery rings (n = 23 from 6 patients) were cut to 2 - 4 mm length and 2 - 4 mm internal diameter. They were mounted on a wire myograph under physiological conditions and were pre-constricted to prostaglandin F2α. Concentration response curves were constructed to Selexipag by cumulative addition to the myograph chambers. The viability of the rings was confirmed with Acetylcholine and potassium chloride. **Results:** The Selexipag caused dose dependent vasodilation to human pulmonary arteries. The range of doses used was from 1 pM to 30 uM, n = 4 were excluded due to non-reactivity and n = 19 were included. Initial significant vasodilation of PAs was noted at 3 uM. Maximal response was achieved at 10 uM of Selexipag (EC50 = 1.21 uM). **Conclusion:** The study demonstrated the vasodilatory effect of Selexipag on PAs. Selexipag may be clinically effective in the treatment of pulmonary arterial hypertension. However, additional data are needed to validate the results of this study and determine its clinical significance.

Keywords

Pulmonary Arterial Hypertension, Selexipag, Human Pulmonary Artery, Prostacyclin, Vasodilation

1. Introduction

Pulmonary arterial hypertension (PAH) is currently defined as a resting mean

arterial pressure > 25 mmHg and a normal pulmonary artery wedge pressure of < 15 mmHg via catheterisation of the right heart [1]. There are five subtypes of the updated World Health Organisation Pulmonary Hypertension (WHO PH) classification. Group 1 describes those with PAH of unknown cause as well as multiple subgroups based on the cause. Group 2 includes patients with PAH with left heart disease. Group 3 is composed of PAH due to hypoxia and/or lung disease and group 4 is classed as PAH due to chronic thromboembolic PH (CTEPH). Finally, group 5 is PAH with unclear multifactorial aetiology [2]. PAH is a chronic progressive disease of small pulmonary arteries that results in right-sided heart failure. The World Health Organisation (WHO) has produced a way of classifying patients with PAH based on how much of their physical activity is limited and the severity of their symptoms. At the point of diagnosis, almost three-quarters of patients fit in the WHO class III or IV. Class III states that patients experience no symptoms at rest but feel limited in activities of daily living, Class IV states that patients experience symptoms even at rest and are exacerbated by any activity [3]. PAH carries a poor prognosis with a median survival of 2.8 years, which can lead to death if left untreated, although disease progression is quite variable [4]. PAH is more prevalent in elderly patients over 65 years of age, varying from 12.8% and, in some areas of the world, as high as 63%. 3-year survival rates have been showed to range from 44% to 75%, however, in patients under the age of 50 the survival rates were higher [4].

The aetiology of PAH is thought to be multifactorial. Studies have shown that patients with mutations of the bone morphogenetic protein receptor type 2 gene (BMPR2) present a decade earlier with PAH than those devoid of the mutation [5]. BMPR2 encodes for a receptor within the transforming growth factor beta (TGF-β) family. This pathway regulates the differentiation and growth of pulmonary vascular endothelial cells and smooth muscle cells. Mutations may lead to uncontrollable aberrant growth of these cells. The growth of the cells leads to vascular remodelling, of which three types have been classified as being part of PAH: plexiform lesions, arteritis and dilation lesions. The plexiform lesions are seen classically in idiopathic PAH. As a result of vascular remodelling, pulmonary resistance increases due to obstruction. This increases the pressure within the right ventricle and leads to right-sided heart failure. Proliferation of cells within the tunica intima and media are common characteristic changes seen in most PAH cases histologically [6]. Other factors include increased presence of endothelin (ET)-1, a mediator for vascular remodelling causing proliferation and dysfunction leading to the changes found in PAH, connective tissue disease, congenital cardiac disease, pulmonary disease and systemic disorders such as Sarcoidosis [7]. Treatment of PAH revolves around the mechanisms implicated in its pathophysiology including the effacement of pulmonary arterioles, microvascular thrombosis, increased vascular reactivity and plexiform lesions [8].

Phosphodiesterase enzyme inhibitors have been found to increase the quantity of cyclic GMP (cGMP) within the pulmonary arterial system leading to vasodilation. The metabolism of cGMP is regulated by several enzymes, of which Phos-

phodiesterase-3 (PDE-3), PDE-4 and PDE-5 are the most prevalent. PDE-5 is the most common enzyme found within the pulmonary vascular system and is the reason why sildenafil, a PDE-5 inhibitor, is used in the management of PAH. Inhibition of PDE-3 leads to an increase in cAMP, promoting vasorelaxation [6].

Endothelin receptor antagonists have also been developed as an alternative to phosphodiesterase enzyme inhibitors in the management of pulmonary vasoconstriction. Plasma endothelin-1 levels are raised in PAH and are associated with worse outcomes due to smooth muscle cell and fibroblast dysfunction [9]. Two subtypes of Endothelin-1 (ET-1) receptors, Endothelin A (ETRA) and Endothelin B (ETRB) are present in pulmonary smooth muscle cells but only the ETRB subtype is expressed on vascular endothelial cells [9]. Activation of these two subtypes in pulmonary smooth muscle cells leads to proliferation. However, activation of ETRB alone leads to the release of nitric oxide and Prostaglandin I_2 which have dilatory effects [3]. Two endothelin antagonists that have showed to improve long term outcomes are ambrisentan and bosentan [10]. Ambrisentan selectively antagonises ETRA and seems to be more effective when used as a combination therapy rather than as monotherapy. The ARIES-1, 2 and 3 (Ambrisentan in Pulmonary Arterial Hypertension, Randomised, Double-Blind, Placebo-controlled, Multicentre, Efficacy Studies) are large randomised controlled trials that have shown that ambrisentan, in combination with spironolactone or other background PAH treatments, improved 6-minute walking distances, reduced symptom severity and increased haemodynamic stability compared to ambrisentan monotherapy [11]. Ambrisentan was also found to improve survival and lower rates of death and hospitalisation when used to treat connective tissue disease-related PAH [12]. Bosentan, a non-selective antagonist, has proven to be beneficial in the management of PAH. Bosentan was shown to improve 6-minute walking distances, severity of PAH and prevent disease progression after 1-year of treatment [13].

Prostanoids refers to (Prostaglandin) PGI_2 agonists. At a cellular level, prostanoids result in an increase of cAMP which inhibits aggregation of platelets and promotes the relaxation of smooth muscle and it is also well known that prostacyclin limits smooth muscle cell proliferation and thrombosis [14]. The therapeutic agents that are in use in patients with advanced PAH (WHO class III and IV) are 3 reconstruct, epoprostenol and iloprost. These synthetic analogues of endogenous prostacyclin have been shown to reduce mortality in PAH when used in conjunction with other agents. For example, epoprostenol was the first of these prostanoids to be licensed and has been shown to reduce mortality in PAH when used in conjunction with sildenafil and bosentan [15]. However, the agent has an extremely short half-life of less than 6 minutes requiring a continuous infusion via intravenous catheter [16]. Subsequently, prostanoids with longer half-lives were developed that had different routes of administrations. Iloprost, another prostanoid developed to resolve the issues surrounding the use of subcutaneous pumps and intravenous catheterisation, has a half-life of approximately 45 minutes. Iloprost, when administered, requires 6 - 9 inhalations per day, each tak-

ing up to 6.5 minutes of inhalation time via a nebuliser [17]. However, non-compliance became an issue with Iloprost, most likely due to the frequency of daily doses [18]. Other prostanoids have shown to be effective with 2-year survival rates up to 91% as found in the TRIUMPH-1 study (*Double Blind Placebo Controlled Clinical Investigation Into the Efficacy and Tolerability of Inhaled Treprostinil Sodium in Patients with Severe Pulmonary Arterial Hypertension*). This study also highlighted the range of adverse effects including headache, nausea and vomiting, cough, diarrhoea, cramps, and severe side effects such as hypotension and accelerated hypotension. Cough, headache, and nausea being the top 3 most common adverse effects [19].

The problems surrounding these agents involve issues with half-life, compliance, and adverse effects, including hepatotoxicity, oedema and anaemia [20]. This has created a need for agent that is cheap, has minimal side effects, a long half-life and an administration route that is effective in providing enough of the agent whilst keeping the patient compliant [6].

Selexipag is highly selective PGI_2 agonist that has been shown to be clinically effective in multiple trials including the GRIPHON (*Prostacyclin PGI2 Receptor Agonist in Pulmonary Arterial Hypertension*) and TRANSIT (*Tolerability and Safety of the Transition From Inhaled Treprostinil to Oral Selexipag in Patients with Pulmonary Arterial Hypertension*) trials [21] [22]. Selexipag is given orally twice daily and is rapidly absorbed. It is metabolised by carboxylesterases in the liver to produce ACT-333679, its active metabolite, which has a half-life 5 times greater than Selexipag [23]. There is a gap of knowledge demonstrating the direct action of selexipag on human pulmonary arteries *in vitro*. The majority of publications characterising the effect of selexipag are primarily based on murine studies. [23] [24]. This study aims to characterise the *in vitro* effect Selexipag has on human pulmonary arteries as a dose-response curve. The results of this study may help support use of selexipag in clinical practice and improve the overall treatment of patients with PAH.

2. Material and Methods

Ethical approval to use human pulmonary tissue was sought from the local research ethics committee and local research and development department (Research Ethics Committee approval reference: 15/NW/0808, HEY R&D reference: R1884). Consent was acquired from patients undergoing lung resection and disease-free regions of pulmonary vasculature was sampled. Patients who could not give informed consent and those under the age of 18 years of age were excluded from the study.

Pulmonary arteries were harvested from patients undergoing lung resection. The arteries were taken from healthy lung tissue and cut into rings with a diameter of 2 - 4 mm and a length of 2 - 4 mm. In total, pulmonary arteries from six patients were cut into 25 small rings and loaded onto the DMT-620M multi-wire myograph system (**Figure 1**) and bathed in Krebs-Henseleit solution

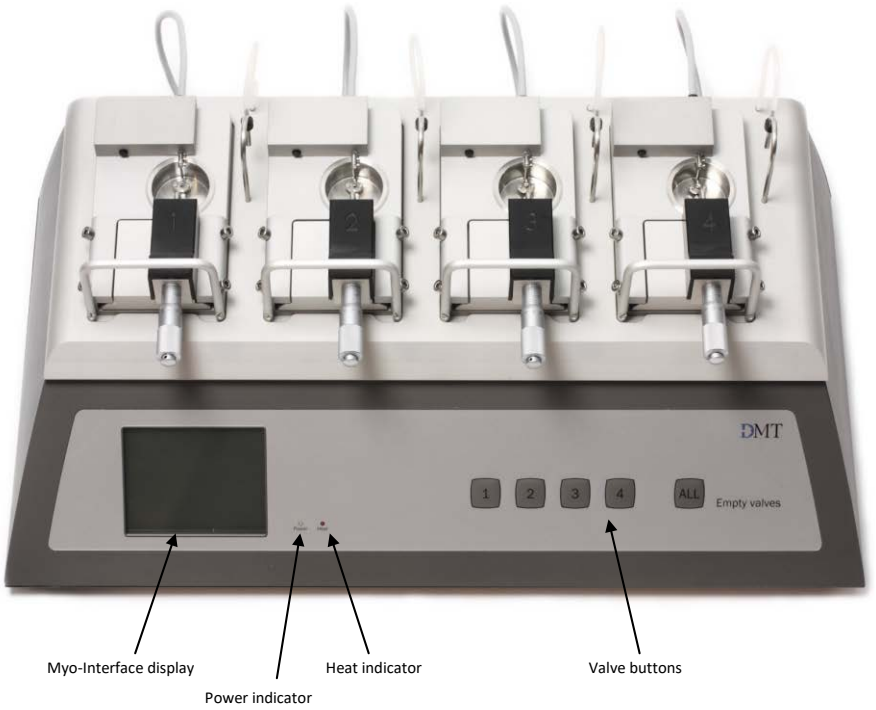

Figure 1. DMT 620M Multi-wire Myograph System mounted 4 vessels at a time, one per each organ bath.

(consisting of 113.8 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 1.2 mmol/L KH_2PO_4, 11.4 mmol/L glucose, and 2.4 mmol/L $CaCl_2$ dissolved in distilled water) aerated with 21% O_2: 5% CO_2 at 37˚C. The system was calibrated according to the calibration program manual before mounting the vessels and running our experiment.

The PA rings were left to equilibrate for 1 hour under a baseline resting tension of 1.61 gram force (gf). This value for the resting basal tension was determined in a previous study conducted by our team [25]. After the vessels were equilibrated, 11.21 μM of $PGF_{2\alpha}$ (EC_{80}) was added to the chambers to mimic the vasoconstriction seen in PAH. Once the tension had plateaued, we added cumulative logarithmic concentrations of selexipag ranging from 1 pM to 30 μM. Stock solution was prepared with EDTA, as per manufacturer recommendation and the serial dilutions were prepared with distilled water. We added the concentrations in a stepwise fashion only adding the next concentration when the tension had plateaued from the current concentration of selexipag in the chamber. We applied the same experiment conditions to our control vessels and applied additional Krebs solutions to the control vessels. A sample trace (**Figure 2**) recorded on LabChart during the experiment shows the sequence of events that took place. The EC50, defined as the concentration needed to produce half-maximal response, was calculated using the AAT Bioquest EC50 Calculator [26]. Finally, the rings were washed multiple times followed by testing the integrity of the endothelium and smooth muscle using 1 μM of Acetylcholine and 1 μM of Potassium Chloride. Only 19 pulmonary rings reacted to these agents in the end

and were therefore included in the results, 4 of the vessels did not react and their data were ignored in the analysis of the results.

Five percent of carbon dioxide/balance air (10 lt cylinders) was sourced from BOC Limited. All reagents were obtained from Fischer Scientific and acetylcholine from Sigma Aldrich.

3. Results

A dose-response curve (Figure 3) was constructed for selexipag by plotting the logarithmic concentrations of selexipag (x-axis) against the active tension (y-axis). Active tension was defined as the difference between the maximum tension after $PGF_{2\alpha}$ pre-constriction and the tension following addition of the specific selexipag concentration. Error bars were constructed by calculating the standard deviations for each concentration of selexipag.

All rings dilated in response to selexipag. Maximum vasodilation was seen at 10 μM of selexipag. Interestingly, the active tension begins to increase at concentrations above 10 μM showing the vasodilatory effect of selexipag diminishing at higher concentrations. This could be related to the exhaustion of tissue or might be the antagonistic effect at high concentrations of selexipag. The EC50 was calculated to be 1.12 μM.

The active tension from 1 pM - 10 nM selexipag (excluding 3 pM selexipag)

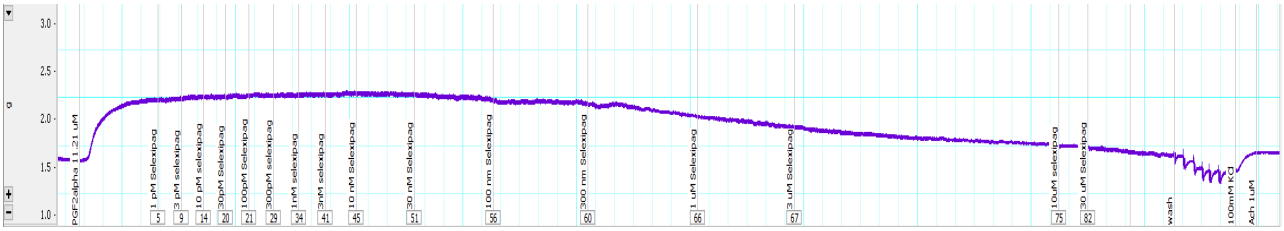

Figure 2. Sample trace of the experiment recorded using LabChart.

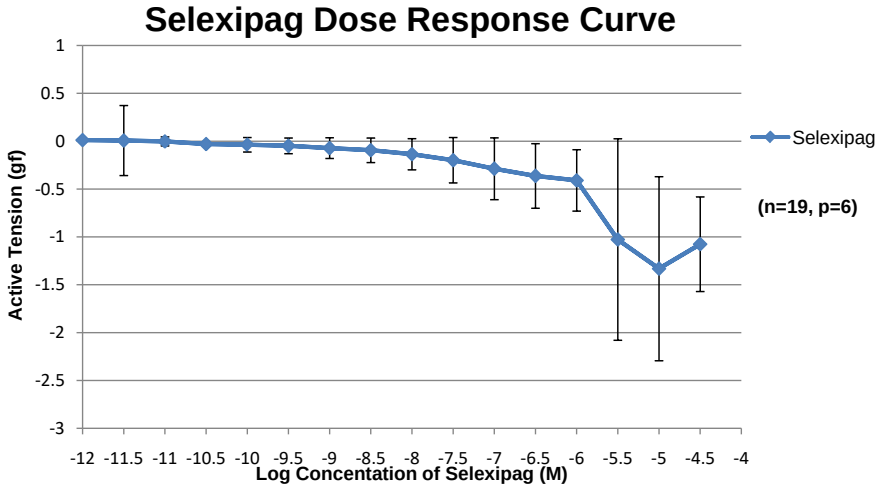

Figure 3. Dose-Response Curve for Selexipag: Active tension plotted on the Y axis and log concentration of selexipag on the X axis. Maximal vasodilation was recorded at 10 μM.

are statistically different from the active tension at 10 μM selexipag. While the active tension from 1 pM - 30 nM selexipag are statistically different from the active tension at 30 μM selexipag. This shows that selexipag at 10 μM and 30 μM have a significant vasodilatory effect compared with the lower concentrations. However, the changes in active tension between 10 μM and 10 nM selexipag are statistically insignificant and therefore may not have a marked vasodilatory effect.

4. Discussion

The primary aim of our study was to characterise the efficacy and potency of selexipag *in vitro.* In our experiment we show that selexipag has a vasodilatory effect that reversed the prostaglandin induced vasoconstriction.

This preliminary study is just the first phase of a series of experiments aimed to compare the effect that selexipag has with other similar agents. The next stage of our study would be to compare the dose-response relationship of the active metabolite of selexipag; ACT-333679, which has 13 times greater affinity for the Inositol Phosphate (IP) receptor with selexipag. ACT-333679 lacks the pure selectivity of selexipag and has been shown to affect EP_2, EP_4, and DP_1 receptors. This may contribute to additional side effects [26]. However, pre-clinical murine data has shown that selexipag fails to activate prostaglandin receptors enough to cause contraction of gastric fundi, even at therapeutic concentrations. This can be attributed to the high selectivity of selexipag for receptors in pulmonary arteries [6].

There are multiple studies looking at the effects of selexipag and ACT-333679 on rat pulmonary arteries. The conclusions of these studies are congruent with the results of our experiment: that selexipag does evoke relaxation of pulmonary arteries. We have shown that murine data can be extrapolated into research involving human tissue with regards to selexipag usage. Although, current data seems to suggest that the distribution of receptors and sensitivity to pre-constriction agents may vary among species, prostacyclin analogues are known to evoke similar IP receptor activation in both humans and rats [27]. Selexipag has already been used in clinical trials, the GRIPHON study was a phase 3 double-blinded RCT that looked at the effect of selexipag compared to placebo in 1156 patients with PAH. This clinical trial showed that selexipag reduces the risk of death from PAH in comparison to placebo since 17.8% of patients in the selexipag arm died while 23.5% died in the placebo arm ($p = 0.003$) at the end of treatment. However, at the end of the study, 100 patients in the selexipag cohort died and 105 patients in the placebo cohort died. This showed that although selexipag reduced the risk of death during the treatment period, it did not improve the morbidity of the patients [28]. The recently completed TRITON (NCT02558231. *A multicentre*, *double-blind*, *placebo-controlled*, *phase 3b study*, *randomized* 1:1 *newly diagnosed*, *treatment-naïve PAH patients to initial triple or initial double therapy*) study compared initial triple therapy with tadalafil, selexipag and macitentan with double therapy with tadalafil and macitentan. The conclusion of the

study was that by week 26 there was no considerable differences between either group regarding treatment outcome [29]. A comparison between selexipag and existing therapeutic agents for PAH using human tissue will also be necessary to assess whether current double therapy regimes may benefit from the addition of selexipag. This will shed some additional insight into the more optimal combination of agents.

This study had limitations which we addressed in the experiment. Ethylenediaminetetraacetic Acid (EDTA) is known to have a vasodilatory effect and was used as part of the solution to make stock solution. We decided to then dilute this solution with distilled water to make up the concentrations of selexipag. In previous experiments we had calculated the EDTA concentration to be 17 μM, which does not produce a vasodilatory response from the pulmonary rings, showing that the vasodilatory effect could not be due to EDTA [30]. Our results show large error bars however this may be due to the sample size. Ideally, we would want a larger sample size however it was not feasible. Continuing the experimenting on a higher number of arteries from a larger population of patients would help to validate our results and possibly improve the accuracy of our data.

Another limitation is the use of $PGF_{2\alpha}$ to mimic vasoconstriction caused by PAH. Considering that PAH is caused by multiple vasoconstrictors, using $PGF_{2\alpha}$ only, for vasoconstriction does not quite mimic the pathophysiology of PAH [30]. The experiment also does not reflect the true conditions that exists *in vivo* with regards to selexipag. Selexipag undergoes hepatic metabolism to produce ACT-333679 therefore, the concentration of the selexipag that evokes maximal relaxation of the resected arteries, in our experiment, may or may not evoke the same response when used *in vivo*. Further studies should look at the effect of selexipag with pulmonary arteries 300reconstructed with multiple vasoconstriction agents, including ET-1, and to investigate the effect of ACT-333679 on human pulmonary arteries to provide further information that may contribute to improving the management of PAH.

5. Conclusion

This is the first study of its kind looking at the *in vitro* dose-response relationship of selexipag on human pulmonary arteries. This study has evaluated the extent to which selexipag dilates human pulmonary vasculature and outlines effective *in vitro* concentrations that may be extrapolated to *in vivo* usage of selexipag. The results of this study may provide further insight into improving management of PAH.

Funding

This work was supported and funded by the Research Funds of the Cardiothoracic Department of Castle Hill hospital.

Author Contribution Statement

All authors have contributed significantly to the content of the article and have

read and approved the submission of the manuscript to The Open Cardiovascular Medicine Journal.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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List of Abbreviations

PAH—Pulmonary Arterial Hypertension,
PH—Pulmonary Hypertension,
EDTA—Ethylenediaminetetraacetic Acid,
WHO—World Health Organisation,
$PGF_{2\alpha}$—Prostaglandin $F_{2\alpha}$,
BMPR2—Bone Morphogenetic Protein Receptor Type 2,
TGF-β—Transforming Growth Factor Beta,
ET—Endothelin,
PDE—Phosphodiesterase,
GMP—Guanosine Monophosphate,
cAMP—Cyclic Adenosine Monophosphate,
ETRA—Endothelin Receptor A,
ERTB—Endothelin Receptor B,
PGI—Prostaglandin I,
EP—Prostaglandin E Receptor,
DP—Prostaglandin D Receptor.