Rationale for Targeting the Serotonin System as a Treatment of Pulmonary Hypertension

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14th May 2019

Pulmonary hypertension (PH) is a broad term referring to several related clinical conditions in which patients may present with signs or symptoms including increased pulmonary arterial pressure (≥25 mmHg) on right heart catheterization, shortness of breath, fatigue, and dizziness. The forms of PH are classified across 5 groups based upon common pathological findings, hemodynamic characteristics, and management:

- Group 1: Pulmonary arterial hypertension (PAH)
- Group 2: Pulmonary hypertension due to left-sided heart disease
- Group 3: Pulmonary hypertension due to lung disease and/or hypoxia
- Group 4: Chronic thromboembolic (blood clots) pulmonary hypertension and other pulmonary artery obstructions
- Group 5: Pulmonary hypertension with unclear and/or multifactorial mechanisms

Pulmonary hypertension is caused by a complex interaction between environmental and genetic risk factors. Each subtype of PH has a unique etiology, but all forms are characterized by pulmonary vascular remodeling that leads to resistance to blood flow, resulting in increased cardiac afterload.

Altavant Sciences is currently advancing rodatristat ethyl[1] through clinical development as a potential complementary treatment to standard of care in patients with pulmonary arterial hypertension (PAH). The discussion below summarizes and discusses the rationale for addressing serotonin as a target in the treatment of PAH.

Pulmonary hypertension Group 1 (PAH) particularly affects the arteries in the lungs and the right side of the heart. The condition is often progressive, with the remodeling of the pulmonary vasculature leading to increasing pulmonary arterial pressure. This increased pulmonary arterial pressure puts greater strain on the right heart and, sadly, for some patients ultimately results in right heart failure. Prognosis for these patients can be poor with a median survival of five years. There is no approved cure and patients may ultimately require lung transplantation (Thenappan et al., 2010). Current therapies aim to alleviate symptoms by lowering pulmonary arterial pressure with a view to improving quality of life. These treatments have focused on three signaling mechanisms: nitric oxide/cGMP, endothelin, and prostacyclin, all of which result in vasodilation of the pulmonary vasculature leading to decreased pulmonary arterial pressure (Thenappan et al., 2018). While able to extend life and provide symptom relief, none of these treatments can fully reverse the overall course of PAH.

The potential benefits of targeting the peripheral serotonin system are currently being evaluated in clinical studies with PAH patients. Dysregulation of peripheral serotonin production has been demonstrated in PAH and attenuation of serotonin production has demonstrated efficacy in nonclinical models of the disease.

The purpose of this review is to present and discuss the key data linking dysregulated serotonin biology to the development and maintenance of PAH. The goal is to provide the reader with a

[1] Name applied for; application pending. Previously known as KAR5585 or RVT-1201.

deeper understanding of the latest advances and rationale supporting the utility of targeting the serotonin system for a potentially disease-modifying therapeutic benefit in PAH.

I. The peripheral serotonin system

Serotonin has numerous roles in regulating peripheral biology (Berger et al., 2009; Mawe and Hoffman, 2013), separate from its well-known roles in regulating central brain activity. Indeed, serotonin was named for its initial source from blood serum (*sero-*) and its vasoconstrictive effect (*-tonin*) (Rapport et al., 1948). Dysregulated serotonin synthesis has been implicated in several diseases, as reviewed by Matthes and Bader (2018) in the publication "Peripheral Serotonin Synthesis as a New Drug Target".

Serotonin is unable to cross the blood-brain barrier and is produced separately in the periphery and CNS. The peripheral pool of serotonin is derived from the dietary amino acid tryptophan via a biosynthetic pathway comprising tryptophan hydroxylase 1 (TPH1) and aromatic L-amino acid decarboxylase (AADC) (Walther et al., 2003a). While the bulk of peripheral serotonin is produced in the enterochromaffin cells of the gastrointestinal tract, serotonin production also takes place in many organs, including blood vessels, bones, immune cells, pancreas, lungs, kidney, and skin (Berger et al., 2009).

The major sink of gut-derived peripheral serotonin is in platelets, which absorb serotonin via the serotonin transporter (SERT) and release it during degranulation to regulate blood clotting and the immune system (Mawe and Hoffman, 2013). In lungs, platelet-derived and locally-produced serotonin from both lung tissue and arterial endothelial cells can act upon the underlying pulmonary arterial smooth muscle cells (PASMCs) to cause acute vasoconstriction and drive immune cell recruitment (Eddahibi et al., 2006).

Serotonin signals through a family of 13 receptors consisting of 12 G protein-coupled receptors (GPCRs) and one ligand-gated ion channel (LIC) (El-Merahbi et al., 2015; McCorvy and Roth, 2015). The signaling pathways downstream of these receptors are important for numerous physiological and pathological responses. In PASMCs, serotonin receptor-mediated signaling activates vasoconstrictive Ca^{2+} pathways and stimulates the transcription of proliferative and fibrotic genes (Varghese et al., 2006). Understanding these mechanistic pathways that regulate PASMC proliferation and fibrosis is believed key for linking dysregulated serotonin biology to the development and maintenance of PAH.

In addition to receptor-mediated mechanisms, serotonin can also function in a post-translational modification process called serotonylation (Walther et al., 2003b). Serotonylation via transglutaminase 2 (TG2) can occur at a glutamine residue in the catalytic domain of small GTPases, such as Rho kinase, and causes constitutive activity (Liu et al., 2011). In addition, serotonylation can occur at α-actin and fibronectin, where it has been speculated to regulate cellular proliferation (Wei et al., 2012). More recently, serotonylation has been shown to occur at histones and regulate gene transcription by acting as an epigenetic marker (Farrelly et al., 2019).

Thus, serotonin may be implicated in PAH via two mechanisms: receptor-mediated signaling and post-translational modifications (serotonylation) leading to vasoconstriction and proliferation of PASMCs. These effects of serotonin signaling ultimately contribute to vascular remodeling and the formation of plexiform lesions, which are the central pathological features of PAH.

II. The serotonin hypothesis of PAH

A. Clinical studies linking dysregulated serotonin biology to PAH

Historically, the serotonin system was linked to the development of PAH following widespread use of anorexigenic diet products, such as aminorex and dexfenfluramine. These weight loss drugs are associated with an increased risk of developing PAH (Abenhaim et al., 1996). It is now understood that these drugs cause serotonin release via SERT (Rothman et al., 1999) and can be metabolized into a 5-HT_2 receptor agonist (norfenfluramine) (Fitzgerald et al., 2000). This serotonin-releasing mechanism has also been linked to amphetamine or stimulant drug-associated PAH (Chin et al., 2006). Studies in mouse models have directly linked the cardio-pulmonary effects of dexfenfluramine to serotonin biology (Dempsie et al., 2008). (These and other preclinical studies are discussed in the next section).

Lung tissue samples from PAH patients have shown elevated amounts of TPH1, which is likely a key driver for pathological levels of serotonin production in PAH (Eddahibi et al., 2006). Furthermore, some groups have reported elevated peripheral serotonin in blood samples from PAH patients (Herve et al., 1995). Together, these findings implicate dysregulated serotonin in the pathobiology of PAH – even in patients who did not take diet pills or use stimulant drugs.

In a study conducted by Eddahibi et al. (2001), serotonin transporter (SERT) over-expression was observed in PASMCs from PAH patients, and serotonin-induced growth of these PASMCs could be blocked by SERT inhibitors (SSRIs) (Eddahibi et al., 2001). This study also found that a SERT polymorphism that causes increased SERT expression was associated with an increased risk of PAH (Eddahibi et al., 2001). Indeed, some researchers have proposed that "…remodeling of the pulmonary circulation, either as a result of, or contributor to, increased pulmonary vascular pressure, depends on the presence of SERT" (Fanburg and Lee, 2000).

Dysregulated serotonylation has also been observed in human PAH, where increased serotonylation of RhoA and fibronectin have been found in PAH patient-derived PASMCs (Liu et al., 2011; Penumatsa and Fanburg, 2014; Wei et al., 2012). In addition, Rho kinase is activated by serotonylation and Rho kinase inhibition has been shown to reduce PAH progression in a serotonin-based mouse model of PAH (Guilluy et al., 2009).

Several reports propose additional mechanisms by which serotonin may be implicated in PAH. The role of serotonylated fibronectin is less understood but it appears to regulate smooth muscle cell proliferation and migration (Wei et al., 2012). Over-expression of the 5-HT_{1B} receptor has been observed in PASMCs isolated from female PAH patients (Wallace et al., 2015). 5-HT_{1B} activation and SERT activity cooperate to induce PASMC contraction and proliferation via activation of downstream proliferative and contractile signaling pathways (MacLean, 2018).

The dysregulated serotonin system is also thought to interact with the BMPR2[2]-based mechanisms of inherited PAH (Long et al., 2006). Specifically, mice lacking a copy of BMPR2 exhibit increased pulmonary artery systolic pressure and pulmonary artery remodeling in response to hypoxia combined with chronic infusion of serotonin (Long et al., 2006). This link highlights a significant role of serotonin biology in a variety of distinct etiologies of PAH such as: inherited, idiopathic, and drug-induced.

[2] Bone morphogenetic protein receptor type 2

Taken together, these studies strongly implicate dysregulated serotonin biology in establishing PAH in humans and the subsequent progression of vasculature remodeling. These observations lend wide support for the therapeutic potential of targeting the serotonin system in PAH. The challenge is how best to address the multiple roles and signaling mechanisms of serotonin.

A selective approach, for example via a specific receptor antagonist or SERT inhibitor, while appearing promising during evaluation in mechanistic in vitro assays, may ultimately fail in the clinic as it may not address all the apparent roles of serotonin in PAH. Altavant Sciences believes that therapeutic activity in PAH patients may be best achieved with an approach that leads to an overall reduction in serotonin via TPH1 inhibition since this would lower the spectrum of PAH-implicated factors associated with serotonin.

B. Preclinical studies linking dysregulated serotonin biology to PAH

There is a wealth of preclinical evidence linking the serotonin system to PAH. Indeed, "[o]ver the last couple of decades there has been an accumulation of convincing evidence that targeting serotonin synthesis or signaling is a novel and promising approach to the development of novel therapies for PAH" - (MacLean, 2018).

Rodent models have allowed for the delineation of how distinct regulators of serotonin biology contribute to the development of PAH. There are 4 main mechanisms whereby the effects of serotonin on rodent models of PAH can be probed: 1) serotonin receptor agonism/antagonism, 2) inhibition of serotonin synthesis, 3) blockade of serotonin uptake via SERT and 4) inhibition of serotonylation via TG2. Each of these mechanisms has been shown to influence the development of PAH in rodent models. The overall message is that peripheral serotonin signaling via receptors and intracellular modifications consistently promotes the development of PAH in a variety of rodent models.

Table 1: Key papers and main findings supporting the roles of serotonin in PAH

Serotonin Receptor Antagonism	
Keegan et al. (2001)	Demonstrated that 5-HT_{1B} knockout or antagonism prevented hypoxia-induced pulmonary vascular remodeling.
Launay et al. (2002)	Demonstrated that 5-HT_{2B} knockout or antagonism prevented hypoxia-induced pulmonary vascular remodeling.
Inhibition of Serotonin Biosynthesis	
Morecroft et al. (2007)	Demonstrated that *Tph1* knockout results in less pulmonary vascular remodeling and pulmonary hemodynamics in response to hypoxia.
Aiello et al. (2017)	Demonstrated that treatment with a peripherally restricted TPH inhibitor can prevent the development of pulmonary vascular remodeling in two rodent models of PAH.
Blockade of SERT	
Eddahibi et al. (2001)	Showed that polymorphisms in the SERT gene that lead to increase expression are a risk factor for PAH in humans. Demonstrated that chronic hypoxia results in increased SERT expression. Furthermore, the authors demonstrated that SERT function was necessary for the mitogenic effect of serotonin in PASMCs.

MacLean et al. (2004)	Demonstrated that mice overexpressing the SERT only in smooth muscle cells have an increased response to hypoxia relative to wild type mice.
Inhibition of Serotonylation	
DiRaimondo et al. (2014)	Demonstrated that hypoxia elevates TG2 activity (and therefore serotonylation) and that pharmacological inhibition of TG2 activity attenuated the elevated right ventricular pressure in a hypoxia PAH model.
Liu et al. (2011)	Showed that serotonylation in smooth muscle cells is blocked by SERT inhibition and that inhibition of serotonylation prevents the proliferative effect of serotonin on smooth muscle cells.

Genetically engineered mouse models have enabled mechanistic studies that probe the contribution of specific serotonin system components to the pathobiology of PAH. Studies using subtype-specific serotonin receptor knockout animals or subtype-selective serotonin receptor antagonists have implicated the role of the 5-HT_1 and 5-HT_2 receptors in the pathogenesis of PAH in rodents (Keegan et al., 2001; Launay et al., 2002). Important to note is that, since the receptors have been altered from birth, these models mostly test the role of serotonin receptor signaling in the initiation or development of PAH. Further, studies using serotonin receptor antagonists may also have this limitation as treatment with these potential drugs usually begins concurrently with disease induction. Moreover, as multiple pathways contribute to serotonin's role in PAH, it is unlikely that antagonizing only receptor, or SERT inhibition alone, will deliver meaningful clinical effect.

Early studies in rodent models of PAH explored the impact of lowering serotonin on disease initiation or progression. Exploratory studies implicating the role of the serotonin system pharmacologically used p-chlorophenylalanine (pCPA) to inhibit TPH1/2 and thereby reduce serotonin levels both peripherally and centrally (Carrillo and Aviado, 1969). pCPA had a beneficial effect in the models, such as reduction in the pulmonary arterial pressure. This result has been subsequently replicated by many groups and provided the first evidence that serotonin is a pharmacologic target for PAH.

Either removing peripheral serotonin production or changing its transport into cells can affect the development of PAH in rodent models. Specifically, knocking out the serotonin biosynthetic TPH1 enzyme in mice (*Tph1*-KO mice) results in significantly reduced hypoxia-induced PAH (Morecroft et al., 2007). Since hypoxia in human lungs is known to cause local vasoconstriction, this model clearly suggests the serotonin link in the manifestation of PAH.

These results with *Tph1*-KO mice are complemented by a PASMC-specific SERT overexpression mouse model that spontaneously develops PAH (MacLean et al., 2004). By overexpressing SERT, the PASMCs have increased intracellular uptake of serotonin likely leading to enhanced TG2 mediated serotonylation of intracellular proteins. In addition, SERT knockout (SERT-KO) mice are protected from hypoxia-induced PAH (Eddahibi et al., 2000). Both studies suggest a role for serotonylation in the pathobiology of PAH in rodent models.

Indeed, elevated TG2 activity (and thereby serotonylation) is observed in hypoxia-induced PAH models (DiRaimondo et al., 2014). Another study using cystamine as an inhibitor of TG2 found a survival benefit in a rodent PAH model (Wang et al., 2018). TG2 activity has also been shown to

amplify PDGFRβ[3] signaling by promoting the proliferation of smooth muscle cells in response to PDGF-BB[4] (Zemskov et al., 2012). Each of these studies corroborate the role of serotonylation in PAH models.

When weighing the relative impact of serotonylation versus receptor-mediated serotonin signaling in PAH, it is useful to consider the role of SERT in mediating uptake of extracellular serotonin into the PASMCs. Without the transport of serotonin provided by SERT, serotonylation would not be able to occur. With this in mind, we can make the link between the process of serotonylation and the evidence of SERT-based mechanisms in humans such as: 1) polymorphisms in SERT resulting in increased expression of SERT and higher risk of PAH (Eddahibi et al., 2001) and 2) the use of anorexigenic diet pills (which partly act via SERT) (Abenhaim et al., 1996). When considered in this light, serotonylation may be the dominant force underlying the contribution of serotonin to PAH in a chronic context due to the close link between PASMC proliferation and intracellular uptake of serotonin (Eddahibi et al., 2006; Eddahibi et al., 2001; Fanburg and Lee, 2000). However, proving this would be difficult and there undoubtedly appears to be an important role of serotonin receptor signaling in the initiation of vascular remodeling and acute responses to elevated serotonin (Launay et al., 2002). Regardless of relative contributions, a serotonin-targeted mechanism of action that down regulates both processes (such as TPH1 inhibition) could have the ability to halt or reverse the overall course of the disease.

III. Serotonin pathway-directed treatments for PAH

Initial clinical efforts to target the serotonin system in PAH were attempted with terguride, which is both a 5-$HT_{2A/2B}$ antagonist and a dopamine D_2 receptor partial agonist (Antoniu, 2011). In Japan, terguride is approved for the treatment of hyperprolactinemia, indicating it has an acceptable safety profile in humans (Ciccarelli and Camanni, 1996). Unfortunately, in a 12-week phase 2 clinical study in PAH, terguride did not show efficacy at a 1.5 mg dose. This was the highest possible dose due to terguride's activity at the D_2 receptor, which when over-inhibited may result in unacceptable central nervous system side effects such as drowsiness, restlessness, and uncontrollable movements (Lythgoe et al., 2016). Afterwards, translation of pharmacokinetic-pharmacodynamic data in rodents to humans suggested that the 1.5 mg dose of terguride did not provide exposures sufficient to block the 5-HT_2 receptors (Lythgoe et al., 2016). In addition, since terguride only acts at the 5-HT_2 receptors and lacks activity at the 5-HT_{1B} receptor, which is the main target on PASMCs, terguride may not have the correct pharmacologic profile at serotonin receptors for optimal efficacy in PAH. Thus, the failure of this clinical study may reflect the safety and receptor selectivity limitations of terguride rather than the value of addressing serotonin dysfunction.

IV. Recent advances in targeting the serotonin pathway in PAH

Despite the lack of success to date for reversing PAH in clinical trials with a specific serotonin receptor antagonist, a strong rationale for targeting the serotonin system in PAH remains. The true therapeutic potential of anti-serotoninergic agents has not been adequately tested and

[3] Platelet derived growth factor receptor beta

[4] Platelet-derived growth factor-BB

overwhelming preclinical and clinical evidence strongly implicates serotonin in both the development and progression of PAH. Therefore, two options remain: to search for a molecule with the correct combination of serotonin receptor subtype antagonism and SERT blockade or to inhibit peripheral serotonin production. The latter approach may better target the underlying biology by reducing the signaling of all serotonin receptors agnostically and it has the unique distinction of simultaneously reducing the amount of serotonin available for serotonylation.

Towards this goal, a series of drug-like, peripherally restricted reversible serotonin synthesis inhibitors were developed by Karos Pharmaceuticals, Inc. (Goldberg et al., 2017; Goldberg et al., 2016). These molecules act as competitive and reversible inhibitors of TPH1/2, with IC_{50} values in the low nanomolar range. Importantly, they are selective for TPH1/2, specifically targeting their effects to inhibiting serotonin synthesis without impacting any of the other aromatic amino acid hydroxylases tested. This contrasts with the tool compound pCPA, which is an amino acid analogue that is an irreversible and brain-penetrant TPH1/2 and phenylalanine hydroxylase inhibitor (Koe and Weissman, 1966). Peripheral restriction is key because a brain-penetrant TPH1/2 inhibitor could be expected to cause an increased risk for psychiatric side effects such as depression and suicidality. Since TPH1 is the rate-limiting enzyme involved in peripheral serotonin synthesis, these molecules specifically block de novo serotonin synthesis without affecting the production of other biogenic amines.

Two lead molecules, rodatristat ethyl and KAR5416, were able to dose-dependently reduce serotonin levels in the serum, gut, and lung of rats, while not impacting brain serotonin (Aiello et al., 2017). The major metabolite of serotonin, 5-HIAA, was also reduced by approximately 35-50% in the urine of rats treated with rodatristat ethyl, indicating an overall reduction in serotonin biosynthesis. This level of inhibition of serotonin synthesis with rodatristat ethyl and KAR5416 was efficacious in the two pre-clinical rodent models of PAH tested (Aiello et al., 2017). Specifically, rodatristat ethyl reduced pulmonary vasculature remodeling, as measured by vessel wall thickness and percent occluded vessels (Figure 1). These results suggest that rodatristat ethyl can reduce serotonin biosynthesis to a potentially efficacious level peripherally without impacting brain serotonin.

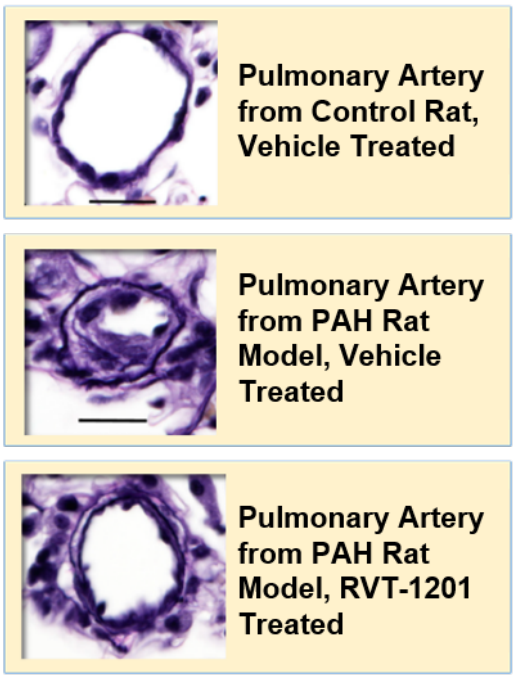

Figure 1: Photomicrographs comparing cross-sections of pulmonary arteries from the rodent monocrotaline model of PAH from either: healthy vehicle-treated, PAH-model vehicle-treated, and PAH-model treated with rodatristat ethyl (RVT-1201).

Altavant Sciences is pursuing the clinical development of rodatristat ethyl (the prodrug for the TPH inhibitor roditristat[4]) and has completed Phase 1 trials to characterize the acute safety and tolerability in healthy subjects. Data shows that once or twice daily repeat administration of rodatristat ethyl over 14 days is generally well-tolerated and reductions in urinary 5-HIAA are achieved that are comparable to those associated with reduction of vascular remodeling in animal PAH models.

A Phase 2a clinical trial to test the safety of rodatristat ethyl in PAH patients on stable standard of care medications for PAH has recently initiated. This study design was developed in light of the low potential for rodatristat ethyl to be involved in drug-drug interactions with other PAH medications, and because nonclinical studies in rodent PAH models have demonstrated at least additivity in efficacy endpoints when rodatristat ethyl is combined with tadalafil or ambrisentan.

In conclusion, many researchers and studies have linked serotonin to PAH at both clinical and preclinical levels. The emerging picture is that dysregulated serotonin through both receptor-mediated signaling mechanisms and post-translational modifications (serotonylation) is an important driver of the underlying pathobiology of PAH. The strong weight of evidence implicating serotonin in PAH and the nonclinical evidence for potential reversal of disease remodeling effects has led to the current clinical development program by Altavant Sciences for rodatristat ethyl in PAH.

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