Author’s response to reviews

Title: NTP42, a novel antagonist of the thromboxane receptor, attenuates experimentally induced pulmonary arterial hypertension

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Version: 1 Date: 12 Feb 2020

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February 12th, 2020

Dear Dr Hughes,

Further to your email of January 22nd, 2020 in which you enclosed the review of our submission - PULM-D-19-00758, we now wish to submit a revised version of the manuscript where we have addressed each of the Reviewers’ comments.
As per your email and Instructions to Authors for manuscripts at revision stage, the enclosures (electronic submission) include: (i) a “Response to Reviewers” document (uploaded as a separate document); (ii) two versions of the revised manuscript, where one has the changes Marked and the other one is Clean/unmarked; and (iii) each of the 6 manuscript figures. In addition, we also upload (iv) two versions of the revised Supplemental Data file, where one has the changes Marked and the other one is Clean/unmarked.

In making this resubmission on behalf of all authors, I trust that the revised version of the manuscript and point-by-point response to the two Reviewer’s comments provided in the “Response to Reviewers” document has adequately addressed the points raised by the Reviewers. However, should you need any additional information from me, I would be happy to assist in whatever way possible.

Yours sincerely and with kind regards,

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B. Therese Kinsella., Ph.D
Corresponding Author

Response to Reviewers:

Vinicio de Jesus Perez (Reviewer 1): 1. Figure 1: I disagree with the authors' impression that TPa and b is observed in SM cells. Staining of TPa and TPb seems to be absent from muscle and localized only to endothelial cells and possibly inflammatory cells. Did the authors inspect plexogenic lesions for their staining patterns? Based on this pattern, I would have elected to use the SuHx rat model given that endothelial injury is the major trigger in this model, whereas MCT is more dependent on SMC driven vascular remodeling. A combination of both models is usually expected for preclinical studies of this type.
Author Response: The Reviewer’s point is acknowledged with thanks. The authors accept that the representative images presented in the original submission made it difficult to discriminate the cell types in the panels in Figure 1, particularly associated with smooth muscle cells. To address the Reviewer’s concern, in the resubmission we show different representative regions obtained from the same histological specimens to more convincingly illustrate the expression, with corresponding commentary in the Results section. (Lines 245-262 in the marked-up resubmitted manuscript). We have inserted both macro images and magnified regions of interest to better highlight the patterns of immunostaining, including demonstrating expression of TPα and TPβ in the vascular smooth muscle. Specifically, within the revised Figure 1, magnified inset images, focussing on the pulmonary arterial walls, demonstrate expression of both TPα & TPβ within the vascular smooth muscle in both control tissue (Figure 1A(ii), 1A(iii) & 1A(v)) and PAH tissue (Figure 1B(ii), 1B(iii) & 1B(v)). To also address the Reviewer’s concerns raised in this point, we have also shown an example of staining for a representative plexogenic lesion in the new Figure 1B(iv), with corresponding commentary in the Results section.

The Reviewer’s point regarding the use of the Sugen 5416/hypoxia (SuHx)-induced PAH rat model is also acknowledged and accepted. While outside the scope of this current submission, investigations in additional preclinical models, including the SuHx-induced PAH model, are being completed and will be the subject of a follow-up manuscript. In the context of this current submission, this limitation of the study to a single preclinical model is noted and has previously been addressed within the Discussion section (Lines 511-533 in the marked-up resubmitted manuscript).

2. Figure 2: Did the authors attempt to do reversal studies? The studies presented are only designed to assess prevention of PH. This limits the impact of the data relative to the potential efficacy of the NTP42 for clinical PAH.

Author Response: The Reviewer’s point is acknowledged. Owing to the rapid progression of disease within the MCT-induced PAH model, this preclinical model is frequently best employed as a prophylactic- or early intervention- model where the test drug is administered either simultaneously with the MCT or within a short duration post-MCT induction, as was the case in this study. As noted in our response to Point 1 above, while outside the scope of this current submission, further investigations are ongoing in alternate preclinical models. This includes the SuHx-induced PAH model using conditions viewed as more reminiscent of a treatment model, rather than an early MCT-based intervention model, and once completed will be the subject of a follow-up manuscript. As stated, limitation of the current study to a single MCT-based preclinical model was addressed within the Discussion section (Lines 511-533 in the marked-up resubmitted manuscript).
However, to address the Reviewer’s query about disease treatment, in the revised manuscript we have included additional data from a second focussed MCT-PAH study, where this data is presented in Supplemental Figure 5 and addressed within the Discussion section (Lines 540-555 in the marked-up resubmitted manuscript). This second MCT-PAH preclinical study, carried out in Wistar-Kyoto rats, was specifically focussed on investigating the effect of NTP42 on cardiac hypertrophy, where NTP42 was delivered at 0.125 mg/kg QD within a delayed treatment protocol whereby the MCT-induced disease was allowed to develop for seven days prior to initiating drug-treatment.

In this second focussed study, NTP42 treatment significantly decreased the MCT-induced rise in the Fulton’s Index (Supplemental Figure 5A). In additional assessments of cardiac hypertrophy within this experimental cohort, RV tissues were stained with anti-CD31 antibody (Supplemental Figure 5B) which enabled autologous quantitation of both CD31 positive vascular endothelial cells, a measure of vascularization/angiogenesis, and also of cardiomyocyte cross-sectional area, a measure of cardiac hypertrophy. Thus, measurement of these two parameters (i) cardiomyocyte cross-sectional area and (ii) vascularisation per unit area provides a direct assessment of ‘metabolic demand’ and ‘metabolic supply’, respectively, within the RV tissue and when expressed as a ratio, can be used as a measure, so-called Metabolic Index, of ‘Maladaptive hypertrophy’ (where ratio of (i)/(ii) is \( \gt \) 1) or ‘Adaptive hypertrophy’ (where ratio of (i)/(ii) is \( \lt \) 1). In accordance with the Fulton’s Index (Supplemental Figure 5A), treatment with NTP42 led to a significant decrease in cardiomyocyte cross-sectional area (Supplemental Figure 5C). While treatment with NTP42 at this dose had no significant effect on RV vascularization per se (Supplemental Figure 5D), considering both RV hypertrophy and vascularisation together within a “Metabolic Index” parameter, treatment with NTP42 led to a significant increase in this indicative parameter of cardiac adaptation (Supplemental Figure 5E).

3. Why is the focus on mast cells alone? There is ample evidence that T cells and macrophages play a role in MCT induced PH. The authors should stain for these cells with validated markers (CD68, CD3, CD45 etc.). Also, they should use a validated marker of mast cell such as CD117 rather than toluidine.
Author Response: The Reviewer is indeed correct that there is growing evidence of the diverse role for inflammatory processes and cell-mediated immunity in the pathogenesis of PAH and indeed in other pulmonary conditions. Of the various immune cells that have been implicated in PAH, mast cells were among the first suggested to potentially play a key role in its pathophysiology, strongly contributing to vascular remodelling and leading to pulmonary fibrosis. Within the scope of this study, we have reported the effect of NTP42 treatment on mast cell recruitment and on the development of pulmonary fibrosis. The effect of NTP42 in reducing mast cell recruitment within the lung, and the possible resulting pulmonary fibrosis, is of particular note and demonstrates a unique benefit of TP antagonism in this regard, impairing both MCT-induced pulmonary inflammation and fibrosis.

In response to the Reviewer’s point regarding T cells and macrophages, we had previously investigated CD3 and CD68 immunostaining for these cell types, respectively, in a subset of lung samples from this study. We found no significant difference in CD3+ T lymphocyte density between the ‘No MCT’ and ‘MCT Only’ groups. This may be a consequence of the level of severity of the disease induced or, more likely, the stage or phase of the inflammatory response to the disease at the time of study termination and lung harvesting. Furthermore, with regards to CD68+ macrophages, while MCT treatment showed a significant increase in these cells, none of the treatments, including the standard-of-care (SOC) drugs Sildenafil or Selexipag, nor NTP42 led to statistically significant alterations in macrophage count in these animals. As noted within the Discussion section (Lines 462-464 in the marked-up resubmitted manuscript), while deemed beyond the scope of the current study, it will be of interest to explore how NTP42 may impact on other pathways and cell types of the innate and/or adaptive immune systems, such as through follow-up studies in alternate preclinical models or in more focussed studies specifically designed to address these parameters including as a function of stage of the inflammatory process.

The Reviewer’s concern regarding the use of Toluidine Blue staining is also acknowledged. In our studies, we elected to use Toluidine Blue as a stain for mast cells as it is a widely and validated cytological mast cell stain. While CD117 can be used as a marker of mast cells in certain tissues, it is not viewed as an adequate immune marker of mast cells in many tissues including the lung, colon, stomach, uterus, and bladder (Ribatti D. The Staining of Mast Cells: A Historical Overview. International archives of allergy and immunology 2018;176:55-60). However, to address the Reviewer’s concern, in the revised manuscript we have noted the limitation of solely using Toluidine Blue within the Discussion section of the submitted revision (Lines 464-467 in the marked-up resubmitted manuscript).
Fabrice Antigny (Reviewer 2): Mulvaney and colleagues have studied the consequences of thromboxane receptor pharmacological inhibition (NTP42) in Monocrotaline-induced pulmonary hypertension. Authors found that preventive NTP42 treatment reduced the MCT-induced PH similarly to Sildenafil treatment. Although the results are very interesting, however this manuscript requires additional experiments or precisions.

Major comments:

1-As demonstrated by the low increase of mPAP, RVSP as well as Fulton index, in these Wistar-Kyoto rats, MCT induce not severe PH but very moderate PH.

Authors should discuss this point or insert a limitation part in their study. Or perform this pharmacological approach (NTP42 treatment) in more severe animal PH (may be another strain of rats or younger Wistar-kyoto rats).

I'm not familiar with Wistar-kyoto rats. Wistar-kyoto developed less severe than Wistar or Spragues Dawley?

Author Response: The Reviewer’s point is acknowledged and accepted. While it is conceded that the level of the MCT-induced disease, as demonstrated by the low but albeit consistent increase in mPAP, RVSP and Fulton’s Index, may not be as severe when compared with some previously reported studies, including in Wistar-Kyoto rats (e.g. Ref. 35 in the marked-up resubmitted manuscript), it should also be acknowledged that the level of MCT-induced PAH disease can vary quite widely across species, strains and even individual animals or research centres. Thus, to address the Reviewer’s concern, we have extensively discussed this point as a possible limitation of our study (Lines 433-443 in the marked-up resubmitted manuscript). Furthermore, and as addressed in our response below to this Reviewer’s Point 3 and Point 4, future investigations of NTP42 efficacy in other animal models and/or through more-focussed preclinical studies are ongoing and may be the subject of additional manuscript submission(s).

2-Regarding the immunostaining presented in figure 1, it is very difficult to see the staining. Author should add more convincing staining!

Immunostaining is not quantifiable approach, and it could be very interesting to quantify the expression of TPa, TPb and IP by Western blot or Quantitative PCR at least.
Author Response: In accordance with Point 1 raised by Reviewer 1, this Reviewer’s point is also acknowledged, where it is accepted that the representative images chosen for the panels in Figure 1 made it difficult to clearly discriminate the cytological pattern of immunostaining. To address the Reviewer’s concern, in the resubmission we show different representative regions obtained from the same histological specimens to more convincingly illustrate the expression, with corresponding commentary in the Results section. (Lines 245-262 in the marked-up resubmitted manuscript). We have inserted both macro images and magnified regions of interest to better highlight the patterns of immunostaining, including demonstrating expression of TPα and TPβ in the vascular smooth muscle. Specifically, within the revised Figure 1, magnified inset images, fociussing on the pulmonary arterial walls, demonstrate expression of both TPα & TPβ within the vascular smooth muscle, in both control tissue (Figure 1A(ii), 1A(iii) & 1A(v)) and PAH tissue (Figure 1B(ii), 1B(iii) & 1B(v)).

We also accept the Reviewer’s point that immunostaining is not a quantifiable approach. The immunostaining for TPα and TPβ conducted herein specifically demonstrated abundant expression of both TPα and TPβ isoforms of the TP in the human lung, both in normal control and PAH disease tissues, as a means of positioning TP antagonism as a valid target for potential therapeutic intervention. Regarding the use of an alternative approach to quantify TPα, TPβ and IP expression in lung specimens from PAH patients and control subjects, we could only obtain a limited number of pre-sectioned lung tissue slides to enable histological staining but, despite repeated attempts from reputable clinical sources, we were unable to procure biospecimens amenable to western blotting or qPCR. Thus, in the current study, the authors have not attempted to quantify (or claim) any possible differences in TPα, TPβ or IP expression in PAH lung biospecimens relative to control specimens, but rather to solely illustrate widespread expression of the TP isoforms in numerous cells type of the lung, surrounding vasculature and inflammatory infiltrate, thereby providing evidence that the TP may be a viable target for pharmacological intervention including for PAH.

3-Authors should also measure (thermo-dilution) or evaluated (echocardiography) the cardiac output. These experiments will determine if RV hypertrophy is adaptive or maladaptive?
Author Response: The Reviewer’s point is acknowledged. In the current study, echocardiography or acquisition of the haemodynamic parameters required to estimate cardiac output were not included as measurable parameters within the study protocol. However, to address the Reviewer’s point on experimentation to determine if the RV hypertrophy is adaptive or maladaptive, in the revised manuscript we have included additional data from a second more focussed MCT-PAH study, where this data is presented in Supplemental Figure 5 and is addressed within the Discussion section (Lines 540-555 in the marked-up resubmitted manuscript). This second MCT-PAH preclinical study, carried out in Wistar-Kyoto rats, was specifically focused on investigating the effect of NTP42 on cardiac hypertrophy, where NTP42 was delivered at 0.125 mg/kg QD within a delayed treatment protocol whereby the MCT-induced disease was allowed to develop for seven days prior to initiating drug-treatment.

In this focussed study, NTP42 treatment significantly decreased the MCT-induced rise in the Fulton’s Index (Supplemental Figure 5A). In additional assessments of cardiac hypertrophy within this experimental cohort, RV tissues were stained with anti-CD31 antibody (Supplemental Figure 5B) which enabled autologous quantitation of both CD31 positive vascular endothelial cells, a measure of vascularization/angiogenesis, and also of cardiomyocyte cross-sectional area, a measure of cardiac hypertrophy. Thus, measurement of these two parameters (i) cardiomyocyte cross-sectional area and (ii) vascularisation per unit area provides a direct assessment of ‘metabolic demand’ and ‘metabolic supply’, respectively, within the RV tissue and when expressed as a ratio, can be used as a measure, so-called Metabolic Index, of ‘Maladaptive hypertrophy’ (where ratio of (i)/(ii) is &gt; 1) or ‘Adaptive hypertrophy’ (where ratio of (i)/(ii) is &lt; 1). In accordance with the Fulton’s Index (Supplemental Figure 5A), treatment with NTP42 led to a significant decrease in cardiomyocyte cross-sectional area (Supplemental Figure 5C). While treatment with NTP42 at this dose had no significant effect on RV vascularization per se (Supplemental Figure 5D), considering both RV hypertrophy and vascularisation together within a “Metabolic Index” parameter, treatment with NTP42 led to a significant increase in this indicative parameter of cardiac adaptation (Supplemental Figure 5E). In combination with the collection of echocardiograms and the determination of cardiac output parameters in future preclinical investigations, these additional approaches should add significantly to the data already generated in this study.

4-Why NTP42 treatment had no consequence on RV remodeling while mPAP is reduced?

Thromboxane receptor are expressed in RV? TP expression are modified in RV from MCT animals? TP inhibition on RV cardiomyocytes could have pro-hypertrophic consequence?
Author Response: As discussed in the response to Point 3 above, Fulton’s Index was the only readout of RV remodelling collected within the scope of this study’s design. By its nature, a lack of change in Fulton’s index is not necessarily an issue as hypertrophy is a necessary, adaptive response of the right heart to work harder due to an elevated pulmonary arterial pressure. While this study has demonstrated that NTP42 reduces mPAP, a detailed evaluation of cardiac structure and function is warranted, such as in follow-up studies as discussed in the response to Point 3 above. However, to address the Reviewer’s point, and as discussed in the response to Point 3 above, we have now elected to include additional data from follow-up MCT-PAH preclinical investigations demonstrating that treatment with NTP42 leads to a significant attenuation of RV remodelling. In addition, to address the Reviewer’s secondary points, as noted in the Discussion section (Lines 407-413 in the marked-up resubmitted manuscript), the TP is indeed expressed in human RV tissue and has already been documented in the literature to be significantly elevated in the RVs from PAH patients compared to non-diseased controls (e.g. Ref 31 and Ref 34 in the marked-up resubmitted manuscript).

5-NTP42 results are similar to the results obtain with sildenafil. I could be very interesting to combine NTP42+sildenafil treatment or use NTP42 in curative treatment.

Author Response: The Reviewer’s point is of course accepted. Use of NTP42 in combination with Sildenafil is, in part, a major focus of an ongoing study employing a different pre-clinical model of PAH. As addressed extensively within the Discussion section of this resubmission (Lines 555-562 in the marked-up resubmitted manuscript), bearing in mind the current PAH treatment guidelines it will be of key importance to assess the efficacy of NTP42 alongside other SOC therapies, including not only the PDI Sildenafil, but also prostacyclin analogues (e.g. Selexipag) or members of the ERA class of PAH therapies.

6-How authors explain the Selexipag results (Fulton index)?

Author Response: The Reviewer is correct in highlighting the Fulton Index data for the Selexipag group. As benefits for Selexipag have been previously reported (e.g. Ref. 36 in the marked-up resubmitted manuscript), we surmise that the lack of benefit for Selexipag in reducing the Fulton’s Index, and indeed in significantly reducing the RVSP, may be a consequence of the moderate level of the PH disease induced in our current study. In addition, it is indeed noteworthy that the Selexipag animal group size was lower than the other treatment groups investigated, which may have had an effect on the overall findings including on statistical powering. In accordance with our response to this Reviewer’s Point 1, in the revised manuscript we have highlighted and discussed the Selexipag findings in the context of these two limitations (Lines 437-443 in the marked-up resubmitted manuscript).
6-The introduction is too long! However, authors should add the actual new definition of PAH, and add additional paper to support their rational

Author Response: Within the revised manuscript, we have shortened the Introduction section. As the Reviewer correctly points out, during the recent World Symposium on Pulmonary Hypertension, the haemodynamic definition of PAH was revised from mPAP ≥ 25 mmHg to 20 mmHg, and this definition has been included along with the appropriate references (Lines 60-61 in the marked-up resubmitted manuscript).

7-The discussion is too too long. Please reduce.

Author Response: The Reviewer’s point is acknowledged. To address this point, we have shortened the Discussion section within the revised manuscript. However, due to our efforts to adequately address the specific comments raised by both Reviewers, we have also had to include additional text but, where possible, striving to keep these as brief as possible.