Author's response to reviews

Title: Characterization of a murine model of monocrotaline pyrrole-induced acute lung injury

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Author's response to reviews: see over
Dear Editor,

Please find our revised version of the manuscript entitled “Characterization of a murine model of monocrotaline pyrrole-induced acute lung injury”.

We provide here comments point-by-point in response to the reviewers concerns.

Reviewer: Pravin B Segal:

A. Major Concerns:

1. As the authors state, because MCTP is known to have a short half-life in aqueous buffers (approximately 3 sec), the exact manner of instillation into the animal is critical. Thus, the comparison between MCT (administered subcutaneously) and MCTP (administered intra jugular vein in DMF as solvent) in producing PAH in the rat is critical in order to validate the technique used (pg. 9 top and Fig. 1). The text on pg 9 states that the two were “comparable”. However, the data in Fig. 1 showing RVH are missing statistical comparison between the MCT and MCTP groups. From the data in Fig. 1 it appears that MCTP as used in this study was not as effective as MCT. This is an issue that must be addressed. Moreover, the text on pg. 9 contains data for RVSP but the Figure does not. Please include RV systolic pressure data in Fig. 1 and do statistical comparisons between the MCT and MCTP groups not just with the DMF controls.

Answer:
Thank you for the very good comment. Indeed, the exact metabolism of MCT and MCTP in mouse and rat is not clear. To address experimentally the MCTP half-life is technically difficult and therefore we overcome this issue by assessing the effects of this compound applied intravenously in rats-animal specie known to be lung sensitive in response to MCT. We also applied MCT in rats classically by subcutaneous injection. Both, MCTP i.v. and MCT s.c. resulted in rats in pulmonary hypertension, demonstrating intravenous application is appropriate to deliver MCTP. MCTP effects in rats proof the concept that MCTP is the active monocrotaline metabolite and our chemically synthesized MCTP is similar to that biologically synthesized. Our aim was in this experiment to compare the type of effects. The degree can not be compared since the two different doses are not equipotent (5mg/kgMCTP versus 60mg/kgMCT). A hypothetical increase of the MCTP dose would probably induce stronger right heart hypertrophy and more severe pulmonary hypertension, but our aim was not to characterize MCTP-induced pulmonary hypertension in rats.

We included the RV systolic pressure data in the figure 1 and performed text changes as follow:

-in the results section:
“Injection of monocrotaline pyrrole (MCTP) in rats..."
Subcutaneous injection of MCT in Spraque Dawley rats at a dose of 60 mg/kg resulted after 4 weeks in dramatic increase of right ventricular systolic pressure (76.87 ± 4.87 versus 25.08 ± 1.35 for control, *p<0.05) and severe right heart hypertrophy (0.64 ± 0.01 versus 0.30 ± 0.01 for control, *p<0.05). The chemically synthesized MCTP was dissolved in dimethylformamide and injected intravenously in rats at a dose of 5mg/kg. This led after 4 weeks to elevated right ventricular systolic pressure (59.60 ± 2.93 versus 25.08 ± 1.35 for control, *p<0.05 as compared to control) and significant right heart hypertrophy (0.47 ± 0.02 versus 0.30 ± 0.01, *p<0.05 as compared to control). (Figure 1 A and B). Both parameters, right ventricular pressure and right heart hypertrophy induced by MCTP injection were lower than those induced by MCT injection.”

-in the discussion section:
“…A positive control experiment included the injection of the synthesized MCTP into rats. This confirms MCTP as being the active metabolite of MCT and demonstrates the efficacy of chemical synthesis. The difference in pulmonary hypertension degree induced by MCTP and MCT correlate with the different bioavailability and dose difference (5 mg/kg for MCTP versus 60 mg/kg for MCT)…”

2. That the particular route of administration intrajugular (in the rat or mouse) delivers a single rapid bolus as opposed to sc MCT in the rat which leads to, in essence, a slow constant release of MCTP from the liver over several hours and perhaps the first day, must be discussed as a limitation of this study. Someone in the future could perhaps use a long-lasting pump device to administer MCTP into the mouse jugular vein slowly over several days and obtain different results. Please discuss (also see item 4 below)"

Answer:
A single rapid bolus is expected to induce acutely strong effects which might not be similar to those of slow constant release. Unfortunately slow intravenous infusion of MCTP in mice is technically limited. However, when injected into rats in rapid bolus MCTP induced pulmonary hypertension. The slow release after subcutaneous injection in rats also resulted in pulmonary
hypertension. Both ways of application in mice did not result in significant pulmonary hypertension. We introduced this issue in discussion as follow:

…”A technical limitation might be related to the way of MCTP application, respectively bolus injection of MCTP versus slow release after subcutaneous MCT injection. However, bolus injection of MCTP in rats results in pulmonary changes which consistently differ from those induced in mice.”…

3. Please carry out mass spec analyses to indicate how much of the MCT was converted to MCTP in the samples used in this study. Also please review the text throughout to fill in the exact concentrations of MCTP injected – many places are left as “9 g/kg body weight” (see pg. 4 for one example)”

Answer: Chemical synthesis of MCTP from MCT was described, analyzed and discussed in detail by Mattocks et al 1989; Toxicon, as referred in our manuscript. We prepared MCTP accordingly. However, to demonstrate efficacy of the chemical synthesis we performed Thin Layer Chromatograms. This revealed high purity of the chemically synthesized MCTP. We corrected the exact MCTP injected concentrations

4. A major issue in this MS is that MCTP administration into the jugular vein of mice produces acute lung injury but not the slower developing PAH. Does MCTP cause “megalocytosis” of murine endothelial cells? This is a simple experiment to carry out in cell culture and would serve as an important positive control for the efficacy of MCTP on mouse cells compared to say rat or human or bovine PAECs”

Answer: The MCTP induced changes in the mouse lung were more related to lung parenchyma. Vascular changes were less impressive and limited in the early phase to some perivascular oedema. The suggested experiment is theoretically simple and ideal to point out the role of endothelium on the interspecies difference in this context. Unfortunately we are limited in performing this experiment by the lack of a murine endothelial cell line. We introduced this issue in the discussion as follow:

…” Our findings demonstrate that interspecies differences in response to monocrotaline and lung diseases are not limited to the liver metabolism of these alkaloids. Future experiments assessing mouse and rat endothelial cells in response to MCTP might elucidate the interspecies variability”...
B. Minor issues:
"Please revisit the lay-out and graphics in all of the line drawing figures. At least in my printed copy, these were not particularly esthetic."

Answer:
This must be a technical issue. Our submitted figures including histograms are accurate. However, we are ready to replace or improve them.

"Please correct typos on pg. 7 – 4th and 3rd line from bottom".

Answer:
We included the adequate changes.

In the future please do not upload a cover letter for Am J Resp Crit Care Med (dated March 21, 2008) when sending the MS to another journal (in this case BMC Pulmonary Medicine).

Answer:
We regret and apologize this event and hope that manuscript revision will not be affected.

Reviewer: Yoshihiro Fukumoto

“This manuscript shows that monocrotaline pyrrole induces acute lung injury in mice, which might be useful as a model of acute respiratory failure and fibrosis. Monocrotaline pyrrole can induce pulmonary hypertension in rats but not in mice. Although this is an interesting paper, there are some minor problems. The aim of the present study was to establish a murine model of pulmonary hypertension; however, monocrotaline pyrrole induced acute lung injury in mice. Moreover, the most of results are from mice of acute injury, although monocrotaline pyrrole induces pulmonary hypertension in rats. Overall, the context is confusing. The authors should write more clearly regarding the differential effects of monocrotaline pyrrole on mice and rats. Also, the authors should show more data of rat MCTP model."

Answer:
Our study represents an attempt to establish a murine model of pulmonary hypertension. It is well known that mice are somehow protected against monocrotaline-induced pulmonary hypertension and it's been suggested that this is due to defective metabolism of these compounds. Therefore we synthesized chemically the active metabolite-MCTP. MCTP injection in mice resulted in acute lung injury, while MCTP injection in rats resulted in pulmonary hypertension. On the other hand, MCT injection in rats results in severe pulmonary hypertension, while in mice results in some lung inflammation and some fibrotic changes. A school of thought explain this interspecies difference by insufficient metabolism of MCT in mouse liver to the active compound MCTP. Different responses to the active metabolite-MCTP in mice and rats are in contradiction to this theory and our study represents
evidence that significant differences are somewhere at the lung level, most likely at the endothelium. We introduced this issue in discussion section as follow:

…” Our findings demonstrate that interspecies differences in response to monocrotaline and lung diseases are not limited to the liver metabolism of these alkaloids. Future experiments assessing mouse and rat endothelial cells in response to MCTP might elucidate the interspecies variability”...

We added RVSP data to the rat MCTP model.

Page 2, para 1, line 1.
“Idiopathic pulmonary hypertension (IPAH)” should be “Idiopathic pulmonary arterial hypertension (IPAH)”. We included the adequate change

Results section:
Page 9, para 1.
The authors should include the data of right ventricular systolic pressure in Figure 1
Figure 1. What is “Dehydromonocrotaline”? MCTP?

Answer:
MCTP is often referred in the literature as “dehydromonocrotaline”. For simplicity we performed the suggested change.

Figures 1, 2, 4, 5-7. The authors should include the number of animals. There are some extra blanks in the text, such as “5 g/kg”.

We included the adequate changes.

All authors have read and approved submission of the manuscript and the manuscript has not been published and is not being considered for publication elsewhere in whole or part in any language except as an abstract.

Yours sincerely,

Ralph Schermuly, PhD