Pulmonary defense mechanisms and their potential role in the prevention of thiourea-induced lung toxicity: histopathological and ultrastructural assessment

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INTRODUCTION

- In rodents, acute toxicity of thiourea and its derivatives is characterised by massive pleural effusion and severe pulmonary oedema and has become a useful model to investigate acute lung injury .
- S-oxygenation of thioureas, mediated by flavin-containing monooxygenases (FMOs), leads to the formation of a reactive metabolite (sulfenic acid) which reacts with glutathione (GSH), undergoes redox cycling and binds to other sulphydrylic groups causing oxidative stress and covalent binding (1) (Fig.1).
- Rats quickly become resistant to the lethal effect of thiourea-related molecules after initial exposure to small, sublethal doses. Pulmonary cell hyperplasia has been hypothesised as the underlying mechanism (2).
- Investigations on thiourea-mediated resistance in rats may bring new insight into new potential therapy for adult respiratory distress syndrome, a common life-threatening lung condition in humans characterised by interstitial and alveolar pulmonary oedema.

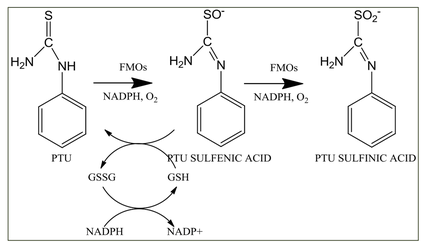

Figure 1. FMO-dependent redox cycling of phenylthiourea (PTU) in the presence of GSH

- The aims of this pilot investigation were to characterise the microscopic and ultrastructural changes induced by a proprietary small phenylthiourea (PTU) derivative in the lung of resistant rats and determine whether pulmonary defence mechanisms are involved in the protection from acute injury .

STUDY DESIGN

- Preliminary studies with high doses of a proprietary small phenylthiourea derivate (PTU) were conducted on Wistar rats to assess the lung by light microscopy and select a resistance-inducing dose.
- In the tolerance study rats were given the protective low dose (LD), alone or in combination with an otherwise lethal high dose (HD), and were assessed at different endpoints (Fig.2). Rats receiving the LD or LD+HD were re-challenged on day 14 with the HD to determine the duration of thiourea-induced resistance.
- A complete morphological work-up (gross and light microscopical examination including immunohistology, transmission electron microscopy) was performed.
- Immunohistology aimed to fully characterise the cell types populating the alveolar unit (Fig.3), and to determine the presence of apoptotic (cleaved caspase 3-positive) and proliferating (PCNA-positive) cells.
- Hepatic and pulmonary glutathione (GSH) levels were measured in each animal.

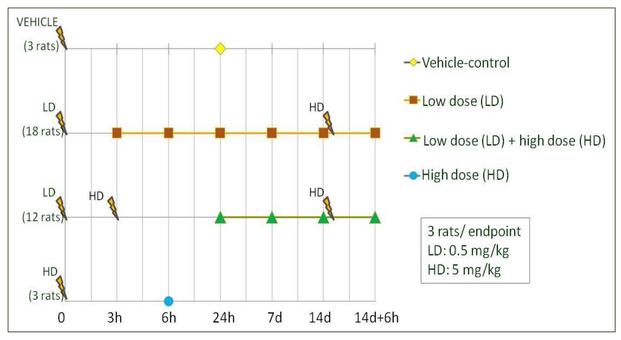

Figure 2. Experimental design

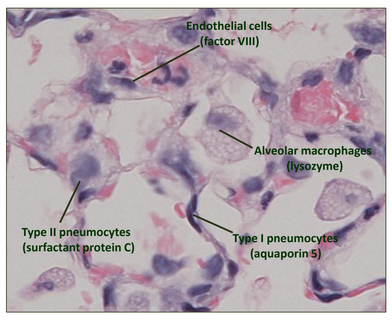

Figure 3. Alveolar cells and, in brackets, immunohistological stains used for their identification and quantification

SUMMARY and CONCLUSIONS

- PTU, when given to Wistar rats at lethal doses (5mg/kg), is a potent selective pulmonary toxicant causing acute respiratory distress, associated with severe hydrothorax and pulmonary oedema.
- Low, non lethal doses are able to protect the lung from the effect of subsequent high (lethal) doses as early as 3hours post low dosing, as demonstrated by the decreased incidence and severity of clinical signs, macroscopic and microscopic changes in this tolerance study.
- Thiourea-induced damage is morphologically characterised by increased vascular permeability (alveolar and interstitial oedema) and mild pneumocyte death (detected only in rats given the HD alone) in the first 24 hours, together with an increase of alveolar macrophages evident as early as 3 hours post dosing. Type II pneumocyte hyperplasia is a later event and was demonstrated in rats at day 7, suggesting its onset on day 2 at the earliest (animals were not examined between 24 hours and 7 days post dosing in this study).
- Previous studies suggested that endothelial cells of the pulmonary capillary network are a primary target of the drug (3). We confirmed these findings and can demonstrate endothelial cytoplasmic blebs and gap formation at the site of intercellular junctions, without evidence of cell death, suggesting that reversible endothelial contraction may be responsible for the observed increased vascular permeability.
- Glutathione (GSH) depletion is a key event in the establishment of thiourea-mediated oxidative stress (1) and pulmonary GSH appears to concentrate mostly in endothelial cells rather than in pneumocytes (4). There is evidence that GSH depletion may lead to increased endothelial contractility and/or cytoskeletal rearrangements (5), which could explain the severe pulmonary oedema that develops with PTU.
- Our results do not provide evidence that type II pneumocyte hyperplasia is responsible for resistance through increased clearance of extravasated fluid as previously suggested (6), since proliferating pneumocytes were not seen up to 24 hours after dosing, while resistance is already effective 3 hours after the administration of the protective dose. Instead, alveolar macrophages are immediately activated and recruited in resistant rats and are more likely to be responsible for clearance of excess fluid.
- Endothelial cells are the earliest target of PTU toxicity, with a subsequent secondary involvement of pulmonary epithelial cells. Their role in the induction of resistance needs to be further investigated.

References: (1) Henderson et al, Chem Res Toxicol. 2004; 17(5):633-40. **(2)** Barton et al, Toxicology. 2000; 143(2):167-81. **(3)** Cunningham et al, J Pathol. 1972;106(1):25-35 **(4)** Hardwick et al, Biochem Pharmacol. 1990; 39(3):581-9. **(5)** Ford at al, Free Radic Biol Med. 2006; 40(4):670-8. **(6)** Guery et al, Am J Respir Crit Care Med. 1997;155(5):1777-84.

RESULTS

- All rats given the HD were electively euthanised starting at 6 hours after dosing due to severe respiratory clinical signs that correlated macroscopically with severe hydrothorax and microscopically with marked alveolar and interstitial pulmonary oedema (Figs. 4-6).
- Rats receiving LD+HD (HD at 3 hours post LD) developed milder clinical signs and had less severe hydrothorax with only mild pulmonary oedema.
- A low degree of pneumocyte death was detected in rats given the HD (Fig. 6), both on days 1 or 14, together with alveolar oedema. Dying cells were mainly shown to undergo apoptosis based on their expression of cleaved caspase 3 (Fig. 7). Staining for aquaporin 5 to highlight type I pneumocytes did not identify any changes in this cell population.
- An increase in the number of alveolar macrophages (lysozyme-positive) was observed in treated groups as early as 3 hours after dosing and reaching the highest intensity at 24 hours (Figs. 6, 8 and 11b).
- Alveolar septa appeared thickened in rats given the LD or LD+HD and euthanised on day 7. This correlated with a statistically significant increase in type II pneumocytes (SPc-positive; Figs. 9a and b, 11a) and was shown to be associated with cell proliferation, since several cells lining the alveolar walls expressed the proliferation marker PCNA (Fig. 9a, inset).
- There was no evidence of damage to endothelial cells in any of the groups including those that had received the HD, as confirmed by both light microscopy (including staining for Factor VIII) and transmission electron microscopy. However, there was ultrastructural evidence of cytoplasmic bleb and gap formation in animals receiving the HD (Fig. 10a and b).
- There was a statistically significant decrease in the glutathione content in liver and lungs of rats given the HD on day 1 or with re-challenge after 14 days (Figs. 12a and b).

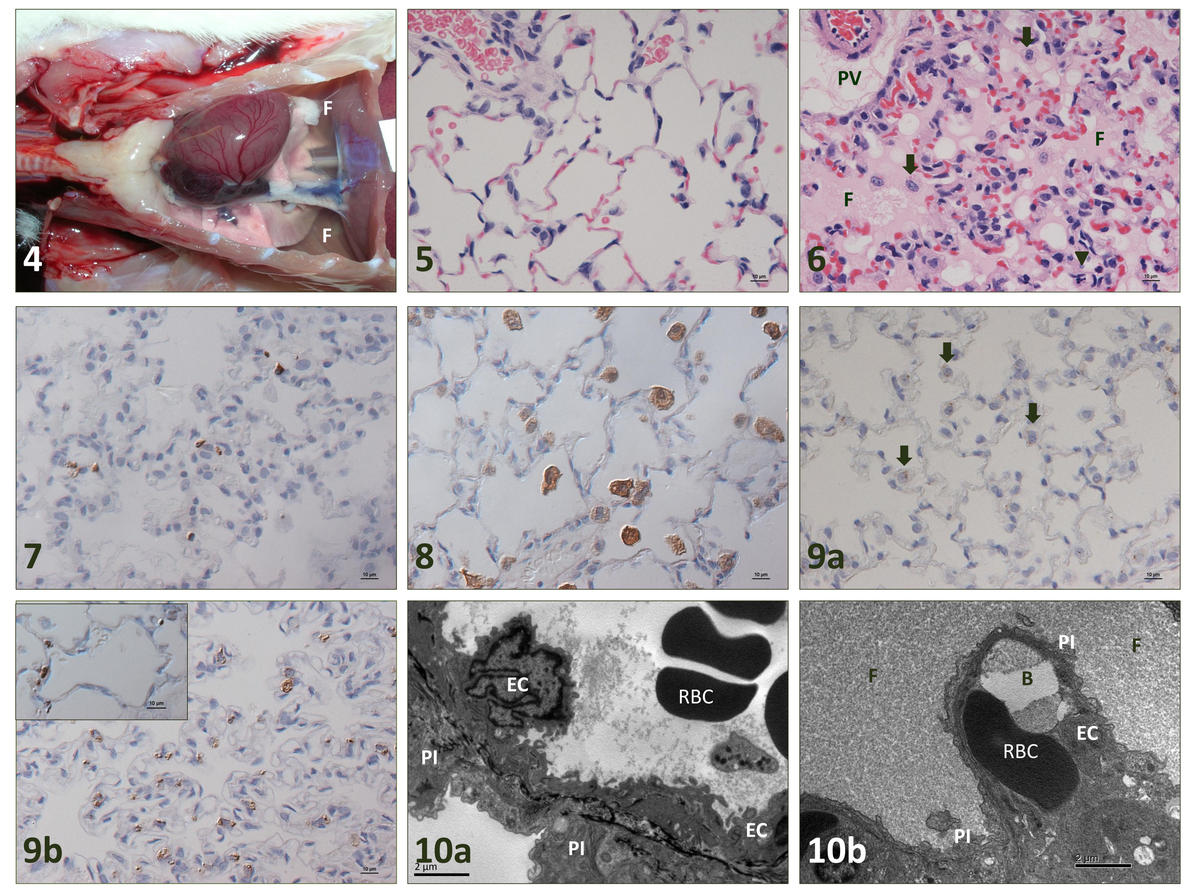

Figure 4. HD, 6 hours. Clear fluid (F) in thoracic cavity. **Figures 5-10**. Lung. **Figure 5**. Control rat, 24 hours. Thin alveolar septa. **Figure 6**. HD, 6 hours. Alveoli contain abundant eosinophilic proteinaceous fluid (F) and scattered large foamy macrophages (arrows). Perivascular spaces (PV) appear distended (interstitial oedema). Alveolar septa are distended and disorganised. Occasional dying pneumocytes (arrowhead) are detected. **Figure 7**. HD, 6 hours. Pneumocyte death is via apoptotis, as indicated by the expression of cleaved caspase 3. **Figure 8**. LD+HD, 24 hours. Alveolar spaces contain numerous intensely lysozyme-positive large alveolar macrophages. **Figure 9**. Identification of type II pneumocytes by staining for SPc. **a.** Control rat. Scattered positive cells are seen along alveolar walls (arrows). **b**. LD, day 7. Type II pneumocytes are more numerous and show more intense SPc expression. **Inset**: scattered cells lining the alveolar walls express the proliferation marker PCNA. **Figure 10.** Ultrastructural features. **a.** Control rat. Normal endothelium (EC). **b**. HD, 6 hours. Endothelium (EC) has separated from alveolar wall to form a bleb (B) projecting in the capillary lumen. Alveoli are filled with proteinaceous fluid (F). PI: type I pneumocyte. RBC: red blood cell.

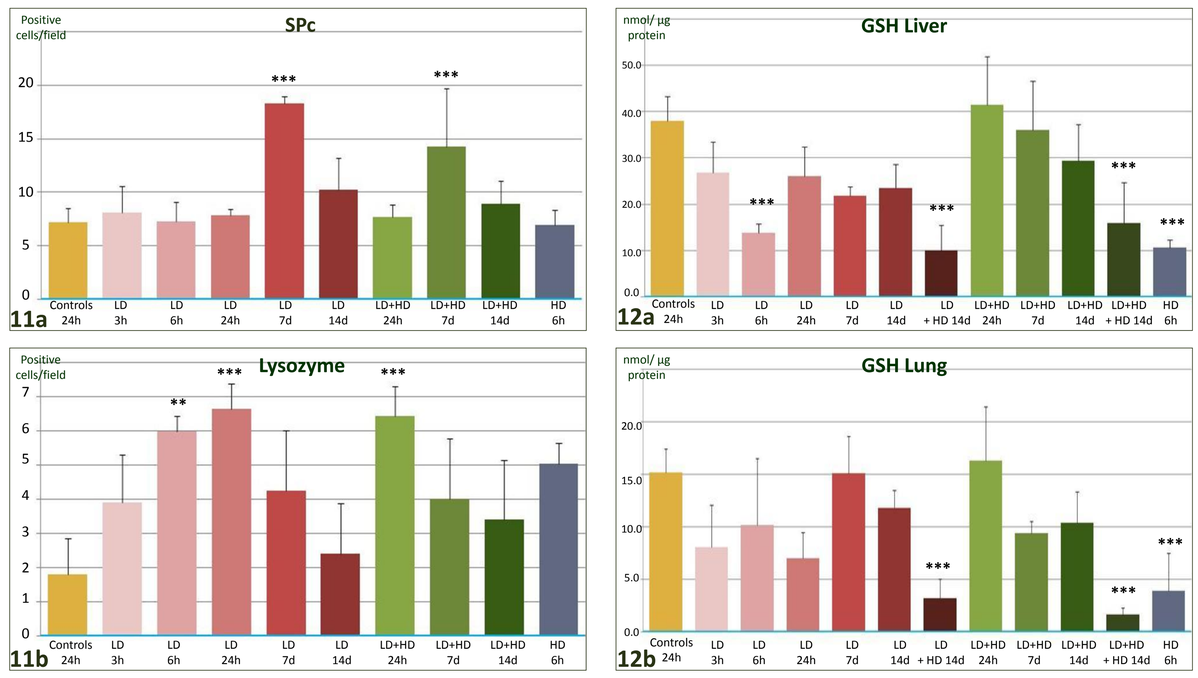

Figure 11. Average score of alveolar cells expressing SPc (type II pneumocytes; **11a**) and lysozyme (alveolar macrophages; **11b**), based on immunohistological stains. Scores are expressed as the average percentage of positive cells of the total cell number per high power field (10 fields/rat).
Figure 12. Levels of glutathione (GSH) in the liver (**12a**) and lung (**12b**), expressed as nmol/ µg of protein.