AIM
To develop a 6 week sub-chronic rat inhalation protocol, with an ex vivo genotoxicity endpoint, which aims to reduce & refine animal use.

INTRODUCTION
- Guidelines for the provision of data for genotoxicity in mammalian cells have been re-defined identifying that an ex vivo study undertaken at the same time as in vivo work is preferable to a second in vitro study in mammalian cells.
- We have explored the potential of the Comet assay to quantitate DNA damage in isolated rat lung alveolar type II epithelial cells (AEC II). AEC II were selected as they are hypothesized to be a target of cigarette smoke (CS) exposure in the lung.
- A 6 week inhalation study protocol will enable data to be available more quickly and may provide sufficient information for British American Tobacco (BAT) to support the use of an ingredient or cigarette technology.
- 30 day inhalation studies would be required when needed for regulatory purposes.

METHODS

- Inhalation protocol
  - Sprague-Dawley rats (5 wk) were supplied by Charles River Laboratories.
  - N = 16 female & 16 male rats, were exposed to 8.8 mg/L CS from 35% reference cigarette (University of Ferrara) or sham air for 6 weeks.
  - The rats were weaned at 10 days to determine CS exposure (AEC II).
  - Lung lobes were processed for AEC II isolation (light) or histopathology (light).

- Isolation of AEC II
  - AEC II were isolated from the right lung lobe using published methods with some modifications:
    - Perfused with 0.15 M NaCl (pH 3.0) at 37°C.
    - Filtrated with 0.45 µm syringe filter before cell viability (35% ± 85% for 15 mm).
    - Percoll was used to 1 mm and filtered through a 150 µm nylon filter (50%)
    - Survival was determined using a Nucleaer stain.

- Identification of AEC II by alkaline phosphatase staining
  - Isolated cells were centrifuged for 5 minutes (300 x g) using a Cytospin (Thermo Scientific) and fixed at room temperature (RT) for 15 hours.
  - Slides were incubated with alkaline phosphatase as previously described, washed with PBS and subsequently counterstained with 1% Mayer's hematoxylin.
  - Cells were counted using light microscopy (x1000) and percentage AEC II calculated.

- Histopathology
  - Left lungs were fixed with 4% neutral formalin and processed.
  - Longitudinal paraffin sections (5 µm) were stained with haematoxylin-eosin or stain block blue and Schiff's reagent.

RESULTS

- AEC II purity & Comet analysis
  - The AEC II in lung lysate was 56.3 - 63.0%.
  - AEC II viability was 95.2 - 99.8%.
  - Mean tail DNA (Tails) were increased following 1 h and 2 h 3MEF CS exposure (Table 1 and Figure 1).
  - There was no difference in % tail DNA between 1 h and 2 h CS exposure (Table 1).

- Histopathology
  - Unprotected macrophages & pigmented macrophages (Figure 2) were increased following exposure to 1 h 3MEF CS and 2 h 3MEF CS (Table 2).
  - Unprotected macrophages and pigmented macrophages were significantly following 2 h 3MEF CS exposure when compared to 1 h 3MEF CS (Table 2).

CONCLUSIONS
- We have developed methods for AEC II isolation & the ex vivo Comet assay which may have potential to use DNA damage resulting from CS exposure.
- The protocol developed reduced animal number by up to 40%, as separate lobes were used for Comet or histopathological analysis.
- This supports a strategy for the reduction & refinement of animal use in product testing.

ACKNOWLEDGEMENTS
- All experiments were performed at Viventecia.
- Histopathological analysis & imaging was performed at Histologic GmbH.

REFERENCES