Introduction
Silthiofam, a fungicide used primarily as a seed treatment for wheat, caused a slight increase in liver tumour incidence in a two-year rat feeding study. The mode of action (MOA) and human relevance of these tumours were previously evaluated in a 2-week rat dietary study and in vitro studies in wild type (WT) male rat and male human hepatocytes. The results from these studies suggested that the MOA for the hepatic effects was via the activation of constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR) nuclear hormone receptors and that the proliferative response noted in rats is not relevant to humans. Additional studies using CAR/PXR double knockout (CARKO/PXRKO) rat hepatocytes and a female human donor have now been conducted to determine if CAR/PXR activation was essential to the proliferative response and to further evaluate the human relevance.

Methods
Hepatocytes isolated from male WT or CARKO/PXRKO rats (Horizon Discovery, Boyertown, PA.) and cryopreserved male and female human hepatocytes were cultured in the presence of silthiofam for 96 hr at concentrations up to and exceeding those that induced substantial cytotoxicity.

Cell proliferation (S-phase) was evaluated using BrdU incorporation with epidermal growth factor (EGF) included as a positive control. CAR and PXR activation were assessed indirectly by evaluating mRNA expression and enzyme activity for CYP2B and CYP3A4. Enzyme assays consisted of pentoxysorurin-O-depentylation (PROD, CYP2B), benzoyloxyresorufin-O-depentylation (BROD, CYP2B) and benzoyloquinoline-O-debenylation (BQ, CYP3A4). Phenobarbital sodium salt (PB) was included for reference.

Values are Mean ± SD (n = 3 for enzyme assays and mRNA analysis, n = 5 for S-Phase). Number in parentheses corresponds to respective DMSO control. A Student’s t-test (2-sided) was performed on the results; * statistically different from control p<0.05; **p<0.01, ***p<0.001.

Results
Cytotoxicity (>20% reduction in ATP) was noted at silthiofam concentrations of ≥100 µM with WT rat and and human hepatocytes, ≥300 µM in CARKO/PXRKO rat hepatocytes and ≥30 µM in human female hepatocytes.

In WT rat hepatocytes, silthiofam increased PROD, BROD and BQ enzyme activities (~2-5X); CYP2B1, CYP2B2 and CYP3A1 mRNA expression (~11-97X); and cell proliferation (~2X).

Exposure of CARKO/PXRKO rat hepatocytes to silthiofam resulted in a slight increase in CYP2B1 mRNA expression (~5X) but no increase in CYP2B2 or CYP3A1 expression or PROD, BROD or BQ enzyme activity. Most importantly, there was no increase in cell proliferation.

Weak CAR and PXR activation, indicated by increases in CYP2B6 and CYP3A4 mRNA expression (~2-5X), was observed in male and female human hepatocytes exposed to silthiofam; but no increase in cell proliferation was observed (only female data shown, male data previously reported).

Conclusions
• Treatment of WT rat hepatocytes with silthiofam resulted in the induction of CYP2B1, to a greater extent, CYP3A4 at both the protein enzyme activity level and the gene expression level.
• Treatment of WT rat hepatocytes with silthiofam resulted in a significant increase in cell proliferation, however, no increase in cell proliferation was observed in either CARKO/PXRKO rat hepatocytes or male or female human hepatocytes.
• Together with the findings from the previously reported two week rat dietary study, these results provide strong evidence that the mode of action for silthiofam-induced cell proliferation in rats is CAR and/or PXR-mediated.
• Therefore, in the absence of increased cell proliferation in human hepatocytes, the increased incidence of liver tumours in the previous two year silthiofam rat study is not considered to be of human relevance.