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Dose-Dependent Effects on Rat Liver miRNAs 200a/b and 429: Potential Early Biomarkers of Liver Carcinogenesis

Abstract# 2455

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Introduction

An increased incidence of liver tumours in the long term rodent bioassay is not an uncommon finding, invariably as a result of a non-genotoxic mode of action. Non-genotoxic liver carcinogenesis has been found to involve activation of certain nuclear hormone receptors (NHR) including the constitutive androstane receptor (CAR), and more recently the induction of specific microRNAs (miRs), has also been demonstrated in studies up to 90days (Koufaris *et al.*, 2012). The stable induction of these tissue specific miRs, namely miR200a, 200b and 429, by liver non-genotoxic carcinogens may serve as early predictors (biomarkers) of heptocarcinogenic potential. To test this hypothesis we used RT-PCR to measure the levels of these miRs in the livers from Wistar rats treated with one known rat hepatocarcinogenic and two non rat-hepatocarcinogenic pyrazole carboxamide succinate dehydrogenase inhibitors (SDHI1, SDHI2 and SDHI3, respectively). The miRs were quantified by RT-PCR in liver RNA samples from three 90 day repeat dose toxicity studies (SDHI1,2 and 3) at the low, mid and high doses relative to control.

Need for an early biomarker that reflects NGC potential/potency.

CAR activation is considered an initiating event in the NGC process, (Elcombe *et al.*, 2014), however not all CAR activators are hepatocarcinogenic in long term rodent bioassays (Rignall *et al.*, 2013). Hence CAR activation is necessary but not sufficient for hepatocarcinogenesis and certain CAR activators are more potent hepatocarcinogens than others. Thus biomarkers that could improve the prediction of the hepatocarcinogenic potential of CAR activator compounds would facilitate risk assessment.

Methods

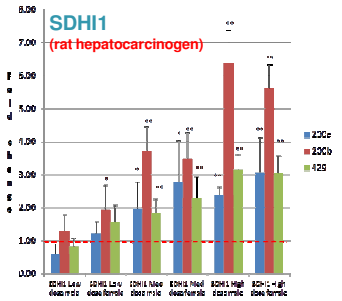
Wistar rat liver FFPE blocks derived from 3 different rat 90 day repeat dose (3 doses – low, medium, high) dietary toxicity studies with 3 different test compounds: SDHI1, SDHI2 and SDHI3 were used for the analysis. 5 males and 5 females from each group, control, low, medium and high dose were analysed for miR 220a/200b/429 levels by RT-PCR. RNA extraction was performed according to the ABI RecoverAll™ Total Nucleic Acid Isolation Kit protocol (Part Number 1975M Rev. C 02/2011). 100ng total liver RNA was converted from miRNA into cDNA in singleplex reactions using the Taqman reverse transcription kit (ABI cat no 4366596) with the 4 specific stem loop primers [001718 (snoRNA), 00502 (miRBase ID: rno-miR-200a-3p), 002274 (miRBase ID: rno-miR-200b-5p), 001077 (miRBAse ID: rno-miR-429)] that amplify mature miRNAs but not precursors. Standard curves were made for each of the targets and the slope was used to assess the amplification efficiency. Singleplex Taqman microRNA assay reactions for miRs 200a, 200b and 429 were performed on 10ng of each of the cDNA samples in duplicate using the probe specific Taqman primers. The data were processed using a method which corrected for variations in PCR efficiencies (Pfaffl, 2001). Fold change values (R values) were calculated for the controls as well as the treated samples relative to a pooled control (calibrator) using the Pfaffl equation:

Ratio(R) = (Etarget) $^{\Delta Ct\ target\ (calibrator\ -\ sample)}$ / (Ereference) $^{\Delta Ct\ reference\ (calibrator\ -\ sample)}$, (Pfaffl 2001).

Statistical comparisons of individual group data (R values) vs respective (sex matched) controls were performed with a two tailed Student's T test using MS Excel.

Results

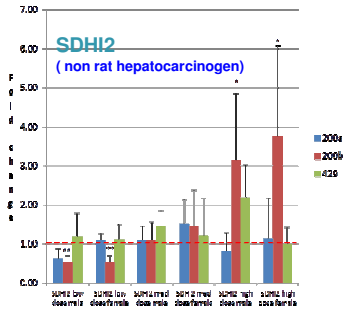
A



•SDHI1 - dose-dependent increase in miR 200a, 220b and 429 in males and females.

•All 3 miRs were induced by SDHI1 at all three doses in females but only at the mid and high doses in males hence the data concurs with an increased potency of this compound at the level of hepatocellular tumours in females.

B

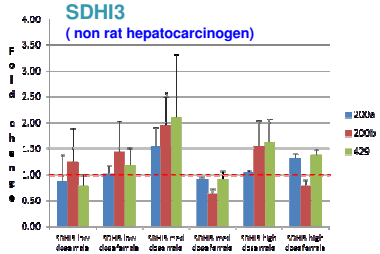


•SDHI2 - miR 200b was significantly ($p<0.05$) higher vs control at the high dose in males and females.

•miR 200b was significantly ($p<0.01$) lower vs control in males and females at the low dose.

•There was no change in either miR200a or miR 429 following SDHI2 treatment.

C

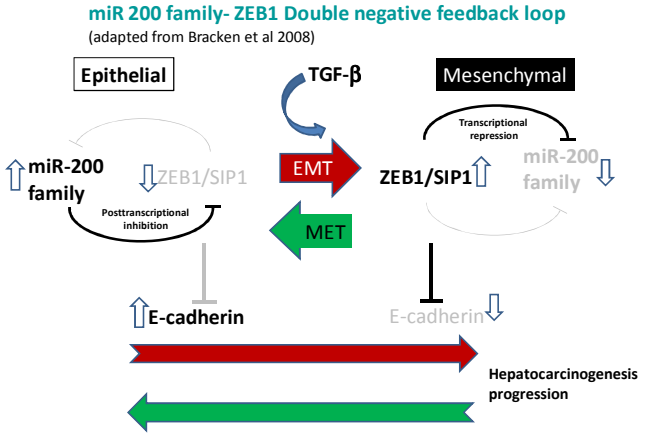


•SDHI3 -no significant treatment related changes in miR 200a, 200b or 429.

Fig 1. Effects of SDHI (1-3) 90d treatment on miR 200a/200b/429 levels. (A) SDHI1; (B) SDHI2; (C) SDHI3.

Data shows relative expression of miR 200a, 200b and 429 in liver (left lobe) of rats treated (dietary) for 90 days with SDHI1, SDHI2 and SDHI3 (low dose; mid dose; high dose). Results are mean (n=5) ± SD fold changes relative to sex matched controls. *, ** significantly different from control by Student's T test, $p<0.05$ or $p<0.01$, respectively. **NB**: Data are normalised to the respective sex matched controls in order to aid visual comparison of the data. Dashed red line shows control value =1.

Figure 2 . Schematic showing hypothesis for the mechanism of miR 200a/200b/429 cluster member involvement in rat hepatocarcinogenesis.



Key: EMT= epithelial mesenchyme transition; MET= mesenchyme epithelial transition

Conclusions

•As only SDHI1 but not SDHI2 or SDHI3 caused rat liver tumours there is concordance between the effect of the SDHIs on all three miRs and their effects at the level of hepatocellular carcinogenesis.

•The effect of SDHI1 on the miRs is more pronounced in females than in males which concurs with an increased potency of this compound at the level of hepatocellular tumours in females.

•Our results suggest that assessment of miR 200a/200b/429 levels has potential as a biomarker of the perturbation of pathways involved in hepatocarcinogenesis in Wistar rats.

This study was supported by Syngenta