Plasma Lipid Profiling in a Rat Model of Hepatocellular Carcinoma: Potential Modulation Through Quinolone Administration

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Background/Aims: **The primary aim of this study was to characterise the blood metabolic profile of hepatocellular carcinoma (HCC) in a rat model, and the secondary aim was to evaluate the effect of the quinolone, norfloxacin on metabolic profiles and exploring the role that gut sterilisation may have on HCC development.** ***Methods:*** **HCC was induced in 10 Fischer rats by administration of intra-peritoneal diethylnitrosamine (DEN) and oral N-nitrosomorpholine. Plasma was collected upon sacrifice. Five of these rats were concomitantly administered oral norfloxacin. Six Fischer non-treated rats acted as healthy controls. Proton nuclear magnetic resonance (NMR) spectra were acquired using a 600 MHz NMR system.** ***Results:*** **Control animals were 120 g heavier than diseased counterparts. Proton NMR spectra from diseased rats displayed significant decreases in lipoproteins, unsaturated fatty acids, acetyl-glycoprotein, acetoacetate, and glucose ($P \leq 0.001$). Plasma citrate and formate levels were increased ($P = 0.02$). Norfloxacin appeared to abrogate this effect slightly.** ***Conclusion:*** **The spectral profiles of plasma in rats with HCC display marked changes with relation to lipid metabolism and cellular turnover. Norfloxacin appears to moderate these metabolic alterations to a small degree. (J Clin Exp Hepatol 2015;5:286–294)**

INTRODUCTION

Human proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy studies of blood in patients with hepatocellular carcinoma (HCC) have identified a number of altered metabolites, implicating changes in hepatic function, lipid metabolism and bile acid metabolism.[1,2] Many rat models of HCC exist, but there are no reports of ^{1}H NMR spectroscopy studies of rat serum or plasma. Characterisation of the serum or plasma metabolic changes in an animal model of HCC would provide valuable translatable information to human disease.

There have been two reports of rat HCC tissue ^{1}H NMR metabolite profiling studies. Tesiram and colleagues performed a meticulous study using ^{1}H NMR spectroscopy to investigate the changes of lipid metabolites in a Fischer rat model of hepatic adenoma, induced with a choline deficient diet.[3] It was demonstrated that a substantial increase in glycerol backbone-containing phospholipids increased throughout adenoma development. HCC did not develop in these animals, but changes described in adenomas are likely to share similarities with HCC. A second ^{1}H NMR spectroscopy study, performed in male Sprague-Dawley rats with diethylnitrosamine (DEN)-induced HCC, reported raised low, very low and high-density lipoprotein (LDL, VLDL and HDL) levels in addition to alterations in branch chain amino acids, acetate, glutamine, choline, trimethylamine-*N*-oxide, glycine, glucose and glycogen.[4] Whether these metabolic profiles are reflected in blood has not previously been investigated using ^{1}H NMR spectroscopy.

Quinolones are administered to patients with decompensated cirrhosis for the treatment of spontaneous bacterial peritonitis (SBP). It has also been reported that quinolones improve survival and decrease incidence of other end-stage complications of liver disease, such as hepatorenal syndrome.[5] Their effect might be mediated through a decrease in gut bacterial translocation and

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Abbreviations: 1-D: one-dimensional; CPMG: Carr-Purcell-Meiboom-Gill 3B; DEN: diethylnitrosamine; FID: free induction decay; ^{1}H: human proton; HCC: hepatocellular carcinoma; HDL: high-density lipoprotein; LDL: low-density lipoprotein; NOESY: nuclear overhauser effect spectroscopy; NMOR: *N*-nitrosomorpholine; NMR: nuclear magnetic resonance; PCA: principal components analysis; PLS-DA: partial least squares discriminant analysis; Q^2: goodness of prediction; R^2: goodness of fit; RD: relaxation delay; RF: radiofrequency; SBP: spontaneous bacterial peritonitis; TLR-4: Toll-like receptor 4; VLDL: very low-density lipoprotein
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circulating endotoxin levels, reducing the pro-inflammatory effectors of liver fibrosis.[6] Increased bacterial translocation, endotoxin and activation of the TLR-4 pathway have also been shown to promote inflammation and HCC.[7] It has been suggested that antibiotic therapy may play a role in reducing HCC incidence in cirrhosis. Several studies of cirrhosis patients and animal models have shown gut sterilisation with antibiotics with quinolones, such as norfloxacin, can decrease bacterial translocation, circulating levels of endotoxin and hepatic inflammation.[8–12] Therefore, the effect this class of drugs may have on HCC development is of interest.[7] This has not been investigated previously.

The primary aim of this study was to ascertain whether altered metabolic profiles could be characterised in a rat model of HCC using ^{1}H NMR spectroscopy. The secondary aim was to investigate the effect of the quinolone, norfloxacin on HCC-induced ^{1}H NMR plasma profile changes.

METHODS

Prior ethical approval was obtained from the University College Hospitals Local Research Ethics Committee, with animal treatment in accordance with the UK Animals (Scientific Procedures) Act 1986.

Fischer Rat Model of HCC

HCC was induced by the administration of two potent carcinogens, DEN (Sigma-Aldrich, Gillingham, UK) and *N*-nitrosomorpholine (NMOR) (Sigma-Aldrich, Gillingham, UK), both of which have rapid rates of hepatic metabolism. This model was chosen owing to the quick development time of HCC (within 16 weeks). However, a washout period of 2 weeks prior to sacrifice was instituted in order to minimise any residual metabolic effects of DEN and NMOR themselves. Male Fischer rats were thus divided into three groups: *Group 1* (n = 5) consisted of rats receiving 100 mg kg^{-1} intra-peritoneal DEN and NMOR at 80 ppm in drinking water ad libitum until 2 weeks prior to sacrifice at 16 weeks; *Group 2* (n = 5) also received the same doses of DEN and NMOR until 2 weeks prior to sacrifice, in addition to 20 mg kg^{-1}/day gavaged norfloxacin until the time of sacrifice; *Group 3* (n = 6) received no treatment and acted as healthy controls (Table 1). All animals received the same standard non-medicated laboratory rodent chow (Teklad® 4% fat fixed-formula diet from Harlan Laboratories, Blackthorn, United Kingdom). All rats in Groups 1 and 2 developed multifocal HCC, Group 1 displaying a greater extent of liver inflammation than Group 2. All rats in Group 3 had normal liver histology. All animals were sacrificed at 16 weeks, whereupon blood was drawn from the abdominal aorta into heparin containing tubes and livers extracted for histological analysis. All samples were centrifuged and snap-frozen in liquid nitrogen and kept at −80 °C until analysis.

Sample Preparation

Samples were prepared according to standard validated protocols.[13] Samples were thawed at room temperature and 200 µL was transferred into 1.5 mL Eppendorf (Eppendorf, Cambridge, UK) tubes to which 400 µL NaCl/D_2O (90%/10%) was added. The mixture underwent centrifugation for 5 min at 13,000 rpm and 550 µL of supernatant was transferred to Norell, 5 mm 507-HP-7 NMR tubes (Norell, Landisville, New Jersey, USA) ready for ^{1}H NMR analysis.

Proton Nuclear Magnetic Resonance Spectroscopy

Samples were run in a random non-grouped order under automation on a Bruker Ultrashield Plus™ 600 NMR system operating at 600.22 Hz ^{1}H frequency (Bruker Biospin, Rheinstetten, Germany), fitted with a super-cooled probe head, containing the receiver and radiofrequency coils, cooled to almost 0 K by use of liquid helium (Cryoprobe™, Bruker Biospin, Rheinstetten, Germany). The system was tuned, matched and frequency locked on to ^{1}H as the nucleus of interest. A representative sample was utilised to set shim gradients to ensure a homogenous magnetic field across the sample, a 90° pulse length and the water suppression offset parameters. These settings were saved and utilised for the whole sample set. Pulse programme and acquisition parameters were set according to optimised protocols for blood from the department of Biomolecular Medicine, Imperial College London. Spectra were acquired using Nuclear Overhauser Effect Spectroscopy (NOESY) and Carr-Purcell-Meiboom-Gill (CPMG) sequences at 300 K. One-dimensional (1-D) NOESY pulse

Table 1 Fischer Rat Groups and Interventions; Groups 1 and 2 Developed HCC.

Intervention *n*:	Group 1 5	Group 2 5	Group 3 6
Diethylnitrosamine (DEN)	100 mg kg^{-1}	100 mg kg^{-1}	0
N-nitrosomorpholine (NMOR)	80 ppm in water	80 ppm in water	0
Norfloxacin	0	20 mg kg^{-1}/day	0