Transcriptomic alterations induced by Monuron in rat and human renal proximal tubule cells in vitro and comparison to rat renal-cortex in vivo

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Monuron (1,1-dimethyl-3-(4-chlorophenyl)urea) is a non-selective phenylurea herbicide, widely used in developing countries although concerns have been raised about its toxicity and carcinogenicity. Monuron was evaluated by the National Toxicology Program in 1988 and shown to be a male rat-specific renal carcinogen. We report that oral administration of Monuron to male rats for 3 days, leads to a larger number of genes being differentially expressed in the renal-cortex than in the liver. Further, we observed up-regulation of cell cycle genes and genes involved in cell proliferation in the renal-cortex while in the liver xenobiotic metabolising enzymes were up-regulated. We also identified one commonly down-regulated gene in both organs – fragile histidine triad gene (Fhit), a putative tumour suppressor gene; however the down-regulation was only significant at the protein level in the liver. In addition, we conducted in vitro whole-genome transcriptomics studies with human and rat renal cortical cells. Rat cells exposed to Monuron showed down-regulation of sterol biosynthesis, spliceosome and cell cycle genes and up-regulation of genes involved in amino acid metabolism and transport. No genes were found to be differentially expressed in human cells exposed to Monuron. Overall, the findings from the in vitro studies showed very little overlap with the whole animal findings.

Introduction

Monuron, 1,1-dimethyl-3-(4-chlorophenyl) urea (MON), is a non-selective phenylurea herbicide that was introduced commercially in 1952 (Fig. 1).

MON is rapidly taken up from the soil by the plant root system and translocates into stems and leaves. The phytotoxicity of MON is due to inhibition of a site close to photochemical system II[1] and also to the release of excited singlet oxygen that eventually leads to the breakdown of the chloroplast membrane.[2] In 1973, MON was banned in the United States for use on food crops, due to the absence of extensive toxicological data and the possibility of carcinogenicity.[3,4] The National Toxicological Program (NTP) partially filled this gap in 1988 with toxicology and carcinogenicity studies in F344/N rats and B6C3F1 mice.[5] In this study, MON fed to rats in the diet at 750 and 1500 ppm for 103 weeks produced a marked cytomegaly of renal tubular cells, mainly proximal convoluted tubules, in the kidneys of rats of both sex. The incidence was 0% in controls and 96% and 100% in males and 24% and 98% in females at the low and high dose respectively. The affected cells also had marked karyomegaly, sometimes with multiple nucleoli. This was associated with an increased incidence of renal tubular cell adenocarcinoma and adenoma in the kidney of male rats with an incidence of 0% in the controls, 6% at the low dose and 30% at the high dose. Renal tubular tumours were not

seen in female rats or mice (fed 5000 or 10 000 ppm) of either sex. Male rats also showed neoplastic nodules and carcinomas in the liver.[5] The basis for the species and sex difference in tumourgenesis is not known, but differences in sex response may be related to the greater increase in renal proximal tubule cell turnover in male versus female rat kidney.[6]

The major route of metabolism of MON in male albino rats, strain not reported, is via oxidative N-demethylation and aro- matic hydroxylation but some chlorinated aniline derivatives are also produced.[7] The principal urinary metabolites are N-(4- chlorophenyl)urea (14.5% dose), N-(2-hydroxy-4-chlorophenyl)- urea (6.5%), N-(3-hydroxy-4-chlorophenyl)urea (2.2%) with some mono- and dimethyl urea and 2-acetamido-2-chloro- phenol. The phenolic metabolites of MON are excreted in the urine as conjugates. Following demethylation of MON the unstable N-hydroxymethyl metabolite decomposes to the N-desmethyl metabolite and formaldehyde.[8] There is also evi- dence that following N-hydroxylation of MON followed by acetylation, or sulphate ester formation that carbonium ion formation would lead to reactions with nucleophilic centre's of cellular components.[9] There is no data in the literature on the pharmacokinetics and dynamics of MON in rats.

MON is classed as a non-genotoxic carcinogen based on a microscreen phage-induction assay[10] and negative results in the Ames Salmonella assay in TA98, TA100, TA1535 and TA1537 strains with or without rat or Syrian hamster liver S9 fraction.[5] We found that MON at 250 μM (IC_{10} at 72 h in rat renal cells) did not induce micronucleus formation.[11] However, studies in Chinese hamster ovary (CHO) cells noted a small increase in chromosome aberrations, which were markedly increased in the presence of Aroclor-induced rat liver S9, indicating MON- induced chromosome aberrations, albeit at a concentration of 1.3 mg ml^{-1} (6.55 mM).[5] MON also caused transformation in Syrian hamster embryo cells in the absence of metabolic acti- vation.[12] While Seiler reported that the putative N-acetoxy metabolite of MON was mutagenic in Salmonella typhimurium TA 100 in neutral aqueous medium.

Elcombe and colleagues studied the effect of a range of car- cinogens over 90 days, including MON, and noted that MON produced a small increase in kidney weight in male F344 rats after 28 and 90 days but not 7 days exposure to 750 and 1500 ppm in the diet, the doses used in the carcinogen bio- assay. They also observed renal tubular basophilia which was not time or dose-related and reported a small increase in renal tubule cell proliferation at 7 days in the high dose male group, which was not seen at later time points.[13] Currently it is unclear how MON causes renal tubule tumours in male rats.

High-throughput whole genome transcriptome analysis might provide insights into biological pathways and molecular mechanisms that are altered after exposure to carcinogen. In this study we aim to identify changes in the whole-genome gene expression profile that occur after short term exposure to MON in vivo and in vitro. In order to do this, we performed microarray analysis using male rat renal-cortex and rat proximal tubular cells NRK-52E.[14] NRK-52E cells are normal epithelial cells derived from a healthy, young, non-inbred Osborne-Mendel rat.[14] NRK-52E cells are largely

glycolytic, with low activities of succinate dehydrogenase, glutamate dehydrogenase and high activities of lactate dehydrogenase. NRK-52E possess less mitochondria, low transport activity for glutathione (GSH) across the mitochondrial membrane, low activities of γ-glutamyltransferase, alkaline phosphatase and low content of brush-border microvilli as compared to freshly isolated rat proximal tubule cells. NRK-52E cells do, however, have levels of ATP close to those found in vivo and only modestly lower activity of Na^+/K^+-stimulated ATPase. GSH dependent enzymes like glutamylcysteine synthetase, GSH peroxidase, glutathione disulfide reductase, and GSH S-transferase were shown to have high activities in NRK-52E cells.[15] However common CYP 450's that metabolise MON have only a residual activity in these cells (Bloch et al., unpublished observation).

In addition to rat cells, we used human proximal tubule cells RPTEC/TERT1[16] as currently it is not clear if MON is a rat-specific carcinogen.

2. Materials and methods

2.1 Materials

MON 99% pure was purchased from Sigma-Aldrich, UK. Trizol reagent and glutamax were obtained from (Invitrogen, UK) and RNeasy Total RNA Mini Kit and RNA later from (Qiagen, UK). All other chemicals were of the highest grade purity available.

2.2 Animal study

All animal procedures were performed in accordance with a license issued under the animals (Scientific Procedures) Act, 1986. Wistar-derived rats where bred in the Life Science Support Unit at Liverpool John Moores University. The rats were housed in plastic cages in a room maintained at 20 °C ± 2 °C and a relative humidity of 50% ± 5% with a 12 h light- dark cycle starting at 04.00 h. Rats were allowed food (rat expanded diet), and water ad libitum. Male rats (190–240 g) 7–9 weeks of age were used, four rats being dosed orally with MON at 40 mg kg^{-1} body weight on day 1 and 400 mg kg^{-1} body weight on days 2 and 3, at 5 ml kg^{-1} in corn oil. Four control animals were dosed orally daily for 3 days with corn oil at 5 ml kg^{-1}. Rats were placed individually in metabolic cages after dosing for the collection of urine, on days 1, 2 and 3 and given food and water ad libitum. Urine was collected from each rat into a container which had 0.1 ml of 10% sodium azide to prevent bacterial growth. Urine volume was determined and the pH measured using a pH meter and then the urine frozen −80 °C for subsequent analyses of Kim-1 and creatinine. Twenty four hours after the last dose all rats were killed by a rising concentration of carbon dioxide. A portion about 50 mg of liver and renal-cortex were quickly harvested and immersed in RNAlater.

2.3 Cell culture

NRK-52E cells (ATCC, CRL-1571) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum, penicillin 100 IU. ml^{-1}, and streptomycin of DMEM and Ham's F-12 nutrient mix. The medium was supplemented with 2 mM

glutamax, 5 µg L^{-1} insulin, 5 µg L^{-1} transferrin and 5 ng L^{-1} sodium selenite, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin, 10 ng mL^{-1} epidermal growth factor and 36 ng mL^{-1} hydrocortisone. Cells were fed three times a week and sub-cultured by trypsinization close to confluence.

2.4 Cytotoxicity-determination of IC$_{10}$

A dose–response curve for MON was determined using the MTT assay.[17] In brief, cells were exposed for 72 h in 96-well plates to a wide range of concentrations of MON dissolved in DMSO and diluted to give a final concentration of 0.1% v/v DMSO. Control cells were exposed to DMSO alone (0.1% v/v). At least three separate experiments were conducted. In addition, LDH activity and lactate were measured in the medium as previously described.[18] The dose that caused approximately 10% cytotoxicity (IC$_{10}$) at 72 h in the MTT assay was selected for the transcriptomics studies.

2.5 Cell treatment

NRK-52E and RPTEC/TERT1 cells were cultured to confluence on 6-well plates. RPTEC/TERT1 cells were maintained at con- fluence for at least 10 days before treatment. For transcrip- tomics studies, cells were exposed to MON dissolved in DMSO (0.1% v/v) at the IC$_{10}$ concentration at 72 h or DMSO (0.1% v/v) alone. After 6 h, 24 h and 72 h the medium was removed and RNA was extracted from cells. For replication, three studies were conducted at each time point.

2.6 RNA isolation and microarray

From in vitro studies total RNA was isolated from DMSO (control) and MON treated cells. From in vivo studies total RNA was isolated from a small about 50 mg portion of the renal-cortex and liver of corn oil (control) or MON treated rats. Trizol reagent was used for RNA isolation. Total RNA was puri- fied using the RNeasy Total RNA Mini Kit (Qiagen) according to manufacturer's instructions. RNA was checked for purity and integrity using Agilent 2001 Bioanalyzer (Agilent Techno- logies GmbH, Germany) before processing. Transcriptomics data was generated using Affymetrix Human Genome U133 Plus 2.0 or GeneChip Rat Genome 230 2.0 Arrays for human and rat respectively. Both arrays provide comprehensive cover- age of the transcribed rat and human genome. Rat genome array comprised of more than 31000 probe sets, analyzing over 30 000 transcripts and variants from over 28 000 well-sub- stantiated rat genes. Human genome array provides complete coverage of over 47 000 transcripts.

2.7 Microarray hybridization

2.7.1

Target preparation. cDNA was prepared using the Affymetrix IVT express kit (Affymetrix, Santa Clara). cDNA syn- thesis and labelling was performed according to the manufacturer's procedures. Subsequent labelling of the samples was conducted by synthesis of Biotin-labelled complementary RNA (cRNA) using the GeneChip IVT

labelling kit (Affymetrix). Purified cRNA was quantified using a spectrophotometer, and unfragmented samples were checked on the Bioanalyzer. Subsequently, cRNA samples were fragmented for target preparation according to the Affymetrix manual and checked on the Bioanalyzer. Samples were stored at −20 °C until ready to perform hybridization.

2.7.2

Hybridization. cRNA targets were hybridized on high-density oligonucleotide gene chips (Affymetrix Human Genome U133 Plus 2.0 and GeneChip Rat Genome 230 2.0 Arrays) according to the Affymetrix Eukaryotic Target Hybridiz- ation manual. The gene chips were washed and stained using the Affymetrix Fluidics Station 450 and Genechip Operating Software and scanned by means of an Affymetrix GeneArray scanner.

2.7.3

Microarray analysis. The intensity values of different genes (probe sets) generated by Affymetrix GeneChip Operat- ing Software were imported into GeneSpring version 11 soft- ware (Agilent) for data analysis. The raw data files (CEL files) containing signal values for individual probes were pre-processed to generate one value per probeset. Pre-processing of arrays was done using GC-RMA (Robust Multiarray Analysis algorithm).

To identify the differentially expressed (DE) probe sets, two sampled t-test (unpaired t-test) with a P-value <0.05 and Benja-mini–Hochberg correction for multiple testing correction and a fold cut off of 1.5 was used for in vitro and in vivo data. Com- parisons were made between control (corn oil in in vivo study and DMSO in in vitro study) and MON treated samples. Three studies were conducted at each time point for in vitro studies. For in vivo studies four rats were used per condition.

2.7.4 Functional annotation.

To understand the biological meaning behind the list of DE genes, Kyoto Encyclopaedia of Genes and Genomes database (KEGG) pathways analysis and Gene Ontology (GO) from DAVID website (The Database for Annotation, Visualization and Integrated Discovery) v 6.7 (http://david.abcc.ncifcrf.gov/) were used. Only GO and path- ways with P-value <0.05 (Benjamin–Hochberg corrected) and with 5 or more genes were analysed and discussed. In addition, Ingenuity Pathway Analysis (IPA) was used. Only pathways and functions with P < 0.05 (Fisher's exact test right- tailed) and with 5 or more genes were analysed and discussed.

2.8 Data deposition

MIAMI-compliant microarray data obtained from rat in vitro and in vivo studies were deposited in the NCBI GEO database under accession number GSE51163.

2.9 Western blots

Protein extracts were prepared from small dissected samples of liver and renal cortex, while NRK-52E cells were collected using a cell scraper. The samples were homogenised

(Ultraturax) in ice-cold radioimmunoprecipitation (RIPA) buffer supplemented with protease and phosphatase inhibitor cocktail set II (Calbiochem) for 30 min to lyse the cells. The samples were then centrifuged (10 000 rpm/30 min/4 °C) and the supernatant protein collected. The supernatant proteins were then resolved by SDS-PAGE and transferred to nitrocellulose mem- brane (Amersham Hybond-ECL). Primary antibodies, fragile histidine triad gene (FHIT) (1 : 2000, Abcam), Kidney injury molecule 1 (Kim-1) (1 : 1000, Abcam), Low density lipoprotein receptor (LDL-R) (1 : 2000, Abcam) and β-actin (1 : 15 000, Sigma) were diluted in blocking solution containing 5% (w/v) non-fat milk powder (Marvel) in 1% Tween-20 in Tris Buffered Saline (TBS-T) at 4 °C overnight. Blots were washed 3×10 min with TBS-T. A secondary antibody (anti-mouse/rabbit) conjugated to horseradish peroxidise (R&D systems) was diluted (1 : 1000) in 5% (w/v) Marvel in TBS-T and the blot was incubated in this on an orbital shaker for 1 hour at room tempera- ture. The following day, the blot was washed in TBS-T (3×10 minutes). Labelled bands were visualized using ECL detection reagent (SuperSignal West Pico, Thermo Scientific). Den- sitometric quantification of band intensity was performed using NIH Image J software. Statistical analysis was performed using Prism software (GraphPad). $P < 0.05$ was considered significant.

2.10 Kim-1 analysis in the urine

Urine samples were thawed, mixed and centrifuged (3000 rpm, 5 min) and measurements performed on the resulting supernatants. Urinary Kim-1 was determined using microsphere- based Luminex technology, as previously described[19] using 25 µl of urine, and analysed in duplicate. Kim-1 was quantified using a 13-point five parametric logarithmic standard curve, the inter- and intra-assay variability was less than 15%. Urinary creatinine was determined spectrophotometrically as described by ref. 20 using 25 µl of urine per analysis, and analysed in duplicate. Urinary levels of Kim-1 were normalized to urinary creatinine concentration.

3. Results

3.1 In vivo studies in the rats

MON was well tolerated following a single low oral dose of 40 mg kg^{-1} (equivalent to about 400 ppm in the diet) so the dose was increased to 400 mg kg^{-1} (equivalent to about 4000 ppm in the diet) for the next two days without any signs of toxicity. Body weight was reduced in the treated compared to the controls rats at the end of the study but this was not statistically significant. Liver weight was significantly reduced compared to controls at termination (Liver/body weight ratio, Control, 4.40 ± 0.12%; MON, 4.06 ± 0.10% p < 0.05) with no change in kidney weight. The transcriptome profile was deter- mined from mRNA isolated from the liver and renal-cortex of control rats given corn oil alone for 72 h and from rats exposed to MON for 72 h, following microarray hybridization with Affymetrix Rat Gene Chips 230 2.0.

After 3 days exposure to MON, 77 DE genes were identified in the renal-cortex. Significant up-regulation of genes involved in the cell-cycle was observed (Table 1). The

only other genes with large increases were Kim-1 (31.1 fold), NQ01 (7.6 fold) and Srxn1 (7.6 fold).

In the liver only six known genes in total were identified (Fhit, Cyp2b1, Cc2d1b, Creg1, Aldh1a1, Ephx1). Interestingly one gene, fragile histidine triad gene (Fhit) was found to be commonly down-regulated in both the renal-cortex and liver of MON exposed rats.

3.2 In vitro studies in human RPTEC/TERT1 cells and rat renal NRK-52E cells

MON showed a similar cytotoxicity in rat and human renal cell lines with an IC_{10} at 72 h of 250 μM and 200 μM respectively (Fig. 2).

For transcriptomic analysis both cell lines were exposed to IC_{10} doses of MON for 6 h, 24 h and 72 h and transcriptomic analysis was performed at all three time points.

In the human renal tubule cell line exposed to MON, no DE genes were found to be statistically significantly different from control at all three time points. Similarly in the rat cell line after 6 h exposure no statistically significant genes were differentially expressed. However, by 24 h exposure, 680 DE probe sets were found with significant enrichment of genes involved in RNA processing, cell death and survival and amino acid metabolism (Table 2). A general down-regulation of genes involved in spliceosome, cell cycle and lipid metabolism were also identified after 24 h in vitro exposure to MON.

After 72 h exposure, 77 DE genes were identified with enrichment of genes involved in cell morphology (Table 3).

A comparison of commonly altered genes identified after 24 h and 72 h exposure to MON in rat cells showed four genes commonly altered: Fgf21, Epha4, similar to mKIAA1924 protein and similar to hypothetical protein MGC47816 (Fig. 3).

The pattern of expression was consistent with time, with Fgf21 being up-regulated after 24 h and 72 h and Epha4, similar to mKIAA1924 protein and similar to hypothetical protein MGC47816 being down-regulated at both time points.

3.3 Overlap of in vivo and in vitro rat data

Six genes (Cdc20, Pcca, Cdca3, Mcm7, Ncaph and LOC689399) were commonly altered in rat renal-cortex in vivo and in renal cells in vitro after 24 h exposure (Fig. 3). However, the pattern of expression was different between in vitro and in vivo. Pcca was found to be down-regulated whereas Cdc20, Cdca3, Mcm7, Ncaph and LOC689399 were up-regulated in vivo, while in the rat cells in vitro the exact opposite was seen with Pcca being up-regulated and the others being down-regulated.

There was no overlap between DE genes identified in the renal-cortex and renal cells after 72 h exposure.

In addition, no commonly altered genes were identified between liver and renal cells in vitro.

3.4 Detection of gene changes at the protein level

To determine if the differences in mRNA levels reflect differences in protein levels, we selected two genes from the in vivo study-Fhit and Kim-1. Fhit was found to be down-regulated in both liver and the renal-cortex in MON-exposed rats. While Kim-1, a known biomarker for renal injury, was the top altered gene found in the renal-cortex, with 31-fold up-regulation.

Western blot analysis in the liver and renal-cortex with an anti- body to Fhit, showed a statistically significant decrease in the Fhit/actin ratio in the liver (control, 2.27 ± 0.08; MON, 1.97 ± 0.12; 1-tailed t-test $P < 0.05$) with no change in the renal-cortex (control, 1.35 ± 0.12; MON, 1.27 ± 0.09; 1-tailed t-test $P < 0.05$), which is in partial agreement with our findings (Fig. 4). However, the up-regulation of Kim-1 was not confirmed at the protein level in the renal-cortex (Fig. 4) or in the urine (Fig. 5) following 1, 2 or 3 doses of MON with Gentamicin as a positive control.

In the rat renal cells exposed to MON up-regulation of Kim-1 was not observed. The only up-regulated renal biomarkers detected in vitro were Vegfa and Vegfb. However down-regulation of lipid metabolism was observed after 24 h exposure to MON and we used an antibody to low density lipoprotein receptor (LDL-R) as a marker of this process (Fig. 6). LDL-R was poorly expressed in both control and MON treated cells after 6 h exposure, by 24 h the MON treated cells has increased the level of expression compared to untreated while at 72 h the expression was similar in both cases (Fig. 6).

4. Discussion

4.1 In vivo studies

We found a nine-fold higher number of genes altered in the renal-cortex than in the liver following MON exposure to male Wistar rats for 72 h. In the renal-cortex, the major finding was up-regulation of genes involved in the cell cycle, in particular G_2M-phase and genes associated with anaphase chromosome separation, alignment and segregation. An increase in the expression of cell cycle genes is a common finding with a number of renal nephrotoxins and renal carcinogens.[21–23] The enrichment of the cell cycle genes after short term exposure is consistent with the small increase in cell proliferation observed in the kidney after 7 days exposure to MON.[13] Some of the gene increase is involved in cell division with role in chromosome stability, alignment and segregation and we cannot at this stage rule out the possibility of adverse chromosomal events in the kidney following MON. Karyomegaly was reported in rat proximal tubule cells following chronic exposure to MON indicating an effect on the nucleus. As dis- cussed earlier some in vitro tests have indicated the mutagenic potential of MON and chromosome aberration and micro- nuclei formation has been reported in the bone marrow of mice given MON by intraperitoneal injection.[24] We reported in vitro in NRK-52E cells the lack of micronuclei formation following exposure 250 μM Monuron.[10] However, we now know that the gene response to MON in vivo is different from that in vitro, so future work should focus on the possibility of chromosomal changes occurring in the renal cortex of

rats.

In contrast to the renal-cortex, in the liver only eight genes were differentially expressed, with four being involved in xenobiotic and endogenous metabolism (Aldh1a1, Ephx1, Cyp1a1, Cyp2b1). These findings suggest that MON is primarily metabolized in the liver, possibly by CYP1A1 leading to ring hydroxylation, N-demethylation and possibly N-hydroxylation, this latter route forming mutagenic and carcinogenic reactive metabolites. No information is available in the open literature on the pharmacokinetics or pharmacodynamics of MON in the rat. In our study using ^{1}H-NMR spectroscopy on plasma 24 h after the last dose of MON, we detected no evidence of either MON or metabolites but they could well be below the limits of detection with this method.

The only common altered gene found in the renal-cortex and liver of MON exposed rats was the putative tumour suppressor gene Fhit. Human FHIT gene is located on the short arm of chromosome 3 which is a common site of chromosomal alterations in many human malignant diseases. Its loss has been reported in cancers of the lung, breast, oesophagus, cervix but also kidney and bladder.[25,26] In human hepatocytes in vitro Josse and colleagues found that FHIT was an early target gene following exposure to the genotoxic carcinogen aflatoxin B1.[27] In the rat, Fhit is located on chromosome 15 and many studies suggest that its lost is an early event in carcinogenesis.[28–31] Although we saw a decrease in expression of Fhit gene in both kidney and the liver, the decrease was only statistically significant in the liver at the protein level (Fig. 4).

MON, is considered to be a non-genotoxic chemical but we cannot exclude the possibility that it may interact with DNA, and hence would be genotoxic. This possibility is considered as the tumour incidence in male rats is higher than that seen with chemicals that cause renal tumours via non-genotoxic mechanisms.[32] Metabolic activation to generate reactive metabolites has been suggested as a mechanism to produce cytotoxicity.[9,10] However, renal tubule cell toxicity was not been observed after exposure of male rats to MON for 3 days, which might have been expected is this was the mechanism of carcinogenesis.[32] We suggest from our findings that an up-regulation of cell cycle genes in association with the morphological changes observed in the nucleus after exposure may be contributing factors.

There are some limitations of our in vivo study. First the transcriptomics analysis was conducted on a mixture of renal- cortical cells rather than renal proximal tubule cells. Second, we used Wistar-derived male rats while F344 rats were used in the NTP bioassay[5] and in the study by Elcombe and co- workers.[13] It is known that different strains of rats can have different sensitivities to chemicals and different activities of CYP 450 isozymes which can affect the response to a chemical insult.[33,34] However, the acute renal pathology was the same in our strain as that observed in the F344 male rat following MON. Third the dose levels of MON and exposure time was different Elcombe et al., looked at the liver and kidneys after 7, 28 and 90 days exposure while the NTP study was for 103 weeks.[5,13] In both of these studies the rats were fed 750 and 1500 ppm in the diet, whereas in this study MON was given by oral gavage at 40 mg kg^{-1} ($\sim$400 ppm) body weight on day 1 and 400 ($\sim$4000 ppm) mg kg^{-1} body weight on days 2 and 3 and

terminated 24 h after the last dose. Our first dose of MON being lower than the low dose of the NTP study while the second and third doses were above the top dose of the NTP bioassay.

4.2 In vitro studies   In vitro in both human and rat proximal tubule cells we observed cytotoxicity after exposure to MON with an IC_{50} of about 1 mM, suggesting that toxicity is either due to the parent compound or metabolites. However, we know that the common CYP 450's enzymes that are involved in MON metabolism have only residual activity in the rat cell line (Bloch et al., unpublished data), suggesting the toxicity may be due to the parent compound. The most unexpected finding was that no statistically significant gene changes were observed in human renal tubule cells after 72 h exposure to an IC_{10} dose of MON. In contrast, in rat renal tubule cells >700 genes were altered over the period of 72 h. The dose of MON was very similar in both cell lines, which suggests that metabolism to a toxic metabolite may be more active in the rat cells. This is consistent with the marked increase in stress response genes, due to the presence of a foreign compound in rat cells but not in human cells. Currently we have no information on MON metabolism in these cells.

We did not observe Fhit down-regulation in rat or human cell renal tubular cells in vitro following MON exposure. One of the interesting findings was the down-regulation of lipid syn- thesis together with concurrent up-regulation of Vldlr, an important cholesterol importer, 24 h after MON exposure. We also observed up-regulation of LDL-R, a transporter that shows considerable similarity to VLDL-R, on both mRNA and protein level (Fig. 6). We also identified two genes Fgf21 and EphA4 that were commonly dysregulated 24 h and 72 h after MON exposure in the rat renal cells. EPH4A is a protein tyrosine kinase receptor that interacts with ligands called ephrins, and has been shown to be down-regulated in kidney cancer[35] which is consistent with our finding. FGF21 is a member of the fibroblast growth factor family and a glucose and lipid metabolism regulator.[36] In our study Fgf21 was up-regulated after 24 h and 72 h MON exposure, which may reflect the lack of effect on lipid metabolism 72 h after exposure.

4.3 Comparison of in vitro and in vivo studies

When comparing in vivo with in vitro studies, cell cycle was identified as the top enriched pathway affected in both studies after 24 h and 72 h exposure (Tables 1 and 2). However, the pattern of expression of cell cycle genes was strikingly different in vivo versus in vitro. Four genes, Cdc20, Cdca3, Mcm7 and Ncaph were up-regulated in the renal-cortex of exposed rats, whereas they were down-regulated in vitro. Ncaph (Non-SMC condensin I complex, subunit H) encodes the regulatory subunit of the condensin complex that is required for conversion of interphase chromatin into mitotic-like condense chromosomes. Cdca3 (Cell division cycle associated 3-box-like) encodes a protein which is required for entry into mitosis, whereas Cdc20 (Cell division cycle 20 homolog) encodes a regulatory protein interacting with several other proteins at multiple points in the cell cycle and is required for two micro- tubule-dependent processes, nuclear movement prior to anaphase and chromosome separation. While Mcm7 (Mini-chromosome maintenance complex component 7) is essential for the initiation of

eukaryotic genome replication.

In parallel to alteration in expression of cell cycle genes, alteration in expression of genes involved in mRNA processing, lipid and amino acid metabolism and transport was observed in rat renal cells in vitro. However, none of these changes were observed in renal-cortex or liver of exposed rats, suggesting fundamental differences between in vitro and in vivo. The reasons for this discrepancy between rat in vitro and vivo is probably related to pharmacokinetics and metabolism, however nothing is known about blood or kidney levels of MON or its metabolites so we do not know if the in vitro dose of MON bears any relationship to that found in the whole animal. The absence of DE genes in human renal tubule cells exposed to MON may support the hypothesis that MON might be a rat-specific renal carcinogen, however caution should be exercised as the metabolism may differ between in vitro and in vivo samples.

5. Conclusion

In summary, our studies have shown that MON administered to rats for 3 days leads to a more marked gene response in the renal-cortex than the liver, with renal cortical genes associated with cell cycle proliferation being up-regulated. In contrast, in the liver, up-regulated genes were mainly related to xenobiotic metabolism. These findings indicate the targeting of MON to the kidney and the possible basis for the renal cancer. Whether this is due to sustained cell proliferation leading to expression of altered genes or to chromosomal damage as a result of production or a reactive genotoxic metabolite is for future work. In vitro human renal tubule cells exposed to an IC_{10} concentration of MON over 72 h showed no DE genes. While DE genes identified in rat renal cells exposed to IC_{10} con- centration of MON showed little overlap with the whole animal findings. Interestingly, the only common genes were mainly involved in the cell cycle and were down-regulated in vitro and up-regulated in vivo. Fhit, a candidate tumour suppressor gene, was down-regulated in both liver and kidney at early stage after MON exposure. While Kim-1, a known renal biomarker, was significantly up-regulated in male rat renal-cortex after short term MON exposure at the transcriptomic level; at the proteomic level no increase was detectable. Overall, our findings show that gene changes seen in vivo, cannot be reproduced in vitro following exposure to MON. In vivo gene changes related to renal tubule cell proliferation were most likely related to the long term exposure to MON seen in the kidney, however we cannot exclude an effect on chromosomal aberration.

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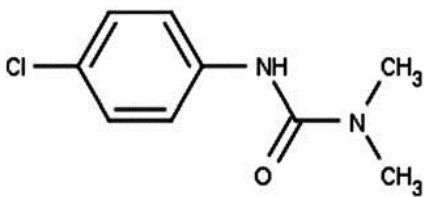
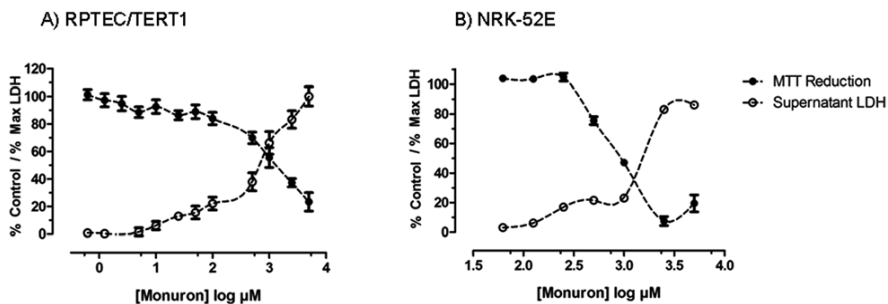Fig. 1 Chemical structure of Monuron, 1,1-dimethyl-3-(4-chloro- phenyl)urea.

Fig. 2 Dose response curves for the cytotoxicity of Monuron in RPTEC/ TERT1 and NRK-52E cells over 72 h. Results are Mean ± SD of three sep- arate experiments.
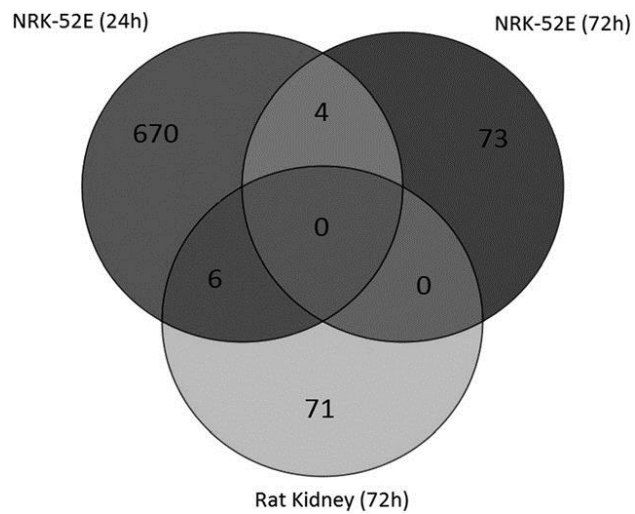

Fig. 3 The overlap of DE genes in rat renal-cortex and rat cells in vitro following exposure to Monuron.
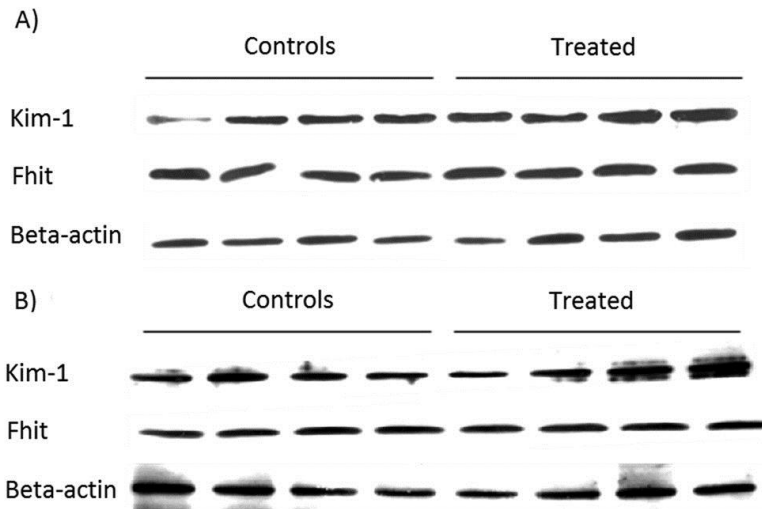
Fig. 4 Western blot analysis of Kim-1 and Fhit in renal-cortex (A) and liver (B) of control rats and rats exposed to Monuron for 3 days.
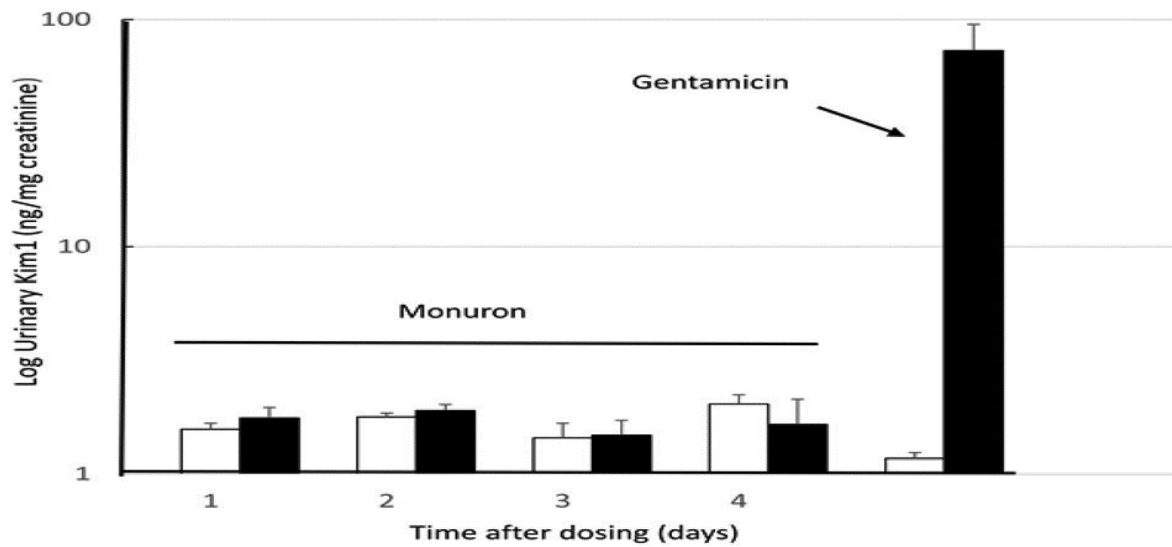

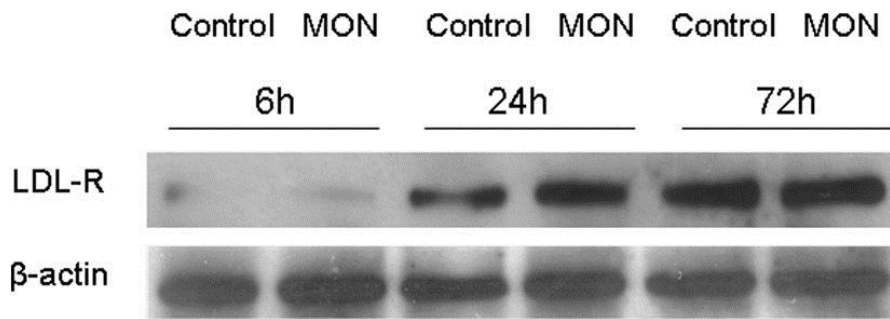Fig. 5 Kim-1 time course in urine in male rats exposed to Monuron.

Fig. 6 Western blot analysis of LDL-R in NRK-52E cells after 6 h, 24 h and 72 h exposure to 250 µM Monuron. Western blots were performed on NRK-52E cells exposed for 6 h, 24 h and 72 h to MON at 250 µM. β-actin was used as an internal control. Band density was quantified using IMAGEJ (National Institutes of Health) and normalized against β-actin.