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Frank C. Richardson[1]
Stephen C. Strom[2]
Deborah M. Copple[1]
Raymond A. Bendele[1]
Gregory S. Probst[1]
N. Leigh Anderson[3]

[1]Toxicology Research Laboratories, Lilly Research Laboratories, A Divison of Eli Lilly and Co., Greenfield, IN
[2]Medical College of Virginia, Richmond, VA
[3]Large Scale Biology, Rockville, MD

Comparisons of protein changes in human and rodent hepatocytes induced by the rat-specific carcinogen, methapyrilene

There is a growing concern that the rodent biossay may not always serve as an appropriate model to assess the carcinogenic risk for humans exposed to certain compounds. Mechanistic research that examines the effects of a compound in rodent and man could help in the interpretation of bioassay results. This paper reports a novel use of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) technology to assess similarities and differences in the response of rodents and humans to the rat-specific hepatocarcinogen, methapyrilene (MP). A sequential examination of rodent and human hepatic proteins was conducted following *in vivo* exposure of rats and mice and *in vitro* exposure of rat, mouse, and human hepatocytes to MP. Results showed that covalent modifications observed in rats and mice *in vivo* were duplicated both qualitatively and quantitatively in the corresponding *in vitro* systems and that these modifications correlated with carcinogenic susceptibility. Covalent modifications in human hepatocytes were minimal despite exposure to concentrations of MP that were 6-fold higher than those used in rodent hepatocytes. These studies suggest that in the case of MP the rat is not the most appropriate model for assessing the human situation. Furthermore, these data show that *in vitro-in vivo* comparisons based on 2-D PAGE may provide adjunctive information for extrapolating rodent toxicity/bioassay results to human risk assessment.

1 Introduction

In 1976, the chronic rodent bioassay was established as the standard method to establish the carcinogenic potential of a compound [1]. Today, there is a growing concern that for many compounds the rodent bioassay and/or the means by which it is conducted, using maximum tolerated doses, may not serve as an appropriate model to assess the carcinogenic risk to humans [2–6]. This is especially true for compounds that do not appear to interact directly with DNA. However, since it is not likely that the rodent bioassay will be replaced in the foreseeable future, it will continue to be necessary to establish the relevance of bioassay results to humans through additional mechanistic and comparative studies.

We report here a novel application of two-dimensional polyacrylamide gel technology (2-D PAGE) to studies aimed at elucidating and comparing the effects of the carcinogen methapyrilene (MP) and rodent and human hepatic proteins *in vitro*. The refinement of 2-D PAGE coupled with computerized data analysis has allowed simultaneous resolution, cataloguing and quantification of over 1000 major hepatic proteins. This can be compared to standard experimental protocols where only one or a few endpoints are examined at a time. Applied to toxicology, 2-D PAGE provides a broad view of the effects of a compound on the molecular anatomy of the cell or tissue of interest [7] and when proteins can be identified, 2-D PAGE can provide mechanistic insight into the changes induced by xenobiotics.

Correspondence: Dr. Frank C. Richardson, Toxicology Labs, Eli Lilly & Co., P.O. Box 708, Greenfield, IN 46140, USA

Abbreviations: CMI, charge modification index; **2-D PAGE**, two-dimensional polyacrylamide gel electrophoresis; **Mitcon**, mitochondrial protein; **MP**, methapyrilene

2-D PAGE has been used to analyze changes in hepatic proteins caused by carbon tetrachloride, cyclohexamide, ibuprofen [8] alcohol [9] and Aroclor 1254 [10] and has been used in *in vitro* systems to determine compound-induced changes in protein-profiles [11–13]. More recently, changes in protein-profiles during hepatocarcinogenesis [14] and liver regeneration [15] have been characterized. Using protein analysis by 2-D PAGE to make cross-species and *in vivo-in vitro* comparisons has remained largely unexplored.

Methapyrilene is an antihistamine (H1 antagonist) that was used in various medications for over 20 years. In 1980, MP was shown to cause a 100% incidence of hepatocellular carcinomas in F-344 rats [16] and it was subsequently withdrawn from the market. Despite a strong response in the rat, no carcinogenic response has been observed in the mouse [17], hamster or guinea pig [18]. In addition, morphologic changes observed in the rat, including mitochondrial alterations [19], were not observed in other species [20]. These results suggest that MP is a rat-specific carcinogen. MP is not genotoxic in routine genetic toxicology tests and does not appear to bind to DNA [21, 22]. Despite positive responses in some specialized tests [23, 24] and a weak response in the Ames assay [25], MP is considered a nongenotoxic carcinogen [26] that may operate by increasing cell replication [27] and/or by other mechanisms that do not involve direct interaction with DNA.

Utilizing 2-D PAGE technology, studies from these laboratories have shown that MP treatment results in the covalent modification of four specific mitochondrial proteins that include the β-subunit of F_1 ATPase (Mitcon: 1), two mitochondrial matrix proteins (Mitcons: 2 and 3) and carbamoyl phosphate synthetase [28]. While it is not clear whether this modification results from MP or a metabolite or from a cellular process such as glycosylation or acetylation, *etc.*, modifying the β-subunit of F_1 ATPase does not appear to alter its function, since the oxidative function of mi-

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tochondria from treated rats is not impaired [29]. Comparisons between 2-D gel patterns obtained from rats and mice treated with MP *in vivo* revealed a correlation between the susceptibility of each species to the carcinogenic effects of MP and the extent of covalent modification of mitochondrial proteins:1–3 [28]. Using these endpoints, we have extended these studies to examine the effects of MP on rodent hepatocytes to determine if and to what degree these modifications occurred *in vitro*.

2 Materials and methods

2.1 Hepatocyte cultures and exposures

Primary hepatocytes were obtained from male F-344 rats, 6 weeks of age, and male CD-1 mice, 6 weeks of age, using collagenase prefusion methods previously described [30]. Rodent hepatocytes were plated on 60 mm collagen-coated plastic culture dishes at a density of ~ 2.2×10^6 cells/plate in plating medium that consisted of Leibovitz's L-15 medium, 28 mM *N*-(2-hydroxyethyl) piperazine-*N*'-(2-ethanesulfonic acid) (HEPES), 10 µM hydrocortizone, 10% fetal bovine serum (heat inactivated), 50 µg/mL gentamicin sulfate, 100 µg/mL kanamycin sulfate, 0.02 units/mL insulin, and 8.3% tryptose phosphate broth. Cells were incubated at 37°C without CO^2. The rodent hepatocytes were allowed to attach for 4 h and then washed with HEPES buffer (2.4 mM HEPES buffer, 140 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2 \times H_2O$, 10 mg/mL Phenol Red, pH 7.4). Rodent hepatocytes were then dosed with 2.5 mL plating medium + 4 µL/mL dimethyl sulfoxide (DMSO) and ± 15 µg/mL MP. Media was changed at 24 h for cells to be exposed 48 h, with the addition of fresh plating media and MP. Human hepatocytes were isolated by collagenase perfusion of pieces of liver tissue as described [31, 32]. Hepatic tissue was obtained from three separate individuals. Case 1 was a 68-year-old male undergoing a hepatic lobectomy for metastatic cancer of the colon. Cases 2 and 3 were a 47-year-old female organ donor and 6-year-old male organ donor. Human hepatocytes were plated at a density of 5×10^6 cells/plate on collagen-coated 60 mm culture dishes in Eagles medium with 5% fetal bovine serum (Hyclone laboratories, Logan, Utah) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 4–5 h. Cultures were subsequently washed 2 × with serum-free media to remove dead and unattached cells and replated in HEPES buffer (2.4 mM HEPES buffer, 140 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2 \times H_2O$, 10 mg/mL Phenol Red, pH 7.4) + 4 µL/mL DMSO ± 90 µg/mL MP. After 6 or 48 h the cells were solubilized in 0.5 mL 2-D PAGE solubilizing solution, which consisted of 9 M urea, 2% Nonidet P-40 (NP-40), 0.5% dithiothreitol, 2% pH 9–11 carrier ampholytes (LKB), then centrifuged at 100 000 × g for 30 min (TL-100 Beckman, TLA-100.3 rotor, 45 000 rpm) [28] and stored at −70°C until 2-D PAGE analysis could be conducted.

2.2 2-D PAGE analysis

Acidic and neutral soluble proteins were resolved based on molecular weight and isoelectric point using sodium dodecyl sulfate-denaturing polyacrylamide gels as previously described [28]. Proteins were separated using the 20 × 25 cm

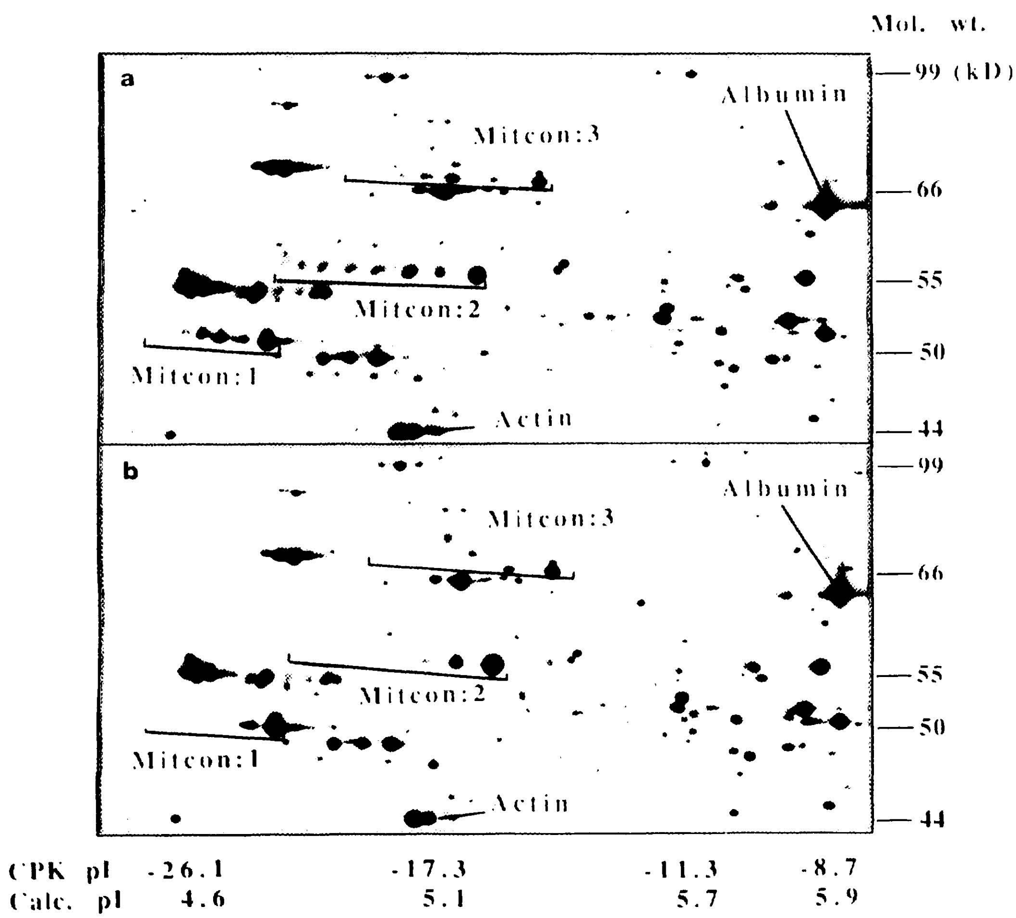

Figure 1. Photograph of Coomassie Blue-stained 2-D PAGE patterns of hepatic proteins from (a) methapyrilene-treated (1000 ppm for 1 week) and (b) control rat. Molecular weights are in kilodaltons (kDa). The isoelectric point has been estimated on standards of carbamylated creatinine phosphokinase (CPK p*I*) [34] or p*I* calculated (p*I* Calc) from CPK charge standard positions. Covalent modifications present in treated samples extend from right to left with unmodified protein on the right. Data analyses were performed using a Kepler® software system (Large Scale Biology Corp.).

ISO-DALT system [33], then fixed and stained using colloidal Coomassie Brilliant Blue G-250 as previously described [28]. Following staining, each stained gel was digitized in red light at 120 micron resolution using either a Molecular Dynamics laser scanner or a Eikonix 78/99 charge coupled device (CCD) scanner. Images were processed using the Kepler software system (Large Scale Biology Corp). The extent of modification of mitochondrial proteins, previously identified using cell fractionation techniques [34], was determined based on the relative abundance of the various charge-modified forms of the parent protein (Fig. 1) and was expressed as a charge modification index (CMI) (Fig. 2). Mitcons:1–3, previously identified as F_1 ATPase, and two mitochondrial matrix proteins were selected as marker proteins with which to calculate the CMI. Overall changes in gene expression caused by MP in the various test systems were also determined by comparing the overall percentage change in protein abundance in each treatment group. Proteins that: (i) had a coefficient of variation of < 20% within a group, (ii) were present in at least all but one of the gel patterns, and (iii) that showed an abundance difference compared to controls that was significant at $p < 0.001$ were summed and normalized to the total number of proteins analyzed in the gel. Due to the limited number of samples in each group, comparisons on individual proteins were not considered statistically reliable.

								Total
Charge (A)	- 6	- 5	- 4	- 3	- 2	- 1	0	
Protein (B)	450	800	1580	2560	4040	7900	12,000	29,330
A • B	- 2700	- 4000	- 6320	- 7680	- 8080	- 7900	0	- 36,680

Charge Index = Total Charges / Total Protein
= -36,680 / 29,330
= -1.25

Figure 2. An example of the calculatinon of the charge modification index (CMI). Spots in the charge train of covalent modification of a specific protein are assigned a relative charge number (row 2). The parent protein has a relative charge of 0. The quantity of protein with each relative charge number, determined by spot size and density, is listed in row 3. Protein x charge (row 4) for each spot is summed and then normalized to the total protein (sum of row 3) to obtain the charge modification index. Successive covalent modifications caused by MP make the protein more acidic and cause an integral change in charge number [28].

3 Results

In rodent hepatocytes each Mitcon protein was modified to approximately the same degree at each time point (Table 1 and Fig. 3). After 6 h of exposure to MP, modifications were observed in the same 4 mitochondrial proteins modified during *in vivo* exposure [28]. Similar to *in vivo* exposures, modifications generated *in vitro* were more extensive in the rat as compared to the mouse, with overall charge indices of –0.3 and –0.07, respetively. By 48 h, overall charge modification indices for Mitcons:1–3 had risen to –0.81 in the rat and –0.33 in the mouse. In comparison, treatment with MP at 1000 ppm in the diet for 10 weeks resulted in a charge index of –1.1 in the rat and –0.14 in the mouse. *In vitro* exposure to 15 µg/mL MP for 48 h also generated covalent modifications in several extramitochondrial proteins (unpublished observations). Unlike rodent hepatocytes, where 25 µg/mL MP has been shown to be toxic [35], human hepatocytes were still 85% viable after 48 h of exposure to 90 µg/mL MP, demonstrated by Trypan Blue exclusion. Concentrations of 90 µg/mL MP, were therefore used in the human hepatocyte studies. Although MP treatment caused similar changes in Mitcons:1–3 within rodent species, changes induced in human Mitcons:1–3 were more heterogeneous

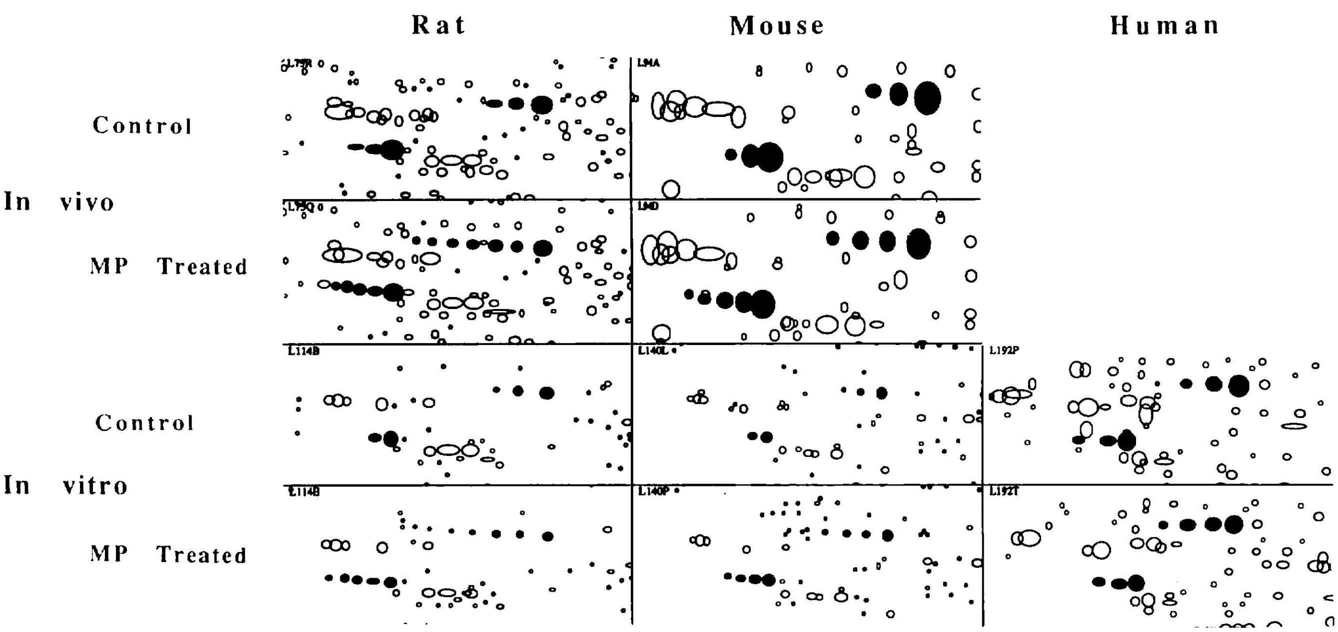

Figure 3. Graphic representations of 2-D PAGE profiles of rat, mouse, and human hepatocytes demonstrating covalent modifications in Mitcon:1 (lower darkened spot) and Mitcon:2 (upper darkened spots) following *in vitro* and *in vivo* exposure to MP. *In vivo* results were previously published [28].

Table 1. Individual and collective CMIs of Mitcons:1–3[a)]

	Time	[MP]	Mitcon:1	Mitcon:2	Mitcon:3	Average	Treated-control
	1W	Control	−0.19	−0.29	−0.23	−0.24	
	1W	1000 ppm	−1.04	−1.42	−1.16	−1.20	−0.96
Rat *in vivo*	1W + 4 WR	Control	−0.13	−0.25	−0.27	−0.22	
	1W + 4 WR	1000 ppm		−0.19	−0.25	−0.22	−0.00
	10W	Control	−0.15	−0.42	−0.45	−0.34	
	10W	1000 ppm	−1.43	−1.52	−1.37	−1.44	−1.10
	10W + 4 WR	Control	−0.14	−0.32	−0.30	−0.25	
	10W + 4 WR	1000 ppm	−0.17	−0.32	−0.34	−0.28	−0.03
Mouse *in vivo*	10W	Control	−0.32	−0.32	★	−0.34	
	10W	1000 ppm	−0.57	−0.46	★	−0.48	−0.14
Rat *in vitro*	6 HRS	Control	−0.14	−0.33	−0.24	−0.27	
	6 HRS	15 ug/mL	−0.39	−0.53	−0.80	−0.57	−0.30
	48 HRS	Control	−0.16	−0.32	−0.40	−0.29	
	48 HRS	15 ug/mL	−1.01	−1.18	−1.11	−1.10	−0.81
Mouse *in vitro*	6 HRS	Control	−0.26	−0.19	★	−0.22	
	6 HRS	15 ug/mL	−0.36	−0.22	★	−0.29	−0.07
	48 HRS	Control	−0.30	−0.27	★	−0.28	
	48 HRS	15 ug/mL	−0.60	−0.62	★	−0.61	−0.33
Human *in vitro*	6 HRS	Control	−0.20	−0.25	−0.23	−0.24	
	6 HRS	90 ug/mL	−0.22	−0.29	−0.30	−0.27	−0.04
	48 HRS	Control	−0.20	−0.29	−0.23	−0.24	
	48 HRS	90 ug/mL	−0.23	−0.48	−0.34	−0.35	−0.11

a) CMI was derived as depicted in Fig. 2. *In vivo* results were previously reported [28]. W = weeks on MP, and WR = weeks off MP prior to sample collection. *In vitro* rodent data is the average of 2–3 animals. Human hepatocyte data is the average of three experiments using hepatocytes from the 3 individuals described in Section 2.1

(Table 1). The extent of covalent modification following 6 h of exposure to 90 μg/mL MP was extremely small (−0.04). The net change in the overall modification indices after 48 h (charge index = −0.11) was due primarily to changes in Mitcon:2 and Mitcon:3 (Table 1). No covalent modification was observed in carbamoyl phosphate synthetase, the fourth mitochondrial protein modified by MP in both rats and mice. Thus it is possible that the slight changes in the charge modification indices observed in human Mitcons:1–3 may have been artifactual.

In addition to causing covalent modifications of mitochondrial proteins in the rodent, MP treatment also appeared to increase the total concentration of these proteins. Covalent modification of Mitcon: 1 resulted in a net decrease in the amount of parent protein; however, a totaling of covalently modified plus parent protein revealed a net increase, consistent with an increase in mitochondrial mass [35] and respiratory function [27].

In a test for changes in overall gene expression at a significance of $p < 0.001$, as described in Section 2.2, 33% (45/136) of rat and 1.4% (2/143) of mouse hepatic proteins demonstrated significant changes in expression following dietary administration of 1000 ppm MP for one week. No changes were observed in human, rat, and mouse hepatocyte proteins following 6 h of treatment as described in Section 2.1. However, following 48 h of MP exposure *in vitro* (concentrations in Table 1), 4% (2/48), 3% (2/64) and 0% (0/26) of rat, mouse, and human hepatic proteins, respectively, showed significant changes in expression.

4 Discussion

In vitro-in vivo systems offer one method to help establish the relevance of rodent bioassay results to man. Traditionally, the results from each experiment are based on one or more endpoints, including ultrastructural changes, DNA binding, nucleolar segregation [36, 37], DNA repair and peroxisomal proliferation [6]. Using 2-D PAGE, the present studies have simultaneously examined the effects of MP on a large number of endpoints comprised of proteins that make up a significant part of the molecular anatomy of hepatic tissue. These results demonstrate that mouse and rat hepatocyte cultures provide models for approximating the species-specific effects of MP on these proteins *in vivo*. Furthermore, the correlation between the *in vitro* and *in vivo* systems supported the use of human hepatocytes to assess the effects of MP on human hepatic proteins.

Comparisons among the overall charge modificatioins showed a ratio of ≈ 8:1.5:1 and ≈ 8:3:1 (rat:mouse:man) at 6 and 48 h, respectively. Considering that a 6-fold greater concentration of MP was used on human hepatocytes, it is concluded that MP treatment is much less effective, if effective at all, at modifying mitochondrial proteins in these cells. The *in vivo* to *in vitro* correlations observed in rodents suggest that the response of human hepatocytes reflects the response likely to occur in man. If so, modifications in humans probably occur at a very low levels, if at all. These studies showed that changes induced by MP treatment *in vivo* and *in vitro* correlate with carcinogenic susceptibility. Although the relationship between carcinogenicity and the mitochondrial modifications observed remains to be established, these data suggest that the rat may not be the most relevant model with which to assess carcinogenic potential of MP to humans.

Preliminary work analyzing overall changes in protein expression was also conducted. During a one-week *in vivo* exposure, rats demonstrated marked changes in protein expression as compared to mice. During *in vitro* exposure, however, protein changes were not as great, with no detectable changes at 6 h and small changes at 48 h. This temporal dependence indicated that continued exposure was required for protein changes to be manifest. Although MP induced equal changes in expression in rat and mouse hepatocytes, no changes were observed in human hepatocytes. While these results provide additional support for a differential effect of MP in rats as compared to humans, further work will be necessary to fully characterize changes induced in hepatocytes by MP because the power of the analysis was severely limited by the small number of samples.

In summary, these studies provide a model by which 2-D PAGE technology can be used to study and compare the effects of xenobiotics on a large number of human and rodent proteins. As more proteins are identified, these comparisons could provide even greater insight into the appropriate use of bioassay data in assessing human risk.

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