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The male rat carcinogens limonene and sodium saccharin are not mutagenic to male Big Blue™ rats

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Limonene and sodium saccharin are male rat specific carcinogens giving rise to renal and bladder tumours, respectively. Both compounds give negative results in genetic toxicity assays suggesting a non-genotoxic mode of action for their carcinogenicity. The α2U-globulin accumulation theory has been invoked to explain the renal carcinogenicity of limonene: the accumulation of micro masses of calcium phosphate in the bladder, coupled with a high pH environment in the male rat bladder, has been suggested to be responsible for the bladder carcinogenicity of sodium saccharin. The implication of these proposed mechanisms is that limonene and sodium saccharin will not be mutagenic to the rat kidney and bladder, respectively. This proposal has been evaluated by assessing the mutagenic potential of the two chemicals to male *lacI* transgenic (Big Blue™) rats. Male BigBlue™ rats were exposed for 10 consecutive days to either limonene in diet, at a dose level in excess of that used in the original National Toxicology Program gavage carcinogenicity bioassay, or to sodium saccharin in diet at the dose known to induce bladder tumours. The multi-site rat carcinogen 4-aminobiphenyl was used as a positive control for the experiment. Limonene failed to increase the mutant frequency in the liver or kidney of the rats, and sodium saccharin failed to increase the mutant frequency in the liver or bladder of the rats. 4-Aminobiphenyl was mutagenic to all three of these tissues. These results add further support to a non-genotoxic mechanism of carcinogenic action for both limonene and sodium saccharin.

Introduction

The US National Toxicology Program (NTP) have reported that gavage exposure of male rats to limonene is associated with dose-responsive increases in the incidences of nephropathy, renal hyperplasia and renal tumours (Kanerva and Alden, 1987; Anon, 1990; Whysner and Williams, 1996; Hard, 1998). The mechanism of tumorigenesis is thought to be a result of an accumulation of α2u-globulin in the cells of renal proximal tubules, and this, coupled with the absence of genetic toxicity for limonene *in vitro* (Watabe *et al.*, 1980; Haworth *et al.*, 1983; Anon, 1990; von der Hude *et al.*, 1990) is consistent with it being a non-genotoxic rodent carcinogen.

The carcinogenicity of sodium saccharin is essentially specific to the bladder of the male rat with minor effects occurring in the female rat bladder (reviewed by Whysner and Williams, 1996). As with limonene, sodium saccharin is negative in almost all genotoxicity assays (reviewed by Ashby *et al.*, 1978; Ashby, 1985; Whysner and Williams, 1996). This has lead to sodium saccharin being classified as a non-genotoxic carcinogen (Ashby *et al.*, 1978; Ashby, 1985; Ellwein and Cohen, 1990). It is suggested that sodium saccharin combines with proteins in the bladder, and this, coupled to a high pH environment which is unique to the male rat bladder, leads to the accumulation of amorphous masses of primarily calcium phosphate in this tissue (Whysner and Williams, 1996). These masses are considered to be responsible for the induction of urothelial hyperplasia, via micro-abrasion, leading ultimately to bladder tumours. In addition, the carcinogenicity of sodium saccharin may be compounded when neonatal rats are exposed during the first three weeks after birth due to the highly proliferative nature of the immature bladder (Cohen *et al.*, 1998).

Transgenic rodent mutation assays present a technique for detecting mutations *in vivo* in any tissue, and hence they may offer insight into the mechanism of action of compounds such as limonene and sodium saccharin. This paper describes the use of male Big Blue™ (*lacI*) transgenic rats in determining whether limonene and sodium saccharin are capable of inducing mutations in the kidney and bladder, respectively, in exposed animals. For these experiments 4-aminobiphenyl was used as a positive control mutagen.

Materials and methods

Chemicals

4-Aminobiphenyl (4AB) was obtained from Lancaster Synthesis (Morecambe, UK) and was prepared for administration as a suspension in corn oil. Animals received a dose of 4AB previously shown to induce a positive response in Muta™Mouse transgenic mice liver and kidney (Fletcher *et al.*, 1998). Limonene and sodium saccharin were purchased from Sigma (Poole, Dorset, UK) and were ground into the standard diet (CT1) using an automatic pestle and mortar to give final levels of 1 and 5%, respectively, in diet. Based on animals consuming ~20 g diet/day, the daily exposure level to limonene was ~525 mg/kg. The original NTP carcinogenicity bioassay employed a gavage dose level of 150 mg/kg/day limonene for 5 days a week over 2 years. Although the dietary dose was in excess of the published gavage dose level, it is known that limonene degrades when in diet (Anon, 1990). Thus, analyses of the diet, as described below, were undertaken at reference time points corresponding to pre, mid and post dosing to ascertain the level of exposure to this compound. A dose of 5% sodium saccharin is commonly recognized as inducing significant bladder epithelial proliferation leading to the development of tumours (Cohen *et al.*, 1998).

Stability of limonene in diet

The concentration of limonene in CT1 diet which had been stored at room temperature was determined immediately following preparation, 24 h prior to use, and after 3 and 10 days of exposure (last day of dosing) by gas chromatography (GC). Samples of diet containing limonene were extracted with methanol by sonication for 15 min followed by mechanical shaking for 30 min and filtration through a PTFE 0.45 μm filter. Aliquots of the supernatant liquid were diluted with methanol, as appropriate, to give sample solution concentrations within the range of the calibration standards used (10–50 μg/ml limonene in methanol). The extracts were analysed by GC using a 15 m×0.53 mm DB 17 column, a helium carrier at a flow rate of 6 ml/min and a flame ionization detector.

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Table I. Analyses of limonene in diet

Nominal concentration (p.p.m.)	Analysis interval (days)	Analysed mean concentration (p.p.m.)	Percent of initial concentration	Approximate limonene dose (mg/kg)
10000	0	9816	100	680
	1	7536	76.8	522
	5 (= day 3 of exposure)	5780	58.9	400
	12 (= day 10 of exposure)	5201	53	360

Analyses of limonene in diet immediately following preparation, 24 h after preparation and days 3 and 10 of exposure. The dose (mg/kg) of limonene was calculated based on a 300 g rat eating ~20 g diet per day.

Table II. Induction of *lacI* mutations in the liver of Big Blue^{TM} rats

Compound	Dose	Animal no.	Total PFU	Mutant PFU	MF$\times 10^{-6}$	Mean ± SD
CT1 diet	–	1	223 665	6	26.8	14.4 ± 8.9
		2	195 500	4	20.5	
		3	218 650	4	18.3	
		4	194 425	2	10.3	
		5	162 325	1	6.2	
		6	239 175	1	4.2	
Limonene	1% in diet	11	165 075	4	24.2	16.2 ± 10.4
		14	190 050	2	10.5	
		15	285 500	6	21	
		16	199 400	4	20	
		17	225 000	0	0	
		19	201 925	6	30	
		20	124 900	1	8	
Sodium saccharin	5% in diet	23	165 875	7	42.2	17.2 ± 14.6
		24	234 125	1	4.3	
		25	286 525	1	3.5	
		26	203 500	1	4.9	
		27	208 425	6	28.8	
		28	198 850	4	20.1	
		29	121 900	2	16.4	
4AB	20 mg/kg	31	212 300	8	37.7	44.9 ± 12.7**
		32	193 900	7	36.1	
		33	183 250	8	43.7	
		34	241 975	8	33	
		37	119 575	8	67	
		40	231 425	12	51.9	

Induction of *lacI* mutations in the liver of Big Blue^{TM} rats 14 days after the last of 10 daily exposures to the appropriate compound. Data were analysed for statistical significance as described herein, $^{**}P < 0.01$.

Animals and study design

Male Big Blue^{TM} transgenic rats were purchased from Stratagene Taconic Farms, Germantown, N.Y., and were housed and maintained under quarantine conditions as described previously (Tinwell *et al.*, 1994). Animals were approximately 12 weeks old at the start of the study and weighed approximately 300 g. Groups of 10 rats received either CT1 diet (negative control), diet containing 1% limonene, diet containing 5% sodium saccharin or 20 mg/kg bodyweight 4AB administered by oral gavage (positive control agent) daily for 10 consecutive days and then killed on day 14 after termination of dosing. This protocol has been shown earlier to give a strong positive response for 4AB (Fletcher *et al.*, 1998). The body weight of each rat was recorded on a daily basis before dosing and prior to termination and all of the animals were observed daily during the study for their physical appearance and activity.

Mutation assay

Animals were killed 14 days after the final dose. The liver, kidney and bladder were removed and flash frozen in liquid nitrogen prior to storage at –70°C. DNA was isolated from liver and kidney tissue using the Recoverease kit (Stratagene) and from pooled whole bladders using the Big Blue^{TM} DNA Extraction kit (Stratagene). The mutation assays were carried out as previously described (Tinwell *et al.*, 1994). Briefly, DNA was packaged using Transpack extracts (Stratagene) and the resultant phage were allowed to infect *Escherichia coli* cultures (SCS-8, Stratagene) for the screening of *lacI* mutants in the form of blue plaques. The mutant frequency (MF) was determined for the liver, bladder (with the exception of animals exposed to limonene) and the kidney (with the exception of animals exposed to sodium saccharin) for each test group. Approximately 200 000 plaque forming units (p.f.u.'s) were analysed for the presence of mutations for liver and kidney DNA samples. In the case of the bladder samples, the number of p.f.u.'s analysed was dependent on the amount of DNA extracted from the tissue, which itself was usually low.

Statistical analyses

The data in Tables II–IV were analysed using simple two-sample comparisons, comparing test MFs with the corresponding CT1 control group. Statistical analyses were performed as per the methods summarized by Piegorsch *et al.* (1997), with one modification: as MFs in transgenic rodents often tend to exhibit statistical overdispersion (Piegorsch *et al.*, 1997), the per-animal MFs could not be pooled into a simple two-sample test for proportions, such as Fisher's exact test (see Weerahandi, 1997), but rather required a more sophisticated approach. Following on similar recommendations given previously (Carr and Gorelick, 1994; Piegorsch *et al.*, 1995), we applied a generalized two-sample score statistic as given in Piegorsch *et al.* (1997). We used the statistic to determine if the null hypothesis of no difference in mutant frequencies between the two groups was plausible, versus an alternative hypothesis of a positive difference. We decided in favour of the alternative if the test's *P*-value dropped below a pre-assigned a-level.

For either tissue, each two-sample comparison was made with respect to the same reference group (CT1 diet), hence the inferences were corrected for

Table III. Induction of *lacI* mutations in the kidney of Big Blue™ rats

Compound	Dose	Animal no.	Total PFU	Mutant PFU	MF×10^{-6}	Mean ± SD
CT1 diet	_	1	224 475	4	17.8	17.75 ± 8.0
		2	221 550	2	9.03	
		3	243 450	5	20.5	
		4	230 075	4	17.4	
		5	299 075	7	20.1	
		6	185 300	4	21.6	
		7	211 275	7	33.1	
		8	198 850	1	5.02	
		9	197 150	2	15.2	
Limonene	1% in diet	11	211 625	3	14.2	21.7 ± 12.8
		12	294 100	6	15.2	
		13	238 775	8	33.5	
		14	190 225	8	42	
		15	199 525	6	30.1	
		16	227 225	1	4.4	
		17	313 525	10	31.9	
		18	254 400	1	3.93	
		19	236 150	6	25.4	
		20	250 225	4	16	
4AB	20 mg/kg	31	227 975	15	65.8	123 ± 106**
		32	227 450	22	96.7	
		33	266 350	19	71.3	
		34	230 375	16	69.5	
		36	36 950	4	108.3	
		37	12 675	2	157.8	
		38	12 050	1	83	
		39	2540	1	394	
		40	15 400	1	64.9	

The induction of *lacI* mutations in the kidney of Big Blue™ rats 14 days after administration of the final dose of compound. Data were analysed for statistical significance as described herein; $^{**}P < 0.001$.

multiplicity. A simple Bonferroni adjustment took each *P*-value and multiplied it by the number of comparisons made from the same set of data (here, two) to arrive at the reported, multiplicity-adjusted *P*-value.

Results

Stability of limonene in diet

The analysis of limonene in CT1 diet, at an initial concentration of 10 000 p.p.m. (1% in diet), when stored at room temperature indicated that it degraded approximately linearly to 53% of the initial concentration after 12 days (Table I). This equated to the animals being exposed to ~522 mg/kg at the start of the experiment and to ~360 mg/kg at the end of the 10 day exposure period (12 days after preparation). The concentration of limonene in diet at the end of the exposure period was therefore at least double that used in the 2 year NTP cancer bioassay (150 mg/kg).

Mutation assays

Limonene

There was no evidence of a significant increase in the MF in either the liver (Table II) or kidney (Table III).

Sodium saccharin

Animals exposed to this compound had small reductions in body weights compared with the control groups, but otherwise appeared healthy during the course of their treatment. There was no significant increase in MF in either the liver (Table II) or bladder (Table IV) of rats exposed to sodium saccharin. Due to difficulties experienced in isolating bladder DNA, the usual 200 000 p.f.u./sample were not obtained for this tissue. However, the results clearly show an increase in mutant frequency in the bladder of rats exposed to the bladder carcinogen 4AB and the absence of an increase for sodium saccharin (Table IV).

Table IV. Induction of *lacI* mutations in the bladder of Big Blue™ rats

Compound	Dose	Total PFU	Mutant PFU	MF×10^{-6}
CT1 diet	_	56 821	1	17.6
Sodium saccharin	5% in diet	40 450	0	0
4AB	20 mg/kg	55 925	4	71.5**

The induction of *lacI* mutations in the bladder of Big Blue™ rats 14 days following administration of the last of 10 daily doses of the appropriate compound. DNA was isolated from the pooled bladders of each group. Data were analysed for statistical significance as described herein; $^{**}P < 0.001$.

4AB

This compound induced a significant increase in MF in both liver (Table II) and kidney (Table III). Despite the difficulties experienced in obtaining high quality DNA from the bladder, a significant increase in MF was also observed in this tissue (Table IV). These results support those seen in transgenic mice (Fletcher *et al*., 1998).

Discussion

Limonene and sodium saccharin are tissue-specific in their carcinogenic properties, but are inactive in conventional genotoxicity assays. Thus, limonene induces renal tumours in male rats only (Anon, 1990) and is devoid of activity in the Salmonella mutation assay (± S9; Watabe *et al*., 1980; Haworth *et al*., 1983), is negative in the rat liver unscheduled DNA synthesis (UDS) assay (von der Hude *et al*., 1990) and does not induce sister chromatid exchange in CHO cells (Anon,

1990). More recently, limonene was reported to give a negative response in the $p53^{+/-}$ transgenic mouse (Carmichael *et al.*, 2000). Sodium saccharin is a male rat-specific bladder carcinogen which is negative in the majority of *in vitro* genotoxicity assays (reviewed by Ashby, 1985; Ellwein and Cohen, 1990; Whysner and Williams, 1996).

The positive control agent 4AB was mutagenic to the liver, kidney and bladder of the Big Blue™ transgenic rats, consistent with effects reported earlier for male Muta™Mice (*lac Z*; Fletcher *et al.*, 1998). The absence of a mutagenic response in the kidney and liver of male Big Blue™ transgenic rats exposed to limonene in diet, at a dose level at least 2-fold that used in the original NTP gavage carcinogenicity bioassay, is consistent with a non-genotoxic mechanism of carcinogenic action for this chemical.

The negative mutation data obtained for sodium saccharin in the rat bladder and liver are also consistent with it operating by a non-genotoxic mechanism of carcinogenic action. It has been proposed that the tumorogenicity of this compound is due to the formation of microcrystals in the bladder. It is thought that these crystals, composed of silicates and proteins (possibly albumin and the male rat-specific protein α2u-globulin) cause microabrasion of the urothelial surface exposing underlying cells to urine. This abrasion, in the context of the high urinary pH in saccharin-treated animals, is proposed to lead to an increase in mild, focal regenerative bladder epithelial cell hyperplasia, leading to tumour induction (Ellwein and Cohen, 1990; Whysner and Williams, 1996; Cohen *et al.*, 1998). It is known that similar mechanical irritation of the bladder epithelium can lead to tumour development through the induction of increased cell proliferation. For example, gallstones have been shown to give rise to lesions in the gall bladder (Lowenfels *et al.*, 1989) and melamine induces bladder tumours through the induction of urolithiasis (Melnick *et al.*, 1984).

More recently, Takahashi *et al.* (2000) have shown that increases in MF could be detected following mechanical irritation of the bladder of Big Blue™ rats through chronic exposure to uracil. In that study increases in the bladder MF were not observed until after at least 10 weeks of exposure to uracil. This suggests that it is not uracil *per se* which is mutagenic, but rather, the sustained hyperplasia induced by it in the bladder. It is possible, therefore, that sodium saccharin would have increased the MF in the bladder had the exposure period been extended to beyond 10 weeks. However, such a late occurring derivative mutagenic effect would be associated with saccharin-induced hyperplasia, as opposed to with sodium saccharin itself.

Whilst the present data cannot be used to define a non-genotoxic mechanism of carcinogenic action for either limonene or sodium saccharin, they support an earlier consideration of such a mechanism (IARC, 1999). In contrast, 4-AB is confirmed as being a multi-site mutagen to the rat, consistent with its potent carcinogenic activity in rodents and humans.

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