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The mutagenic activity of select azo compounds in MutaMouse target tissues *in vivo* and primary hepatocytes *in vitro*

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ABSTRACT

The mutagenicity of Direct Black 38, Sudan I, and Para Red were evaluated in the *in vivo* MutaMouse assay and the *in vitro* MutaMouse primary hepatocyte (PH) assay. Direct Black 38 is an International Agency for Research on Cancer (IARC) Group 1 carcinogen and a prototypical benzidine-based azo compound that requires azo-reduction to yield a DNA-reactive metabolite. Sudan I and Para Red are structurally related azo compounds that have been detected as illegal contaminants in foods. Sudan I is an *in vivo* mutagen, and both it and Para Red are known to be mutagenic *in vitro*. Sudan I is oxidized by hepatic and/or bladder enzymes to yield a mutagenic metabolite, but little is known about Para Red. In the present study, Direct Black 38 elicited a significant mutagenic response in the bone marrow, glandular stomach, small intestine and colon *in vivo*, and in PHs *in vitro*. Sudan I elicited a weak positive response in the bone marrow and a marginally significant treatment effect in the bladder ($p = 0.059$); it did not elicit a significant response in PHs *in vitro*. Para Red elicited a positive response in the colon, as well as in PHs *in vitro*, albeit at a cytotoxic concentration. The findings are well aligned with the known mechanisms of action of Direct Black 38 and Sudan I; they suggest that intestinal azo-reduction plays an important role in the activation of Para Red. The MutaMouse pH results illustrate the ability of this assay to detect chemicals requiring azo-reduction; however, they also demonstrate a gap in applicability domain, as MutaMouse PHs elicit a negative response following exposure to Sudan I. Elucidation of the mechanisms underlying this gap will require further study.

1. Introduction

Approximately 60–80% of all commercially used colourants are azo compounds [1]. Azo colourants are synthetically manufactured, and used extensively in paints, textiles, personal care products, and inks [2].There is evidence that some azo compounds may pose a genotoxic and/or carcinogenic hazard; the International Agency for Research on Cancer (IARC) has classified 37 azo compounds with respect to their carcinogenicity. Three compounds, described as dyes metabolized to benzidine *via* azo-reduction, have been classified as Group 1 carcinogens (known human carcinogens), eleven compounds have been classified as Group 2B carcinogens (possible human carcinogens), and twenty-three compounds have been classified as Group 3 agents (not classifiable as to their carcinogenicity to humans) [3]. The government of Canada's Chemicals Management Plan (CMP) recently completed an assessment of 358 aromatic azo- and benzidine-based substances [2]. The assessment concluded that, although azo compounds pose a hazard, and Canadians are exposed through their use in commercial products,

Abbreviations: IARC, International Agency for Research on Cancer; CMP, Chemicals Management Plan; CYP, cytochrome P450; FMN, flavin mononucleotide; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PH, primary hepatocyte; UDS, unscheduled DNA synthesis; MN, micronucleus; *HPRT*, hypoxanthine phosphoribosyltransferase; SCE, sister chromatid exchange; CHO, Chinese hamster ovary; TGR, transgenic rodent; DMEM, Dulbecco's modified Eagle's medum; PBS, phosphate-buffered saline; FBS, foetal bovine serum; EGF, epithelial growth factor; HBSS, Hank's Balanced Salt Solution; DMSO, dimethylsulphoxide; P-Gal, Phenyl-β-D-galactopyranoside; SFM, Serum-Free Medium; RINC, relative increase in nuclear counts; RICC, relative increase in cell counts; SSC, side scatter; FSC, forward scatter; MF, mutant frequency; pfu, plaque-forming unit; NQO1, NAD(P)H quinone oxidoreductase; BDI, benzenediazonium ion; AhR, aryl hydrocarbon receptor; HRP, horseradish peroxidase; LOEL, lowest observable effect level; +ve, positive; −ve, negative; E, equivocal; NA, not available; PO, oral administration; MLA, mouse lymphoma assay; CA, chromosome aberration; Subcu, subcutaneous administration; BM, bone marrow; GS, glandular stomach; SI, small intestine; Co, colon; Lv, liver; Bl, bladder

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the exposure is too low to pose a significant risk. Nevertheless, it also concluded that these substances would be a concern if exposure levels increase.

Numerous cohort and case-control studies conducted over the last 50 years have shown a correlation between exposure to benzidine, and some benzidine-based dyes, and a higher incidence of cancers, especially bladder cancer [4]. These data are further supported by animal carcinogenicity studies, toxicokinetic studies, and *in vitro* mechanistic studies. Thus, effective health hazard/risk evaluation of azo compounds must consider metabolism, the potential for enzymatic cleavage to yield carcinogenic aromatic amines and/or benzidine. Benzidine-based dyes make up only a small subset of azo bond-containing compounds currently used in consumer products. For the most part, there is a paucity of information regarding the carcinogenicity, genotoxicity, and metabolism of azo compounds in general, thus it is imperative to apply tools that involve metabolically competent cells. The need for effective genetic toxicity screening tools is particularly acute, since azo compound data are lacking, and testing is complicated by low solubility and the metabolic limitations of available tools [5]. Despite the low solubility that complicates genetic toxicity screening, it should be noted that human exposures to insoluble azo pigments can occur *via* direct contact with very common consumer products (*e.g.*, plastics and polymers, paper, surface coatings) [5].

Azo-reduction, rather than oxidation, is thought to be the major route of bioactivation of most azo compounds. It has been estimated that out of the several thousand azo compounds that have been produced, at least 500 can potentially yield carcinogenic aromatic amines through azo-reduction [6]. In mammals, azo-reduction can occur in either the gut or the liver, and may also be catalysed by bacteria on the skin [6]. Azoreductase activity in the liver is mediated by cytochrome P450 (CYP) isozymes in the microsomal fraction, and NAD(P)H quinone oxidoreductase (NQO1) in the cytosolic fraction [5]. Moreover, it is believed that anaerobic bacterial azoreductase activity is important in the gastrointestinal metabolism of soluble azo dyes. Dye metabolites can subsequently be absorbed *via* the gut and further processed in the liver. Once released, the aromatic amine can undergo *N*-hydroxylation and *O*-acetylation to eventually yield DNA-reactive nitrenium or carbenium ions [7,8].

The aforementioned complex routes of metabolic bioactivation of azo compounds pose a problem for conventional *in vitro* genotoxicity assays, thus necessitating modifications of standard assay protocols. One notable protocol modification is the so-called Prival variation of the Ames/Salmonella mutagenicity assay. The modification utilizes uninduced hamster liver S9, instead of the more conventional Aroclor 1254-induced rat liver S9, and flavin mononucleotide (FMN) in the cofactor mix [9–11]. FMN serves as a reducing agent to facilitate azo reduction. Unlike induced rat liver S9, uninduced hamster S9 does not preferentially detoxify benzidine and benzidine-based compounds, thus yielding mutagenic metabolites [9]. Conventional mammalian cell genetic toxicity assays typically use cell lines that are not metabolically competent, thus relying on the addition of Aroclor 1254-induced rat liver S9. Aroclor 1254-induced rat liver S9 enables CYP mediated Phase I oxidation reactions, and typically the S9 mix does not include the cofactors necessary for Phase II conjugation reactions (*e.g.*, 3′-phosphoadenosine-5′-phosphosulfate [PAPS] for sulfotransferase enzymes) or reductive metabolism (*e.g.*, FMN) [9,12,13]. Additionally, induced rat liver S9 is known to cause cytotoxicity in cultured mammalian cell lines [14,15]. An assay that incorporates cells that are metabolically competent, such as primary hepatocytes (PHs), would serve as a helpful tool for the assessment of azo compounds, due to their metabolic activation mechanisms requiring both reductive and oxidative metabolism.

Direct Black 38 is a prototypical benzidine-based azo dye (Fig. 1 A); its metabolism is known to release benzidine. Based on the results of epidemiologic studies in occupational settings, and carcinogenicity studies in experimental animals, Direct Black 38 and similar benzidine-based azo dyes, have been classified by IARC as known human carcinogens (Table 1) [16–20]. Direct Black 38 elicits positive responses in the Ames/Salmonella mutagenicity assay *in vitro*, and these responses are substantially more potent in assays modified to include reductive metabolism (Table 1). Direct Black also yields positive responses in the unscheduled DNA synthesis (UDS), micronucleus (MN), and comet assays *in vivo* (Table 1). Direct Black 38 has not been tested in any *in vitro* genotoxicity assay in mammalian cells, however. Direct Black 38 is thought to be metabolically activated mainly *via* azo-reduction by anaerobic bacteria in the intestinal tract [20–25].

Sudan I and Para Red are structurally related, as they are both 1-amino-2-naphthol-based azo compounds (Fig. 1 B and C). Sudan I is well-studied, in part because it is the simplest in a series of azo compounds that are extensively used world-wide in oils, waxes, printing inks, textiles, and cosmetics [5,26]. In 2003, European authorities reported Sudan I contamination in chilli products [27,28]; this report was followed by numerous additional reports of foodstuffs contaminated by Sudan I and related dyes, including Para Red [29–33]. Sudan I has been designated by IARC as Group 3; the available information did not permit classification of its carcinogenicity to humans (Table 1) [34,35]. Sudan I has elicited positive responses in the Ames test, *in vitro* mammalian cell genotoxicity assays (*i.e.*, the hypoxanthine phosphoribosyltransferase forward mutation assay [*HPRT*] gene mutation assay in AHH-1 and MCL-5 cells, the MN assay in AHH-1, MCL-5, and HepG2 cells, the sister chromatid exchange [SCE] assay in Chinese hamster ovary [CHO] cells, and the comet assay in HepG2 cells), as well as several *in vivo* genotoxicity assays (Table 1). Para Red has also elicited positive responses in the Ames test, as well as the *in vitro* MN assay and the *HPRT* forward mutation assay (Table 1). Unlike Direct Black 38 and many other azo compounds, the main route of Sudan I activation is not *via* hepatic or bacterial azo-reduction, but rather *via* hepatic CYPs and/or bladder peroxidases [26]. Very little data is available concerning the metabolism and mutagenic mode of action of Para Red; however, its structural similarity to Sudan I suggests that its activity may be similar [36].

In the present study, the mutagenicity of azo compounds Direct Black 38, Sudan I, and Para Red are assessed *in vivo* in several tissues of the MutaMouse, and *in vitro* in MutaMouse PHs. Although Direct Black 38 and Sudan I are relatively well-studied, neither has been assessed in a transgenic rodent (TGR) assay; Para Red is an under-studied azo compound. Mutagenicity assessment in several MutaMouse tissues will provide insight into the modes of action of the studied compounds; indeed, information to determine whether the mechanism of action of Para Red is, as predicted, similar to Sudan I (*i.e.*, oxidative metabolic activation), or rather similar to Direct Black 38 (*i.e.*, reductive metabolic activation). These mutagenicity assessments will provide additional insight into the metabolic activation of the selected azo compounds. Preliminary characterization and validation studies have demonstrated that the *in vitro* MutaMouse PH gene mutation assay is capable of detecting mutagens requiring diverse types of metabolic activation [37,38]. This comparison of *in vitro* and *in vivo* methodologies will provide important information regarding the utility of the *in vitro* MutaMouse PH assay to accurately assess compounds that undergo complex metabolism *in vivo*.

2. Materials and methods

2.1. Materials and reagents

Direct Black 38 (95% dye purity) and Sudan I (95% dye purity) were synthesized by TC Scientific (Edmonton, Alberta). Para Red (95% dye purity) was obtained from Sigma-Aldrich Canada (Oakville, Ontario). The composition of the impurities in the azo compounds is not known. Dulbecco's modified Eagle's medium (DMEM), William's E medium, phosphate-buffered saline (PBS), foetal bovine serum (FBS), epithelial growth factor (EGF), penicillin-streptomycin reagent, Hank's balanced

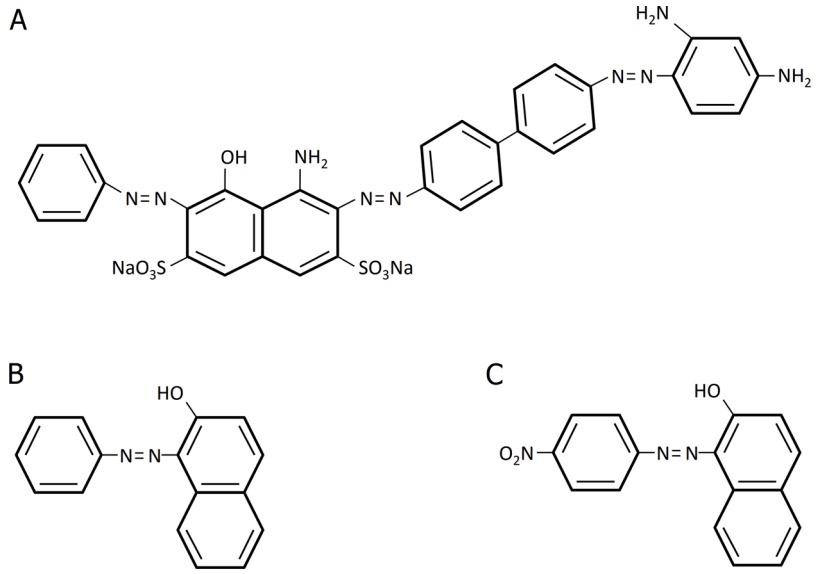

Fig. 1. Structures of Direct Black 38 (A), Sudan I (B), and Para Red (C).

salt solution (HBSS), proteinase K, trypan blue, and SYTOX® green were obtained from Life Technologies (Burlington, Ontario). Corning® Biocoat™ type I collagen-coated culture dishes were obtained from VWR International (Mississauga, Ontario). CIzyme™ collagenase HA (high activity) and BP (*Bacillus polymyxa*) protease were obtained from VitaCyte LLP (Indianapolis, Indiana). Dexamethasone, human insulin, dimethylsulphoxide (DMSO), olive oil, Percoll®, bovine serum albumin (BSA), and IGEPAL CA-630 were obtained from Sigma-Aldrich Canada (Oakville, Ontario). Phenyl-β-D-galactopyranoside (P-Gal) was obtained from MJS BioLynx (Brockville, Ontario). TransPak Packaging Extract was obtained from Agilent Technologies Canada (Mississauga, Ontario).

2.2. Animal treatment

All MutaMouse animals used in this study were bred and maintained locally under conditions approved by the Health Canada Animal Care Committee. Adult male MutaMouse animals aged 9 to 10 weeks were housed individually on a 12 h light / 12 h dark cycle, and provided standard rodent chow and water *ad libitum*. The animals were dosed daily *via* oral gavage for 28 days. Each dose group, including vehicle controls, contained 5 animals. Direct Black 38 was dissolved in water (250, 500, and 1000 mg/kg body weight/day). Sudan I was dissolved in olive oil and Para Red were dissolved in olive oil with 1% DMSO. Sudan I was tested using doses of 100, 200 and 300 mg/kg body weight/day and Para Red was tested using doses of 100, 200, and 400 mg/kg body weight/day. Doses were selected based on preliminary range-finding studies performed for each chemical; however, despite being well-tolerated in the range-finding study, the animals in the 300 mg/kg Sudan I dose group displayed unacceptable toxicity and were euthanized by cervical dislocation before the end of the main-study gavage period. Partway through the study, due to aspiration of the chemical into the lungs, one animal in the Sudan I 100 mg/kg group died shortly following gavage dosing. This reduced the group size to 4. All remaining animals were euthanized 3 days following the end of the gavage period [39] by cardiac puncture under isoflurane anaesthesia, followed by cervical dislocation and chest cavity opening. Tissues, including the bone marrow, glandular stomach, small intestine, colon, liver, and bladder, were collected, processed, flash frozen in liquid nitrogen, and stored at -80 °C according to previously established methods [40].

2.3. Isolation, culture, and exposure of PHs

MutaMouse PHs were isolated as described previously [37]. Briefly, cells were obtained using a two-step collagenase technique, with the addition of a Percoll® isodensity purification step [41,42]. The cells were plated at a density of 1.2×10^6 cells/dish onto 100 mm collagen-coated culture dishes using Attachment Medium (20 U/L human insulin, 4×10^{-6} mg/mL dexamethasone, 10% FBS, and 100 U/mL penicillin-streptomycin in DMEM), and incubated at 37 °C and 5% CO_2. Two hours (t = 2 h) following plating, the Attachment Medium was replaced with Serum-Free Medium (SFM; 10 mM HEPES, 2 mM L-glutamine, 10 mM pyruvate, 0.35 mM L-proline, 20 U/L human insulin, 4×10^{-6} mg/mL dexamethasone, 0.01 µg/mL EGF, and 100 U/mL penicillin-streptomycin in Williams Medium E), and the plates were incubated at 37 °C and 5% CO_2.

MutaMouse PHs were exposed to test chemicals as described previously [38]. Briefly, stock solutions of the azo compounds were prepared in DMSO. After 18 h of culture, PHs were exposed to the Direct Black 38, Sudan I, and Para Red at concentrations of 5, 10, 25, 50, and 100 µg/mL in SFM with 1% DMSO for 6 h at 37 °C and 5% CO_2. Three biological replicates (*i.e.*, separate experiments using PHs from three different donor mice) were used for each test chemical. Following exposure, the medium was replaced with fresh SFM, and the hepatocytes were incubated for a further 72 h prior to lysis and DNA isolation.

2.4. Cytotoxicity measurement

Cytotoxicity was measured using the relative increase in nuclear counts (RINC) metric. The RINC metric is analogous to the widely accepted relative increase in cell counts (RICC) metric [43]. RINC was quantified by flow cytometry using a method described previously [37,38,44–46]. Briefly, cultured hepatocytes were lysed, and fluorescently labelled polystyrene microspheres added to each sample to normalize nuclei counts [37,38]. Each microsphere-lysate sample was diluted 1:10 prior to flow cytometric analysis. Data were acquired using a BD Biosciences FACScalibur flow cytometer (BD Biosciences, Mississauga, Ontario) equipped with a 488 nm laser. Instrumentation settings and data acquisition were facilitated using CellQuest Pro software (BD Biosciences). Data analysis was performed using Flowing Software version 2.5.1 (Turku Centre for Biotechnology, Turku, Finland). SYTOX® green and bead fluorescence emission were captured in the FL1 channel (530/30 band-pass filter). Events were scored as nuclei following the application of key criteria (*i.e.*, within a side scatter (SSC) *vs.*

Table 1
Summary of genotoxicity and carcinogenicity data for Direct Black 38, Sudan I, and Para Red.

Chemical (CAS)	Genotoxicity profile			Carcinogenicity findings	References
	Ames Test[a]	*In vivo* genotoxicity tests	*In vitro* mammalian cell tests		
Direct Black 38 (1937-37-7)	+ve[b] in TA1538 (+S9) and TA98 (+S9). -ve[c] in TA1538 (-S9), TA98 (-S9), TA100 (±S9), and TA1535 (±S9).	+ve for UDS[d] in rat liver and MN[e] in rat bone marrow. +ve for comet in mouse stomach, colon, liver, bladder, brain and bone marrow.	NA[f]	IARC Group 1 -Increased risk of human bladder and colorectal cancer. Mice (P.O.[g]) – liver and mammary gland tumours. Rats (P.O.) – liver, bladder, and colon tumours.	[20,91–104]
Sudan I (842-07-9)	-ve in TA97 (-S9), TA98 (-S9 & Prival), TA100 (±S9 & Prival), TA1535 (±S9), TA1537 (±S9), TA1538 (±S9). +ve in TA97 (+S9), TA1538 (+ hamster S9). Weakly + ve in TA98 (+S9) in 1 study; –ve in 2 other studies.	E[h] or -ve for UDS in rat liver. +ve for MN in rat bone marrow. Weakly + ve for MN in mouse bone marrow in 1 study and -ve in 1 other.	+ve for *HPRT*[i] in AHH-1 and MCL-5 cells. +ve for MN in AHH-1, MCL-5, and HepG2 cells. E or -ve in MLA[j] (+S9). +ve for SCE[k] in CHO[l] cells (±S9). -ve for CA[m] in CHO cells (±S9). +ve for comet in HepG2 cells.	IARC Group 3. Mice (PO) – No increase in tumours. Mice (Subcu[n]) – liver tumours Mice (bladder implantation) – bladder tumours. Rat (P.O.) – No increase in tumours.	[34–36,61,62,65,82,83,105–116]
Para Red (6410-10-2)	+ve in TA98 (+S9) and TA1538 (+S9). -ve in TA98 (-S9), TA1535 (±S9), and TA1538 (-S9).	NA	+ve for *HPRT* in AHH-1 cells. +ve for MN in AHH-1 and MCL-5 cells.	NA	[36,117]

[a] Standard Ames test (*i.e.*, not Prival method), unless indicated.
[b] +ve, positive.
[c] −ve, negative.
[d] UDS, unscheduled DNA synthesis assay.
[e] MN, micronucleus assay.
[f] NA, not available.
[g] PO, oral administration.
[h] E, equivocal.
[i] *HPRT*, hypoxanthine phosphoribosyltransferase forward mutation assay.
[j] MLA, mouse lymphoma assay.
[k] SCE, sister chromatid exchange assay.
[l] CHO, Chinese hamster ovary cells.
[m] CA, chromosome aberration.
[n] Subcu, subcutaneous administration.

forward scatter (FSC) region, within a region that excludes doublets, and within an FSC *vs* FL1 region). RINC values were calculated as previously described [37,38].

2.5. DNA isolation and mutant frequency (MF) analysis

Bone marrow, glandular stomach, small intestine, colon, liver, and MutaMouse PHs were lysed as previously described [38,40]. Bladder tissue was homogenized in lysis buffer (1 mM EDTA, 100 mM NaCl, 20 mM Tris – HCl, pH 7.4) using a glass Dounce tissue grinder, 1 mg/mL proteinase K and 1% SDS were added to the suspensions, and the homogenate was incubated overnight at 37 °C with shaking. DNA for all tissues was isolated by phenol-chloroform extraction as previously described, with an additional chloroform extraction step [38,40]. DNA was precipitated with ethanol, spooled onto a sealed Pasteur pipette, washed with 70% ethanol, dried, dissolved in TE^{-4} buffer (10 mM Tris pH 7.6 and 0.1 mM EDTA), and stored at 4 °C.

The frequency of *lacZ* mutants was determined using the P-Gal positive selection method as previously described [40,47–49]. Briefly, TransPak was used to retrieve and package λgt10*lacZ* vectors from MutaMouse genomic DNA. *E. coli* cells (*E. coli* C lacZ -, galE -, recA -, Kanr, pAA119) [50] were allowed to adsorb the phage particles; cells were plated with P-Gal selective medium and incubated overnight at 37 °C. Plaques were scored manually, and MF was calculated as the ratio of mutant plaque-forming units (pfu) to total pfu determined from non-selective plates (*i.e.*, without P-Gal). N = 3 for all *in vitro* results, except for Para Red at 25 μg/mL, where N = 2 due to cytotoxicity. Although all of the compounds were tested at 5, 10, 25, 50, and 100 μg/mL; however, cytotoxicity was too severe above 10 μg/mL and 25 μg/mL for Direct Black 38 and Para Red, respectively, to obtain sufficient DNA for scoring. N = 5 for all tissues, except for the Sudan I 100 mg/kg dose group, wherein N = 4 due to a premature death (see Section 2.2), and the Direct Black 38 1000 mg/kg glandular stomach sample group, the Para Red 200 mg/kg glandular stomach sample group, the Direct Black 38 control small intestine sample group, the Direct Black 38 control colon sample group, and the Para Red 200 mg/kg colon sample group, wherein N = 4 due to low DNA yields.

2.6. Statistical analyses

The *lacZ* mutant frequency data were analyzed in RStudio version 1.0.136 (RStudio, Boston, MA, USA) using the glm function. The quasi-Poisson distribution family was used to account for over-dispersion, and the offset was designated as the natural log of total pfu. Type 1, or sequential analysis, was employed to examine the statistical significance of the chemical treatment (*i.e.*, Chi squared test), and custom contrasts statements were employed to evaluate the statistical significance of responses at selected doses or concentrations [51]. The resulting p-values were corrected for multiple comparisons using the Bonferroni method.

3. Results

3.1. Mutagenicity in various MutaMouse tissues in vivo

MF for the *lacZ* transgene was evaluated in 6 tissues *in vivo*: bone marrow, glandular stomach, small intestine, colon, liver and bladder. Following Direct Black 38 exposure, a significant treatment effect was observed in the bone marrow (χ^2 = 17.1, p < 0.001), glandular stomach (χ^2 = 20.2, p < 0.005), small intestine (χ^2 = 12.3, p < 0.005), and colon (χ^2 = 20.1, p < 0.005), with maximal 5.9-, 5.3-, 3.1-, and 8.5-fold increases in MF over control, respectively (Fig. 2 A). For all tissues, at least one dose elicited a MF that was significantly elevated over control (p < 0.05). Sudan I only elicited a significant treatment effect in bone marrow (χ^2 = 36.1, p < 0.005) (Fig. 2 B). The 7.1-fold increase over control at the 200 mg/kg dose was marginally significant with a p-value of 0.052. A marginally significant treatment effect was observed in the bladder of mice exposed to Sudan I (χ^2 = 10.9, p = 0.059), but there was no significant dose-specific increase in MF over control. Para Red exposure induced a significant treatment effect in the colon (χ^2 = 8.9, p < 0.001), with a statistically significant 1.4-fold increase above control at the 400 mg/kg dose (p < 0.05) (Fig. 2 C). The *in vivo* results are summarized in Table 2.

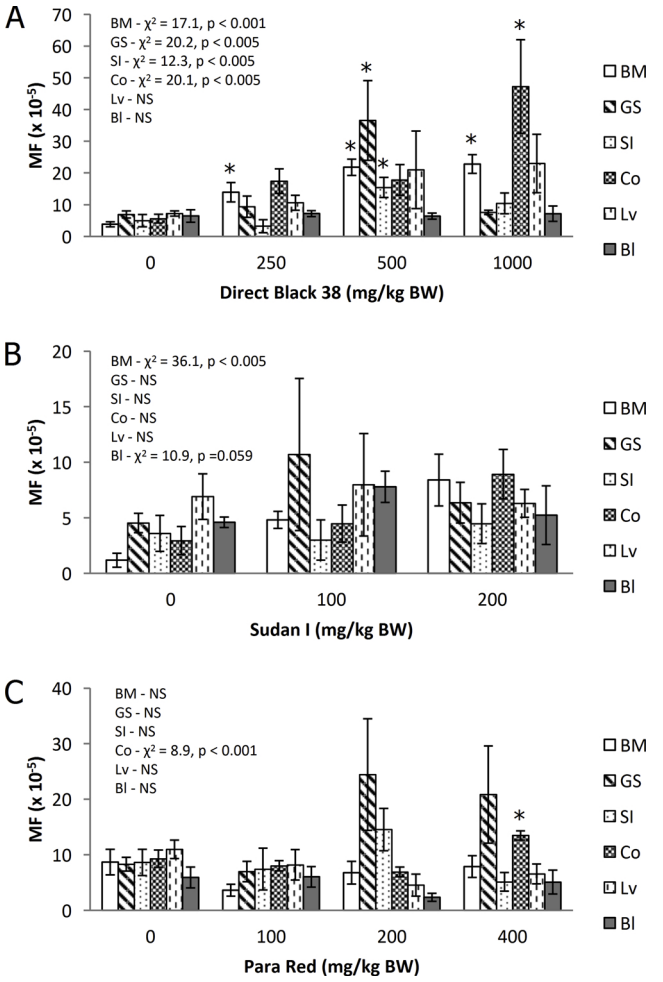

Fig. 2. Induced *lacZ* MF in tissues from MutaMouse specimens exposed to Direct Black 38 (A), Sudan I (B), and Para Red (C). Bars represent average MF ± SEM. Asterisks indicate MF values that are significantly elevated relative to the concurrent control (p < 0.05). Inset boxes show statistical results for the overall treatment effect. N = 5 for all observations, except in cases noted in the Materials and Methods. NS, not significant; BM, bone marrow; GS, glandular stomach; SI, small intestine; Co, colon; Lv, liver; Bl, bladder.

3.2. Mutagenicity in MutaMouse PHs in vitro

MF was also evaluated following *in vitro* of exposure of MutaMouse PHs. Direct Black 38 induced a significant treatment effect (χ^2 = 4.2, p < 0.005), with a maximal MF increase of 2.2-fold over control at 10 μg/mL (p < 0.05) (Fig. 3 A). Sudan I exposure did not elicit any significant treatment effect (*i.e.*, increase in MF) up to a test concentration that decreased RINC to 0.18 (Fig. 3 B). Para Red induced a marginally significant (χ^2 = 12.6, p = 0.055) treatment effect; a statistically significant MF increase of 5.0-fold over control was observed at 25 μg/mL. However, this concentration elicited a marked cytotoxicity (*i.e.*, RINC < 0.20). The *in vitro* results are summarized in Table 2.

Table 2
Summary of mutagenicity results for Direct Black 38, Sudan I and Para Red - various MutaMouse tissues *in vivo* and MutaMouse primary hepatocytes (PHs) *in vitro*.

	Direct Black 38	Sudan I	Para Red
In vivo			
Bone marrow	+[a]	+/−[c]	–
Glandular stomach	+	–	–
Small intestine	+	–	–
Colon	+	–	+
Liver	-[b]	–	–
Bladder	–	+/−	–
In vitro			
PHs	+	–	+/−

[a] +, significant treatment-related effect and at significant increase in MF for at least one dose.
[b] -−, no significant treatment-related effect, and no significant increase in MF above control.
[c] +/−, not clearly positive or negative, see text for details.

4. Discussion

The present study investigated the mutagenic activity of Direct Black 38, Sudan I, and Para Red, both *in vitro* and *in vivo*. The selected tissues are either known targets of these chemicals (Table 1), and/or sites of contact in the gastrointestinal tract. The doses and concentrations tested are much higher than Canadian oral exposure estimates for benzidine-based azo compounds related to Direct Black 38 (*e.g.*, 0.027 μg/kg body weight per day in infants exposed to Acid Red 97-containing textiles) and Sudan I (*i.e.*, up to 8.1 μg/kg body weight per day in children exposed to Sudan I-containing ballpoint pen ink); however, the data generated by the assays in this study are intended for hazard identification and must account for chronic exposures. The responses of the chemicals varied greatly; Direct Black 38 elicited the strongest response in the widest range of tissues, as well as a clear positive response in PHs *in vitro* (Figs. 2 A and A, Table 2). Sudan I elicited a positive response in bone marrow, a marginally significant treatment effect in the bladder, and a negative response in PHs *in vitro* (Fig. 2 B and B, Table 2). Para Red, the least well-studied of the three chemicals, elicited a positive response in the colon, and a positive response in PHs (Figs. 2 C and C, Table 2). The pattern of results obtained offer insight into the mechanisms of action of these compounds. Moreover, the ability to appropriately detect mutagenic activity using an *in vitro* assay based on PHs.

Direct Black 38 is a human and animal carcinogen that targets the bladder, colon, liver, and mammary glands (Table 1); as mentioned in the Introduction, the mechanism of action of involves azo bond cleavage and release of benzidine. Azoreductase activity is present in both the liver and the intestine; in the former it is mediated by CYPs and NQO1, in the latter by intestinal microflora [5,52–54]. Benzidine released following azo-reduction can then be transported to other tissues where further modifications *via* acetyl- or sulfotransferases result in the formation of electrophilic species (*e.g.*, nitrenium, carbenium) that can readily react with DNA bases (*e.g.*, guanine) to form adducts [24,55]. Interestingly, bacterial azo-reduction of Direct Black 38 has been shown to be much more efficient than hepatic azo-reduction [23,25].

The strongest Direct Black 38 response was manifested in the colon, a known site of bacterial azo-reduction; this was followed by bone marrow, glandular stomach, and small intestine. The bone marrow effect is particularly is interesting since it is distal from the gastrointestinal tract, thus requiring systemic circulation of Direct Black 38 metabolites. Interestingly, the highest dose (*i.e.*, 1000 mg/kg) yielded a lower MF than the 500 mg/kg dose in glandular stomach and small intestine. The drop in MF in these tissues at the highest dose is presumably due to cytotoxicity in these tissues. Despite Direct Black 38 being linked to bladder tumours in humans and rats, and liver tumours

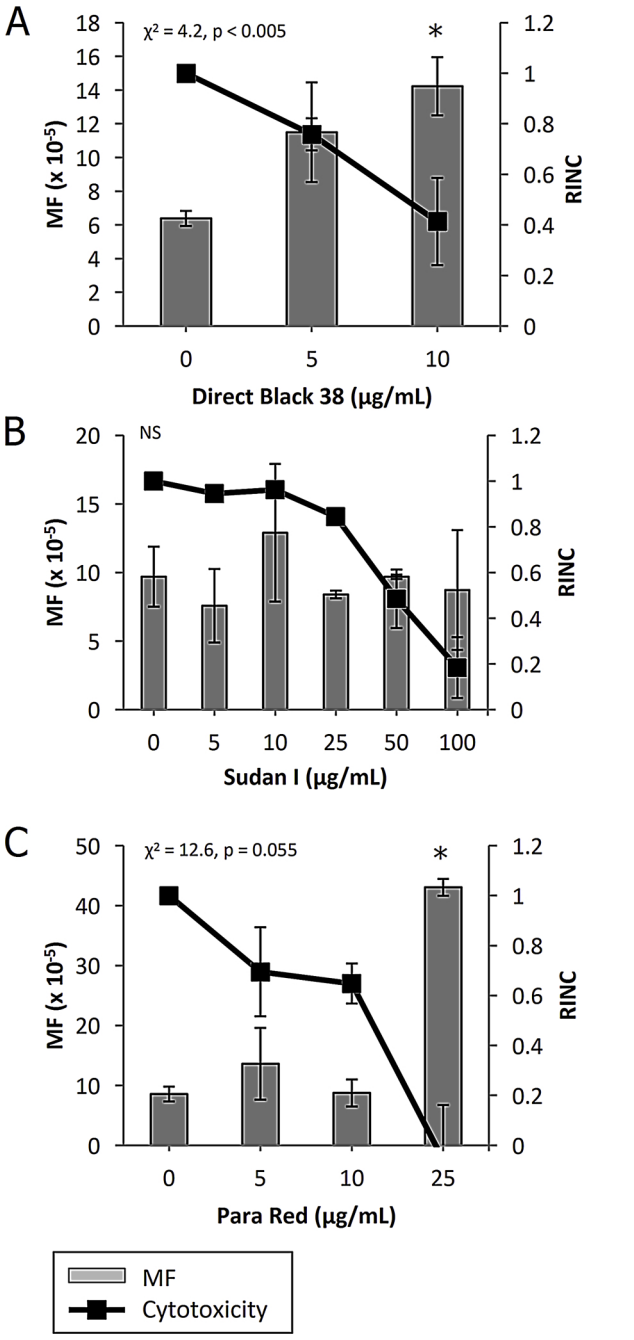

Fig. 3. Induced *lacZ* MF in MutaMouse PHs exposed to Direct Black 38 (A), Sudan I (B), and Para Red (C). Grey bars represent MF ± SEM and black squares show relative increases in nuclear counts (RINC) ± SEM, a measure of cytotoxicity. Asterisks indicate MF values that are significantly elevated relative to the concurrent control ($p < 0.05$). Inset boxes show statistical results for the overall treatment effect. N = 3 for all observations, except for 25 μg/mL Para Red, wherein N = 2 due to cytotoxicity. NS, not significant.

in mice and rats, no significant response was observed in either of these tissues. Lack of response in these tissues may be a consequence of low cellular turnover rates (*i.e.*, mitotic index) that dramatically reduce the likelihood of mutation fixation in MutaMouse specimens [56]. Indeed, urothelial cells (*i.e.*, the cells that line the bladder) and hepatocytes *in vivo* have been shown to have extremely slow turnover rates. The turnover rate of mammalian urothelial basal cells is approximately 3 to 6 months, the slowest of any mammalian epithelial cells, and the turnover rate of murine hepatocytes is approximately 6 to 13 months [57–60]. Thus, future studies of compounds such as Direct Black 38

should enumerate DNA adducts in tissues with low cellular turnover, and/or mutant frequency after far longer sampling times. Indeed, OECD test guideline 488 indicates that longer sampling times (*i.e.*, 28 days instead of 3 days) may be required for tissues with low rate of cellular turnover (*e.g.*, liver, bladder, *etc*) [39].

A significant response was also observed in PHs exposed to Direct Black 38 *in vitro*. This is not unexpected since, unlike hepatocytes *in vivo*, *ex vivo* MutaMouse PHs proliferate relatively rapidly [37]. Moreover, as noted in a previous study, murine PHs express a full complement of CYPs and NQO1, which can confer azoreductase activity [37]. This positive *in vitro* result echoes what has been observed in experiments using rat PHs. More specifically, Bos et al. [1984] demonstrated that Direct Black 38 incubated with rat PHs resulted in the formation of diacetylbenzidine, a DNA-reactive, reductive metabolite, without the addition of acetyl coenzyme A. The results confirm that, despite difficulty of detecting *in vivo* mutagenic hazard in known tumour sites, MutaMouse PHs can reliably detect a carcinogenic azo compound known to be activated *via* azo-reduction. Unfortunately, due to a paucity of published information, the ability to detect Direct Black 38 mutagenicity in MutaMouse PHs *in vitro* cannot be compared to results obtained using other mammalian cells (Table 2).

Sudan I is hepatocarcinogenic in mice following subcutaneous administration; it produces bladder tumours in albino and (C57 X IF)F_1 mice following wax pellet implantation [61–65] (Table 1). However, the wax pellet implantation studies are no longer deemed appropriate since the pellet itself can irritate the bladder epithelium [66]. As mentioned in Section 1, Sudan I, unlike Direct Black 38, does not require azo-reduction to become metabolically converted to a DNA-reactive agent. In fact, azo-reduction seems to mainly lead to detoxification of Sudan I with the production of aniline and 1-amino-2-naphthol [5]. There are two main routes of activation for Sudan I: hepatic oxidation by CYP 1A1 and CYP 3A, and metabolism by bladder peroxidases [5,26]. Cytochrome P450-mediated oxidation of Sudan I leads to the formation of the benzenediazonium ion (BDI). BDI is formed following metabolism by rat liver microsomes [67,68], and it forms 8-(phenylazo)guanine adducts with calf thymus DNA *in vitro* and rat liver DNA *in vivo* [69,70]. CYP 1A1 and CYP 3A metabolism also leads to the formation of *C*-hydroxylated detoxification by-products [67,71,72]. Sudan I is an aryl hydrocarbon receptor (AhR) agonist and strongly induces CYP 1A1 and NQO1 in rats [73,74]. Peroxidases, including horseradish peroxidase (HRP) and mammalian prostaglandin H synthase, can metabolize both Sudan I and *C*-hydroxylated Sudan I metabolites to DNA-reactive species [75–79]. High levels of peroxidases (*i.e.*, prostaglandin H synthase) are found in the urinary bladder, where CYP activity is known to be very limited [80]. The same DNA adducts observed *in vitro* following HRP-mediated metabolism have been detected in the urinary bladder of Fisher 344 rats following oral administration of Sudan I [81]. Thus, the metabolic processes underlying the mutagenicity and/or hepatocarcinogenicity of Sudan I likely include several enzymatic systems that collectively catalyse the generation of several DNA-reactive species (*e.g.*, BDI, peroxidation products of *C*-hydroxylated Sudan I).

In this study, Sudan I elicited a significant overall treatment effect and marginally significant MF increase in the bone marrow, as well as a marginally significant overall treatment effect in the bladder (Fig. 2 B). The bone marrow result suggests that Sudan I was metabolically activated and systemically circulated; this result echoes the positive *in vivo* MN assay results observed in both rat and mouse bone marrow [82,83]. Although the observed bladder response is only marginally significant, it is consistent with the aforementioned role of bladder peroxidases in metabolic activation. Moreover, published information regarding Sudan I activation in the liver and bladder, and information regarding Sudan I cancer target tissues (*i.e.*, liver and bladder), support an expectation of liver and bladder effects. The marginal overall treatment effect for bladder, and the accompanying lack of a significant MF increase at any of the tested doses, may be due, as noted for Direct Black 38, to the slow turnover of bladder epithelial cells. Moreover, pattern of results suggests that the experiment was underpowered. In order to improve the ability to detect an effect in the bladder, future work should employ increased numbers of animals per dose group, particularly for the most relevant dose(s) (*e.g.*, 100 mg/kg). Interestingly, no response was observed in the liver. Similar to the bladder results, this may be due to low cell turnover necessitating, as noted for Direct Black 38, a longer sampling time. Moreover, as noted for Direct Black 38, DNA adduct analyses of the liver and bladder could provide important information to elucidate the respective ability of the liver and bladder to activate Sudan I *in vivo*. Although this study has provided evidence of mutagenic activity in the bone marrow and bladder, additional investigations are needed to fully characterise genetic toxicity; moreover, to understand mechanism of action and tissue-specific metabolism.

Sudan I did not elicit a significant increase in MF in MutaMouse PHs exposed *in vitro* (Fig. 3 B). This lack of response is interesting, considering that Sudan I elicited a positive response in AHH-1 and MCL-5 cells in the *in vitro HPRT* gene mutation assay [36]. Indeed, published information about the metabolism and activation of Sudan I, combined with available information about MutaMouse PH metabolic capacity, suggest that the substance should be converted into a DNA-reactive metabolite in MutaMouse PHs (*e.g.*, CYP 1A1-mediated catalysis to DNA-reactive BDI). Moreover, the rapid proliferative capacity of MutaMouse PHs should lead to fixation of elevated *lacZ* transgene mutations. The discrepancy in response between these *in vitro* systems may be related to the strong CYP 1A1 and NQO1 induction capabilities of Sudan I [74]. AHH-1 is an immortal human B lymphoblastoid cell line that inducibly expresses CYP 1A1. MCL-5, which is derived from AHH-1, expresses particularly high levels of CYP 1A1, and has been transfected with 2 plasmids: one containing 2 copies of CYP 3A4 cDNA and 1 copy of CYP 2E1, and one containing 1 copy each of CYP 1A2, CYP 2A6, and microsomal epoxide hydrolase. Although these cell lines show induced fold-change increases in CYP 1A1 activity that is similar to MutaMouse PHs, the absolute magnitude of both their basal and induced activity are approximately 10-fold lower than that of MutaMouse PHs [37,84–86]. Thus, failure to elicit a positive response in MutaMouse PHs, when combined with the results for MCL-5 and AHH-1 cells, and the observation of CYP 1A1 generation of detoxified *C*-hydroxylation products, suggest that the level of CYP 1A1 activity in MutaMouse PHs is preferentially producing detoxified metabolites. Additionally, the induction of NQO1 in MutaMouse PHs, which has azoreductase activity, and is not known to be present in AHH-1 or MCL-5 cells, may also be contributing to the detoxification of Sudan I. Indeed, although convincing evidence has yet to be published, there has been speculation that NQO1 may lead to the reduction and detoxification of Sudan I [74]. The roles of CYP 1A1 and NQO1 in Sudan I metabolism and mutagenicity *in vitro* could be confirmed by retesting Sudan I in the presence of CYP 1A1 and NQO1 inhibitors, such as α-naphthoflavone and dicumerol, respectively [87,88]. Furthermore, it may also be useful to perform a follow-up study that assesses the frequency of Sudan I adducts. Such follow-up studies could evaluate hypotheses regarding the inability to elicit mutations in MutaMouse PHs, which, by extension, would shed light on the utility of MutaMouse PHs for identifying the mutagenic hazards of azo compounds.

As mentioned in the Introduction, very little is known about the mechanism of action of Para Red; although, due to its structural similarity, it is presumed to act in a similar fashion to Sudan I. In other words, oxidation mediated by hepatic and bladder enzymes that lead to production of DNA-reactive metabolites. Interestingly, it is known that human intestinal microflora can catalyze azo-reduction of Para Red, yielding 1-amino-2-naphthol and 4-nitroaniline. In contrast, azo-reduction of Sudan I yields 1-amino-2-naphthol and aniline [89]. Unlike aniline, which is not mutagenic in the Ames test, 4-nitroaniline induces mutations in Salmonella TA98 in the presence of induced rat liver S9 [90]. Thus, although there is a paucity of information about the metabolism and mutagenicity of Para Red, there is some evidence to

suggest that intestinal azo-reduction may play a more important role in comparison with Sudan I. Indeed, the results obtained show that Para Red can elicit a positive response in the colon *in vivo* (Fig. 2 C, Table 2), which in turn suggests that colonic azoreductase activity is yielding 4-nitroaniline, *i.e.*, a mutagenic metabolite. This is in stark contrast to the mutagenic mechanism of Sudan I, which requires oxidation.

Para Red also yielded a positive result in MutaMouse PHs *in vitro*, albeit only when tested at a highly cytotoxic concentration (*i.e.*, 25 μg/mL) (Fig. 3 C). Para Red also elicited a positive response in the *HPRT in vitro* gene mutation assay in AHH-1 cells, with a lowest observable effect level (LOEL) of 7.5 μg/mL in the absence of cytotoxicity [36]. These observations suggest that CYP 1A1 mediated catalysis likely plays a role in the mutagenic activation of Para Red. Although Para Red is mutagenic in both systems, the discrepancy with respect to the active concentration is likely related to the different metabolic profiles of MutaMouse PHs and AHH-1 cells [37,84]. Further studies involving select enzyme inhibitors, such as the CYP 1A1 inhibitor α-naphthoflavone, would lead to an improved understanding regarding the metabolic activation and mutagenicity of Para Red. Identification and quantification of Para Red-induced DNA adducts would similarly contribute to mode of action determination.

An important goal of the present study was generation of information about the *in vivo* mutagenicity and mechanisms of action of selected azo compounds. None of the tested compounds had previously been analysed in a TGR gene mutation assay, and, using the MutaMouse system, the work examined *in vivo* mutagenic activity in selected tissues. The target tissues identified in the Direct Black 38 experiment support the important role of intestinal microflora in the metabolic activation (*i.e.*, azo reduction) of this potent carcinogen. The effects observed in the bone marrow and bladder, and the absence of effects in the colon, of Sudan I-exposed animals supports the proposed oxidation-mediated activation of this food contaminant; reaffirming that bacterial azo-reduction is not a major route of activation [26]. Importantly, the study investigated the mutagenicity of the food contaminant Para Red, and found that, despite close structural similarity to Sudan I, it targets different tissues (*i.e.*, the colon). This finding suggests that azo-reduction by anaerobic bacteria in the colon is likely leading to the production of mutagenic 4-nitroaniline; demonstrating that, in comparison with Sudan I, azo-reduction plays a more important role in the activation of Para Red. Overall, the present study's *in vivo* evaluation of Direct Black 38, Sudan I, and Para Red offers insight into the proposed mechanisms of action of these chemicals, and identifies data gaps that could be filled by, for example, DNA adduct analyses, additional TGR assays with longer sampling times for key tissues such as the liver and bladder, and enzyme inhibition experiments. Additionally, investigating the mutation spectra of these chemicals in their target tissues could elucidate further details regarding their mechanisms of action.

The study was also designed to evaluate the utility of the *in vitro* MutaMouse PH assay to reliably assess the mutagenicity of compounds that undergo complex metabolism *in vivo*. Previously, our group showed that MutaMouse PHs are metabolically competent and capable of detecting mutagens with a variety of metabolic activation requirements (*i.e.*, polycyclic aromatic hydrocarbons, aromatic amines, mycotoxins, nitroarenes, and nitrosamines) [37,38]. The chemicals previously examined require both Phase I oxidation reactions and Phase II conjugation reactions; however, none necessitated azo-reduction for generation of DNA-reactive metabolites. The current study illustrates the ability of the MutaMouse *in vitro* PH gene mutation assay to detect chemicals requiring azo-reduction (*e.g.*, Direct Black 38 and Para Red). However, it also demonstrates a gap in the applicability domain of this assay with respect to azo compounds that do not require azo-reduction for metabolic activation (*i.e.*, Sudan I). The negative Sudan I response was unexpected in light of the fact that this compound elicits positive responses in other *in vitro* mammalian cell gene mutation assays; it indicates that further studies are warranted regarding the roles of key metabolic enzymes in the detoxification *versus* activation of compounds such as Sudan I (*i.e.*, CYP 1A1 in MutaMouse PHs) [36]. This apparent limitation and possible lack of sensitivity requires further study. Precise determination of the ability of the MutaMouse PH assay to assess the mutagenicity of azo compounds will require testing of additional azo compounds, including some well-studied compounds with existing *in vivo* and *in vitro* data. Nevertheless, the results presented here indicate that this assay is capable of reductive metabolism, and thus is a useful tool for the assessment of azo compounds.

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