Metabolic influences for mutation induction curves after exposure to Sudan-1 and para red

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Sudan-1 and para red are industrial dyes that have been illegally added to some foodstuffs, leading to withdrawal of the adulterated products throughout the UK since 2003. This resulted in international concern that arose because Sudan-1 is classified by International Agency for Research on Cancer as a Category 3 carcinogen. However, little is known about the dose response of this chemical at low, more biologically relevant, doses. The study therefore aimed to characterize the dose response for gene mutation and chromosomal damage induced by two azo dyes, namely Sudan-1 and para red. Gene mutations were analysed using the hypoxanthine phosphoribosyltransferase forward mutation assay and chromosomal damage was measured using the cytokinesis-blocked micronucleus assay. Two cell lines were used in these investigations. These were the AHH-1 cell line, which inducibly expresses CYP1A1, and the MCL-5 cell line derived from a subpopulation of AHH-1 cells that expresses a particularly high level of CYP1A1 activity. The MCL-5 cell line has also been transfected with two plasmids that stably express CYP1A2, CYP2A6 and CYP3A4 and all four of these CYP enzymes are known to metabolically activate Sudan-1. AHH-1 cells were used to investigate the dose response of the azo dyes, and MCL-5 cells were used to see if the dose response changed with increased metabolism. Sudan-1 induced a non-linear dose–response curve for gene mutation and chromosomal damage in AHH-1 cells. The genotoxic activity of Sudan-1 was greatly increased in MCL-5 cells. This indicated that the oxidation metabolites from Sudan-1 were both more mutagenic and more clastogenic than the parent compound. Para red also demonstrated a non-linear dose response for both gene mutation and chromosome damage in AHH-1 cells, and an increase in micronuclei induction was observed after increased oxidative metabolism in MCL-5 cells. Sudan-1 and para red are genotoxic chemicals with non-linear dose responses in AHH-1 but not in MCL-5 cells, and oxidative metabolism increases the genotoxic effect of both compounds.

Introduction

Sudan-1 (1-phenylazo-2-naphthol) is an azo dye that has been illegally used to colour chilli and chilli products and has been found at levels ranging from 24 to 5591 μg/ml (1). In February 2005, a recall of ~575 products was made, which consisted of foodstuffs ranging from ready-made meals to sauces, such as Worcester sauce (2). It has been characterized as being a liver and urinary bladder carcinogen in mammals (3) and has been given a Category 3 classification by the International Agency for Research on Cancer. This means that the agent (mixture or exposure circumstance) is not classifiable as to its carcinogenicity to humans. A similar scenario emerged for para red [1-(4-nitrophenylazo)-2-naphthol]. Para red possibly acts via a similar mode of action to Sudan-1 and they have both been shown to have increased genotoxic activity after metabolic activation (4,5). Azo dyes are aromatic compounds characterized as having one or more azo bonds (\(N=N\)). These chemicals have a wide range of applications in the textile, leather, paper, food, pharmaceutical and cosmetic industries (6). Some azo dyes have been associated with bladder cancer in humans and also splenic sarcomas, hepatocarcinomas and nuclear anomalies in experimental animals, along with chromosomal aberrations and gene mutations in Chinese hamster cells (CHL and V79-MZ) and mouse lymphoma cells (7–10).

A recent study investigated the clastogenicity of Sudan-1 and its metabolites in the cytokinesis-blocked micronucleus (CBMN) assay. Human hepatoma HepG2 cells were used because these cells retain many of the functions of normal human liver cells (11) and express many functional phases I and II xenobiotic-metabolizing enzymes (12). The first finding of the study in HepG2 cells was that Sudan-1 induced micronuclei (MN) at 25 μM (6.2 μg/ml) and above in a dose-dependent manner (11). In further in vitro studies, Sudan-1 was also reported to induce gene mutations in the mouse lymphoma assay (9,13,14). In the study by Cameron et al. (9), Sudan-1 showed a potential no observable effect level (NOEL) at 24 μg/ml without metabolic activation and possible NOEL at 2.1 μg/ml with S9 metabolic activation in the mouse lymphoma \(TK^+/−\) assay. However, the NOEL was not sufficiently assessed in these studies as no doses were tested below the NOEL without S9 and there were only four doses tested with S9. The importance of an NOEL for risk assessment has been highlighted by the recent report by the European Medicine Agency (15). Our study aimed to address this by testing more concentrations in the low-dose region. NOEL has recently been shown for the azo dyes Disperse Red and Disperse Orange in the MN assay in HepG2 cells (16) using similar methodology to parts of our test strategy.

Alongside the standard battery of in vitro assays used in Genetic Toxicology, more mechanistic investigations have also been carried out for Sudan-1. These have reported that the Sudan-1 (Figure 1) oxidative metabolites produced by CYP1A1, CYP1B1, CYP2A6, CYP2C19, CYP2D6 and CYP3A4 in human microsomes can potentially form DNA
adducts (3,5). One example is the microsome-dependent enzymatic splitting of the azo group that produces a benzene-
diazonium ion (BDI) (5,17). This BDI can react with DNA in vitro and the major DNA adduct formed is 8-(phenyl-
azo)guanine (5). Another is the formation of the 6-OH-
Sudan-1 following oxidation by CYP1A1 or CYP3A, which
is activated by peroxidase to form DNA, RNA and protein
adducts (5). Reduction of Sudan-1 by azoreductases produces
aromatic amines, including aniline, and these compounds can
also have a genotoxic effect (18,19). More recent studies have
shown that Sudan-1 acts via reactive oxygen species (ROS)
being formed at higher doses of 12.5–25 μg/ml (ROS levels
analysed using the 2’’,7’’-dichlorofluorescein diacetate assay)
and causing DNA strand breaks (observed in the Comet assay
and CBMN assay) and 8-hydroxydeoxyguanosine (8-OHdG)
adducts (identified using immunoperoxidase staining) in
HepG2 cells (11). However, strand breaks were formed at the
lower doses tested (6.25 μg/ml) and ROS were not. These
previous studies suggest that ROS are of no relevance at low
doses and the predicted mode of action is the BDI adduct.
Therefore, when Sudan-1 is oxidized, it acts through an indirect
mode of action. Compounds with indirect modes of action have
been shown to have non-linear dose responses (20–24).

The metabolism of para red (structure shown in Figure 1) has
not been well characterized. However, it is a structural
analogue of Sudan-1 and therefore potentially microsome-
dependent enzymatic splitting of the azo group could produce
a structural analogue of BDI, which may also be DNA reactive.
This and other genotoxic metabolites could be predicted for
para red; however, these cannot be substantiated because of the
lack of published literature on this particular azo dye. There is
some published literature on the genotoxic effects of para red,
which was shown to be genotoxic in the Salmonella typhimurium
strains TA 1538 and TA 98 after metabolic activation but was
negative without metabolic activation (4). There are very limited
data to substantiate these findings; however, there have been
reports of the known aromatic amine metabolite of para red,
p-nitroaniline, being genotoxic in vitro (25). It was found to be
positive in S.typhimurium strain TA98, with and without
metabolic activation, to induce chromosomal aberrations in
CHO cells with S9 and also to induce gene mutations in the
mouse lymphoma assay without S9 (25). Therefore, para red
metabolites have been reported to be genotoxic in vitro in
a number of assays but the mode of action and the effects of
low-dose exposures have not been previously investigated.

Living organisms can be predicted to tolerate low levels of
DNA damage through activity of protective mechanisms, such
as DNA repair. Furthermore, mammalian cells have evolved
a series of protective mechanisms, whereby induced DNA
lesions may be repaired and heavily damaged cells are
eliminated by apoptosis and/or necrosis. Therefore, it has been
predicted that thresholds may exist for at least some DNA-
reactive genotoxins. We have recently confirmed these
predictions by showing that some DNA-reactive genotoxins,
e.g., methyl methanesulphonate and ethyl methanesulphonate
(EMS), exhibit a threshold dose response depending on the
mode of action while others, e.g. methyl nitrosourea and ethyl
nitrosourea (ENU), exhibit a more linear dose response
(26–28). These findings were supported by recent in vitro tests
where EMS was shown to have a threshold dose response and
ENU was linear, at both the gene mutation and the chromo-
somal damage using MutaMouse and the bone marrow MN
test, respectively (29,30). Therefore, each compound should
be investigated on a case-by-case basis until it is accepted that
DNA-reactive genotoxins exhibit both linear and non-linear
dose responses depending on the mode of action.

Both azo dyes are metabolized into genotoxic agents and as
a consequence should be assessed after metabolic activation.
Studies were carried out in two different human cell lines with
different metabolic capabilities. This would allow the geno-
toxic activity of the metabolites produced by each cell line to
be compared. Sudan-1 and para red were investigated using the
hyoxanthine phosphoribosyltransferase (HPRT) forward mu-
tation assay to assess gene mutations and the CBMN assay
to quantify chromosomal damage. These assays are both sensitive
in the low-dose region of exposure due to the high number of
cells being scored, and they were employed to quantify the
 genetic damage induced following exposure to low levels of
both azo dyes. The CBMN assay and the HPRT assay have
good historical basis for quantification and we have optimized
and validated the sensitivity of these assays in the low-dose
region of exposure (27). AHH-1 and MCL-5 cells have been
recommended for use when undertaking in vitro genotoxicity
testing to reduce false positives (31), and they were designed
for the purpose of testing for the effects of oxidation (32–34).
Therefore, both assays were utilized in these two human cell
lines to determine whether the two azo dyes exhibit an NOEL
for genotoxic activity after metabolic activation.

Materials and methods

AHH-1 cell line (Gentest™)

AHH-1 is a human B lymphoblastoid immortal cell line that was derived from
the RPMI 1788 cell line (35,36). Freedman et al. (35,36) reported that the cell
line RPMI 1788 contained aryl hydrocarbon hydroxylase activity. Furthermore,
clonal RPMI 1788 isolates with the desired metabolic activity that have been
shown to be free of mycoplasma have now been obtained, and AHH-1 is
derived from this cell line (37). RPMI 1788 cell line was isolated from a healthy
donor and transformed with the Epstein–Barr virus. The AHH-1 cell line is a
homogenous (clonally derived) cell line and it inducibly expresses
CYP1A1 and no other CYP enzymes (38). In contrast with RPMI 1788, AHH-
1 and its derivatives (the metabolically competent cell line MCL-5) have been
consistently found to be mycoplasma free in our laboratory.

MCL-5 cell line (Gentest™)

MCL-5 cells are derived from L3 cells, a subpopulation of AHH-1 cells that
express a particularly high level of CYP1A1 activity (32,38). The MCL-5 cell line
has also been transfected with two plasmids: one containing two copies of
CYP3A4 complementary (cDNA) and one copy of CYP2E1 cDNA and a second
containing one copy of each CYP1A2, CYP2A6 and microsomal epoxide
hydrodase cDNA (32,38). Therefore, MCL-5 cells stably express all five CDNAs
and also have increased levels of CYP1A1 compared to AHH-1 cells.

AHH-1 cells were used to investigate the dose response of the azo dyes, and
MCL-5 cells were used to test the influence of the above additions on azo
metabolism.

AHH-1 and MCL-5 cell culturing

Cells were cultured in 80-cm² flasks in a 37°C CO₂ incubator. Cell density was
maintained at 1.5 × 10⁵ cells/ml. Neither cell line was cultured for more th
the recommended 5 weeks. Both cell lines have a cell cycle time of 22–24 h (32). The human lymphoblastoid cell line AHH-1 was cultured in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 1% l-glutamine (Life Technologies) and 10% donor horse serum (BDGentest, Oxford, UK). The cells were maintained at a concentration of 1–2 × 10⁶/ml. The MCL-5 cell line was cultured in the same medium as MCL-5 but with the addition of 200 µg/ml bygromycin-B (Invitrogen).

Chemicals. Sudan-1 and para red were purchased from Sigma (Dorset, UK). All chemical dilutions were freshly prepared from stock solutions with propylene glycol using a sonicating water bath.

CBMN assay. AHH-1 and MCL-5 (10 ml) suspension with cells at 1 × 10⁵/ml were seeded for 24 h at 37°C, 5% CO₂. Each was dosed with appropriately diluted test chemical (in duplicate) and 3 µg/ml cytochalasin B for one cell cycle. Sudan-1 dose range was 0, 0.5, 1, 0.5, 1.25, 1.5, 2.5, 5, 7.5, 10 and 10 µg/ml in AHH-1 cells and 0, 0.5, 1, 1.25, 1.5, 2.5, 5, 7.5 and 10 µg/ml in MCL-5 cells. Para red dose range was 0, 2.5, 5, 7.5, 10, and 20 µg/ml in AHH-1 cells and 0, 0.5, 1, 1.25, 1.5, 2.5, 5, 7.5, 10, 15 and 20 µg/ml in MCL-5 cells. Treated cells were harvested, cytospun onto polished glass slides, fixed in 90% methanol, stained with acridine orange (12.5 mg/100 ml phosphate buffer, pH 6.8) and viewed under an Olympus BX50 fluorescence microscope. MN were scored per treatment (1000 per duplicate). Relative cell viability was calculated using the cytokinesis-block proliferation index (CBPI) measure of cytotoxicity (40).

HPRT forward mutation assays. To remove background mutants within the AHH-1 cell population, 5 × 10⁴ cells/ml were grown for 3 days in the hypoxanthine (2 × 10⁻⁴ mol/l)–aminopterin (8 × 10⁻⁷ mol/l)–thymidine (3.5 × 10⁻⁵ mol/l) (HAT) media, followed by 24 h in HT media (as HAT media but without the aminopterin) and then transferred to normal growth media for 3–4 days. Cell suspensions (10 ml) at 5 × 10⁴/ml were exposed to the test chemical at the appropriate concentration, for 24 h at 37°C, 5% CO₂. The cells were washed to remove the azo dye, resuspended in 50 ml fresh media and incubated for 13 days. Sudan-1 dose range was 0, 0.5, 1, 1.25, 1.5, 1.75, 2, 2.5 and 5 µg/ml in AHH-1 cells and 0, 0.5, 1, 1.25, 1.5, 2.5 and 5 µg/ml in MCL-5 cells. Para red dose range was 0, 2.5, 5, 7.5, 10, 15 and 20 µg/ml in AHH-1 cells. Then the 96-well plates were loaded with 4 × 10⁴ cells per well in selective media containing 0.6 µg/ml 6-thioguanine (HPRT assay). In addition, plates without selection containing 20 µg/ml cytochalasin B for one cell cycle were used. Subsequent colony formation (>20 cells in a well) was scored to determine the mutation frequency (MF) of each dose, calculated as described by Furth et al. (41).

Statistical analysis. The questions being tested were as follows: (i) Do Sudan-1 and/or para red induce gene and/or chromosomal damage? (ii) Do these azo dyes induce non-linear dose responses and exhibit an NOEL? and (iii) Do these NOEL change with increased metabolism?

A one-way analysis of variance followed by a Dunnett’s posthoc test was used to determine if any of the treatment doses were significantly different from the zero dose. Data were square root transformed before statistical analysis to standardize the data. The NOEL was the highest dose that did not cause a significant (P < 0.05) increase in adverse effect. A lowest observable effect level (LOEL) was the lowest dose that caused a significant (P < 0.05) increase in adverse effect.

Point of departure (POD) was the first dose to produce an effect above that of the historical control mean + 1 SD. This was obtained from within our laboratory (Table I) and it was used to support the NOEL and LOEL.

**Results**

**Gene mutation studies**

**HPRT forward mutation assay methodology** was used to investigate the mutagenic activity of the azo dyes, Sudan-1 and para red. The aim of this part of the study was to assess whether these azo dyes had NOEL of mutagenic activity under different levels of metabolic activity.

**Sudan-1.** Two different cell lines AHH-1 and MCL-5 were used in the Sudan-1 study to show how different levels of metabolic activation produced by the different levels of oxidative enzymes in these two cell lines affect the mutagenic activity of this azo dye. A change in dose response between these two cell lines would indicate that increased oxidation of Sudan-1 through altered expression of CYP1A1 or the addition of extra cytochromes in MCL-5 increases the genotoxic potential of the azo dye.

Sudan-1 was shown to induce gene mutations at low levels in human lymphoblastoid cell lines (Figure 2). These data indicate that Sudan-1 shows a non-linear dose response in AHH-1 cells (NOEL at 1.5 µg/ml and LOEL at 1.75 µg/ml) with a change in dose response in MCL-5 cells (NOEL at 0.5 µg/ml and LOEL at 1 µg/ml). However, more concentrations are required <0.5 µg/ml before one can define the dose response for MCL-5 cells.

**Para red.** Para red was used to investigate whether there was a similarity in genotoxic activity and dose response between this and Sudan-1. AHH-1 cells were used to see if the oxidative metabolites produced by metabolism with CYP1A1 were genotoxic and whether there was a non-linear dose response.

Para red was shown to induce gene mutations at low concentrations in human lymphoblastoid cell lines (Figure 3). These data indicate that para red shows a non-linear dose response in AHH-1 cells (NOEL at 6 µg/ml and LOEL at 7.5 µg/ml) with an LOEL at a higher concentration for para red than for Sudan-1 (1.75 µg/ml).

In every instance, apart from MCL-5 with the HPRT assay where a historical control was unavailable, the NOEL and LOEL corresponded with the POD (Table I). In other words, the first statistically significant dose (P < 0.05) was the same as the first dose to produce an effect greater than the average background +1 SD.

**Chromosomal damage studies**

**Sudan-1.** AHH-1 cells were used to investigate the dose response of Sudan-1, and MCL-5 cells were used to see if the dose response changes with increased metabolism.

Sudan-1 was shown to induce MN at low levels in human lymphoblastoid cell lines (Figure 4). These data indicate that Sudan-1 shows a non-linear dose response in AHH-1 cells (NOEL at 2.5 µg/ml and LOEL at 5 µg/ml) with a change in dose response in MCL-5 cells (NOEL at 1.25 µg/ml and LOEL at 1.5 µg/ml).

**Para red.** Para red was used to investigate whether there was a similarity in clastogenic activity and dose response between this and Sudan-1. AHH-1 cells were used to see if the oxidative metabolites produced by metabolism with CYP1A1
were genotoxic and whether there was a non-linear dose response.

Para red was shown to induce MN at low levels in human lymphoblastoid cell lines (Figure 5). These data indicate that para red showed a potential non-linear dose response in AHH-1 cells (NOEL at 7.5 µg/ml and LOEL at 10 µg/ml) with a change in dose response in MCL-5 cells (NOEL at 2.5 µg/ml and LOEL at 5 µg/ml).

In every instance, the NOEL and LOEL corresponded with the POD (Table I). In other words, the first statistically significant dose (P < 0.05) was the same as the first dose to produce an effect greater than the average background +1 SD.

**Discussion**

The azo dyes Sudan-1 and para red were shown to be genotoxic and to have the potential to induce gene mutations and chromosome damage in human cell lines. Sudan-1 had an increased mutagenic and clastogenic effect in the MCL-5 cell lines compared to the AHH-1 cell line (Figures 2 and 4, respectively) because of the different metabolic capabilities of the different cell lines. There was a clear NOEL for Sudan-1 in AHH-1 cells for the induction of both gene mutations and chromosome damage, and the NOEL shifted to a lower concentration in MCL-5 cells for chromosome damage.
However, more doses would be required <0.5 μg/ml to further investigate the dose response of Sudan-1 for HPRT gene mutations in MCL-5 cells (Figure 2). MCL-5 cells have a higher level of CYP1A1 and other oxidative enzymes and therefore, more metabolites are formed in this cell line. Therefore, the differences in metabolism are potentially responsible for the difference in genotoxic effect of the Sudan-1 metabolites. Sudan-1 can be oxidized by cytochrome P-450 monooxygenases, with CYP1A1 producing the genotoxic BDI metabolite and 6-OH-Sudan-1 (5). Human microsomes also have CYP1A2, CYP2A6 and CYP3A4 (expressed in MCL-5 cells and not in AHH-1 cells) and human microsomes have the capability of producing genotoxic metabolic products from Sudan-1 (3,5,42–45). This hypothesis is supported by Figures 2 and 4 because MCL-5 cells induced an increase in genotoxic response compared to AHH-1 cells, possibly by the
additional cytochromes or higher expression of CYP1A1 in MCL-5 cells.

The mode of action for the genotoxic activity of Sudan-1 has not yet been fully characterized. However, these data indicate that the dose response is dependent on the level of metabolism, which means that Sudan-1 acts via an indirect mode of action. Genotoxic agents with indirect modes of action have non-linear dose–response curves (20–24); therefore, the non-linear dose responses observed for Sudan-1 are in line with previous findings. The predicted mechanism of action is the stable 8-(phenylazo)guanine adduct formed by BDI reacting as one of the electrophilic species derived from Sudan-1 (11,17). DNA adducts are potentially mutagenic and clastogenic, and their genotoxic activity depends largely on how well the lesion is repaired by the specific DNA repair pathway. Therefore, the exact mechanism of action is currently not known and further studies are required to define this for the dose responses.

Sudan-1 was mutagenic and clastogenic and exhibited its effect in a dose-dependent manner with an NOEL for both gene mutation and chromosomal damage end points (Figures 2 and 4). This effect was greatly increased when Sudan-1 was metabolized, and therefore, the metabolites of this azo dye were more genotoxic than the parent compound.

Para red was used here as a comparative azo dye to Sudan-1; however, it has not been widely investigated. Its detection in the food products meant that it was a relevant model azo dye to use. Genotoxic assessment of para red showed a non-linear dose response for both gene mutations and MN induction in AHH-1 cells (Figures 3 and 5). However, the NOEL for para red was higher than that of Sudan-1 for MF and also for MN induction (Figures 2 and 4), and therefore, it was shown to be less genotoxic. As with Sudan-1, the NOEL for para red shifted to a lower concentration in the CBMN assay in MCL-5 cells. The NOEL for para red was higher than that for Sudan-1 in the HPRT assay. Therefore, it was not tested in MCL-5 cells. The mode of action is not known for para red; however, the predicted pathway is production of a DNA-reactive metabolite (25).

There is yet to be conclusive evidence that azo dyes exhibit a threshold response under different conditions, but data are slowly emerging (16). Our findings show that Sudan-1 is likely to have a non-linear dose response under certain conditions depending on the metabolic capabilities of the system being used. The observed effects reported here are clearly driven by enzymatic processes. The production of genotoxic metabolites will follow Michaelis–Menten type kinetics and the 'tissue' risk for genotoxicity will be a function of uptake (partitioning between blood and tissues) in that tissue (dose) and the enzymatic activity of enzymes in that tissue towards these substrates. Therefore, we cannot assume that there is a threshold in vivo and we would predict different dose responses in tissues, which exhibit different enzymatic activity. Tissues with high metabolic activity would have a lower NOEL or possibly linear dose response compared to tissues with low metabolic activity. MacGregor (46) and the in vitro genetic toxicity quantitative subgroup are currently devising approaches to normalize data sets for comparison between in vitro to in vivo test systems. The initial findings are very promising and this approach could lead to more emphasis being put on the in vitro data sets for hazard and risk assessment in the near future. To validate these approaches, in vitro and in vivo data sets are required within the low-dose region of exposure, and the data presented here would be suitable for these purposes. At the current time, the concentrations are hard to relate to in vivo as different factors are present in living organisms and different target organs are affected in different ways. However, by having these pieces of the puzzle available to such expert groups, there is the potential to devise such comparisons. These findings do show that Sudan-1 and para red have the potential to induce non-linear dose response for genotoxic activity in vitro, yet metabolic activation such as that carried out in the human liver can activate these compounds to become more genotoxic and saturate the DNA repair mechanism even at very low levels.

Conclusions

For Sudan-1, MCL-5 cells induced an increase in genotoxic response compared to AHH-1 cells, possibly by the additional cytochromes or higher expression of CYP1A1 in MCL-5 cells.

Sudan-1 was mutagenic and clastogenic and exhibited its effect in a dose-dependent manner with NOEL and POD for both gene mutation and chromosomal damage end points (Figures 2 and 4). This effect was greatly increased when Sudan-1 was metabolized, and therefore, the metabolites of this azo dye were more genotoxic than the parent compound.

Para red was mutagenic and clastogenic and exhibited its effect in a dose-dependent manner with NOEL and POD for both gene mutation and chromosomal damage end points (Figures 3 and 5). The clastogenic effect was greatly increased when para red was metabolized, and therefore, the metabolites of this azo dye were more genotoxic than the parent compound.

Sudan-1 proved to be a genotoxic carcinogen and the data are now available to aid in the risk estimation process. The data are not yet clarified as to the mechanisms of interaction with the genetic material and further data are necessary.

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References

Azo dye thresholds and metabolism


