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Toxicol Ind Health 2009; 25; 5
DOI: 10.1177/0748233709103037

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Toxicology and Industrial Health 2009; **25:** 5–13
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Genotoxicity studies of a desealant solvent mixture, SR-51®

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The Royal Australian Air Force (RAAF) has reported that personnel involved in F-111 fuel tank maintenance were concerned that exposure to a range of chemicals during the period 1977 to mid-1990s was the cause of health problems, including cancer. Particular concern was directed at SR-51®, a desealant chemical mixture containing the following four solvents: aromatic 150 solvent (Aro150), dimethylacetamide, thiophenol (TP), and triethylphosphate. The present study examined the mutagenic potential of SR-51® using a range of well-known mutagen and genotoxin assays. The tests used were i) a modified version of the Ames test, ii) the mouse lymphoma assay, iii) the comet assay (a single-cell gel electrophoresis assay), and iv) a mouse micronucleus test. The modified Ames test used mixed bacterial strains in liquid suspension media. The Ames test results showed that SR-51® (tested up to the cytotoxic concentration of 36 μg/ml, 30 min incubation) in the presence and absence of S9 metabolic activation was not mutagenic. The mouse lymphoma assay used cultured mouse lymphoma cells in a microwell suspension method. The mouse lymphoma assay was also negative with SR-51® (tested up to the cytotoxic concentration of 22.5 μg/ml, 3 h incubation) in the presence and absence of S9 metabolic activation. The Comet assay, using cultured mouse lymphoma cells, showed no evidence of DNA damage in cells exposed up to the cytotoxic concentration of SR-51® at 11.25 μg/ml. The in-vivo mouse micronucleus test was undertaken in wild-type C57Bl6J male mice dosed orally with SR-51® for 14 days with a single daily dose up to 360 mg/kg/day (the maximum-tolerated dose). No increases were observed in micronuclei (MN) frequency in bone marrow collected (24 h after final dose) from SR-51®-treated mice compared to the number of MN observed in bone marrow collected from untreated mice. Tissues collected from treated mice at necropsy demonstrated a significant increase in spleen weights in the high dose mice. Gas chromatography analysis of SR-51® identified more than 40 individual components and an oxidation product, diphenyldisulfide derived from TP under conditions of mild heating. In conclusion, there was no evidence that SR-51® is mutagenic. *Toxicology and Industrial Health* 2009; **25**: 5–13.

Key words: *Ames test; comet assay; cytotoxicity; genotoxicity; micronucleus test; mouse lymphoma assay; solvents; solvent formulations*

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Los Angeles, London, New Delhi, Singapore
10.1177/0748233709103037
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Introduction

A study undertaken by the Royal Australian Air Force (RAAF) reported that a number of their personnel involved in F-111 fuel tank maintenance were concerned that occupational exposure to a range of chemicals during the period 1977 to mid-1990s was the cause of past and current health problems, including cancer (RAAF, 2001; SHOAMP, 2003, 2004; D'Este, *et al.*, 2008). The maintenance program, known as the Deseal/Reseal (DSRS) program, involved maintenance crew (over 600) working within the leak-prone fuel tanks and desealing and resealing the riveted metal joints. As a consequence of this work, personnel were exposed to a range of chemicals (RAAF, 2001). Of particular concern was a desealant formulation, SR-51®, which consisted of the following four components: 75% aromatic 150 solvent (Solvesso 150), 10% dimethylacetamide (DMA), 10% thiophenol (TP), and 5% triethylphosphate (TEP). As a consequence of the cancer concerns by the maintenance personnel, a study was undertaken to examine the mutagenicity potential of SR-51® using three in-vitro screening assays, (Ames test, mouse lymphoma assay, and the comet assay) and one in-vivo test (mouse micronucleus test).

The DSRS program was undertaken at the RAAF Airbase at Amberley (Queensland, Australia) where summer temperatures regularly exceed 35 °C during the months October to March. Containers of SR-51® were frequently left on the runway and reached an estimated temperature of 45–60 °C. The possibility that these high environmental temperatures might have changed the composition of SR-51® was also investigated using gas chromatography (GC). DSRS maintenance was mostly carried out within aircraft hangers and the personnel were routinely exposed to the vapour from the heated SR-51®.

Materials and methods

The solvent mixture – SR-51®

SR-51® was prepared according to archival specifications provided by the manufacturer, the Eldorado Chemical Company, Texas (the company ceased production of SR-51® in the mid-1980s). SR-51® consisted of the following four components: an aromatic solvent 150 solvent (Solvesso 150), DMA, TP, and TEP. Aro150 was donated by Exxon Chemicals, Australia. DMA, TP, and TEP were all purchased from Sigma Aldrich, Australia. To prepare SR-51®, the four components were mixed in the following ratio, Aro150: DMA:TP:TEP (15:2:2:1).

GC analysis

GC was used to determine the components of SR-51® and the components in the liquid and vapour phase at room temperature (20 °C) and at 60 °C. The separation was carried out on a Hewlett Packard 5890 Series II gas chromatograph fitted with a split/splitless capillary injector (split ratio approximately 50:1) and flame ionisation detector with helium as carrier gas. The following column and separation conditions were used for the analyses: column head pressure, 110 kpa; column (non-polar) Phenomenex ZB-5 length 30 m × 0.25 mm internal diameter, film 0.25 μm; detector temperature, 270 °C; injector temperature was 270 °C for analysis of liquid SR-51® and 250 °C for analysis of headspace vapour SR-51®. Oven temperature program for analysis of liquid SR-51® was 40 °C for 1 min, to 320 °C at 20 °C per min, remaining at 320 °C for 5 min. To analyse the liquid SR-51®, vials containing 200 μl SR-51® were heated for 30 min at 20 °C or 60 °C. A 0.04 μl aliquot of each heated liquid was injected into the GC. Experiments were performed in duplicate. To analyse the vapour, a series of headspace analysis vials containing 3 ml of SR-51® were heated at 20 °C or 60 °C for 30 min. During the heating, a Supelco StableFlex® fibre (DVB coated, thickness 65 μm) was inserted through the vial septum into the SR-51® vapour and left in place for the 30 min for absorption of vapour components onto the fiber to occur. The fiber was then placed in the GC injection port for 1.5 min for desorption to occur. Experiments were performed in duplicate.

S9 metabolic enzymes

An exogenous metabolic activating system (S9 Enzymes) was used in both the Ames test and mouse lymphoma assay. S9 metabolic enzymes were purchased from In Vitro Technologies (Maryland,

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Baltimore, USA). and supernatant extracts from Sprague Dawley rat livers were collected from rats treated with the polychorinated biphenyl, Aroclor 1254, a known inducer of enzymes in rats.

The Ames test

The Ames II™ Manual System Kit was purchased from Xenometrix (Boulder, Colorado, USA). The kit contained the tester strains of *Salmonella typhimurium*: mixed strain stocks (TA7001-TA7006) and a single strain stock of a traditional Ames Strain, TA98. In a preliminary range-finding experiment, the cytotoxicity of SR-51® was determined. The concentration found to be cytotoxic was 36 μg/ml SR-51®. The test was performed according to the manufacturer's instructions. Due to the volatility of SR-51®, all incubations were performed in closed eppendorf tubes in order to reduce the vaporization. Six concentrations of SR-51® diluted in dimethyl sulfoxide (DMSO) were tested in triplicate (i.e. 1.125, 2.25, 4.5, 9, 18, and 36 μg/ml) for 90 min in a medium containing histidine. This incubation was done in the presence or absence of S9 enzyme fraction. After incubation, the cultures were diluted and aliquoted into multi-well plates (384-well plate) containing histidine-free media. The negative control was 4% DMSO. The positive control in the absence of S9 activation was a mix of 4-nitroquinoline-1-oxide and 2-nitrofluorene in DMSO (62.4 and 250 ng/ml, respectively). In the presence of S9 activation, the positive control was aminoanthracene (5 μg/ml). The negative control, each concentration of SR-51®, and the positive control samples were each aliquoted into 48 wells per plate. The number of wells containing revertant colonies was counted. The results were assessed using XenoMatrix™ software and criteria for a valid assay applied as recommended in the Ames II Manual System User's Manual. The criterion for the negative control (DMSO only) was ≤5 positive wells per 48 wells indicating spontaneous mutations. The criterion for the positive controls was ≥13 positive wells per 48 wells.

The mouse lymphoma assay

SR-51® was tested in a mouse lymphoma assay, that used cultured mouse lymphoma cells in a microwell liquid suspension method as described previously by Honma, *et al.*, (1999). Sealed 25 cm^2 tissue-culture flasks were used for the incubation of cells with the chemical in order to reduce the vaporisation of SR-51® (200 μl of chemical solution was added per 20 ml of culture). The mouse lymphoma cells (strain L5178Y $tk^{+/-}$ −3.7.2 C) were sourced from the American Tissue Culture Collection and purchased from Cryosite, Australia. The cell cultures were purged of spontaneous mutants a week prior to the mutation experiments by adding methotrexate to media (supplemented with thymidine and hypoxanthine and glycine) followed by growth without methotrexate. The positive control in the experiment without enzymatic activation was 4-nitroquinoline-1-oxide at a final concentration 0.1 μg/ml (Clements, 2000) and cyclophosphamide at a final concentration of 3 μg/ml in the experiment with S9 activation (Honma, *et al.*, 1999). The negative vehicle control was 1% DMSO. In a preliminary range-finding experiment, the cytotoxicity of SR-51® was determined with and without S9 enzymatic activation. Cells were exposed for 3 h to concentrations up to 90 μg SR-51®/ml. The mutagenicity experiment was performed for four concentrations of SR-51® (2.8, 5.6, 11.25, and 22.5 μg/ml), a negative control, and a positive control. Two independent experiments were conducted: one in the absence of S9 and one in the presence of S9, both with 3 h exposures. Each test was performed with duplicate cultures. After the incubation, all plates were checked for mutant colonies. The number of colonies was counted using a light board.

The comet assay – the single cell gel electrophoresis assay

DNA damage was evaluated using a single cell gel electrophoresis assay (the Comet assay). Mouse lymphoma cells (Cryosite, Sydney, Australia) were exposed *in vitro* for 3 h to SR-51® (solubilised in DMSO at 1% (v/v) at concentrations of 2.8, 5.6, and 11.25 μg/ml. Sealed tissue-culture flasks were used for the incubation due to the volatility of SR-51®. Cell cytotoxicity was tested immediately at the end of the 3-h incubation using the Trypan blue exclusion dye method. The highest concentration of SR-51® tested (11.25 μg/ml) resulted in >85% viable cells. Negative control cells were exposed to 1% (v/v) DMSO only. Positive control cells were exposed to 200 μM (30 min, 4 °C) hydrogen peroxide, a known *in vitro*

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inducer of DNA damage. Comet assays were performed using alkaline unwinding of DNA electrophoresis following an adapted protocol of Donnelly, *et al.*, (2000). The DNA damage was visualized with a Zeiss Axioplan 2 upright fluorescence microscope equipped with an excitation filter (515–560 nm) and barrier filter (590 nm) at 100× magnification. A Zeiss AxioCam HR digital monochrome CCD camera using Zeiss AxioVision 4.0 image acquisition software was used to store comet images. All images were obtained using the same exposure time (763 ms). The comet images were subsequently analysed using the Comet Assay Software Project (CASP) online software (http://www.casp.sourceforge.net) to obtain a range of measurements including percent DNA in tail, olive tail moment, and tail length (Konca, *et al.*, 2003). For each concentration, 100 nonoverlapping comets per two slides were randomly captured at a constant depth of the gel, avoiding edges and damaged gel regions. The parameters of olive tail moment (tail length integrated over intensity of the tail) and percent DNA (percent migrated DNA) in the tail were used as indicators of the severity of DNA damage. Each experiment was performed in duplicate. Data from the two experiments were pooled.

The mouse micronucleus test

Twenty-five male C57Bl/J6 wild-type mice (Animal Resource Centre, Perth, Australia) were housed individually under standard laboratory conditions. The mice were divided into five different groups ($n = 5$) and dosed via oral gavage daily for 2 weeks, 5 days per week with three different doses of SR-51® (90, 180, or 360 mg/kg). Body weights were recorded daily. In preliminary dose-finding experiments, the highest dose was determined to be the maximum tolerated dose based on the animal ethics criteria, in particular weight loss was not greater than 10% during the period of dosing. The next highest dose tested (450 mg/kg) caused greater than 10% weight loss. The SR-51® was dissolved in peanut oil. There was a negative control group (daily 0.1 ml peanut oil) and a positive control group, that received a single intraperitoneal dose of cyclophosphamide (25 mg/kg) on day 14. All mice were killed with CO_2 24 h after the last dose. Bone marrow was collected from the femur of each mouse and four slides per animal were prepared and stained with May-Grunwald–Giemsa stain. The slides were scored and 1000 polychromatic erythrocytes (PCE) per animal were analyzed for the presence of micronuclei (MN). The ratio of normochromatic (NCE) to PCE was determined on 200 erythrocytes per animal. A total of 1000 PCE were counted per animal and the number of MN-PCE were calculated. After the mice were killed for the collection of bone marrow, an autopsy was performed and spleen, kidneys, and liver were collected and weighed.

Statistical analyses

Statistical analyses were performed using SPSS Version 10 software. Results were expressed as means ± SE. The difference was considered significant when $P < 0.05$. Data were tested for homogeneity of variance using Leverne's test. If variance was homogenous, one-way analysis of variance was carried out for the variable. If any significant differences were detected, possible inter-group differences were assessed using the Bonferroni technique. If variances were heterogeneous, the Kruskal–Wallis test was carried out on the variable. If any significant differences were detected, possible inter-group differences were identified using the Dunnett T3 multiple comparisons test.

Results

GC Analyses of SR-51®

SR-51® consists of four components, DMA, TP, TEP, and Aro150. The chromatogram of liquid SR-51® at room temperature, 20 °C (Figure 1) shows the presence of more than 40 chemicals, most of these are components of Aro150, which is a hydrocarbon fluid mixture. The TP peak (represented 7.0% peak area) and the presence of a small diphenyl disulphide (DPDS) peak (1% peak area) demonstrated that even at room temperature there is some oxidation of TP to DPDS. Heating the SR-51® for 30 min at 60 °C did not significantly alter these peaks. The vapour composition was also examined since this was the prime mode of exposure of maintenance personnel (i.e. via inhalation). At room temperature (20 °C), TP was not increased in the headspace (7.4% peak) but DPDS increased to 3.2% peak area. After heating SR-51® for 30 min at 60 °C, TP peak area increased to 9.0% and DPDS to 12.5%.

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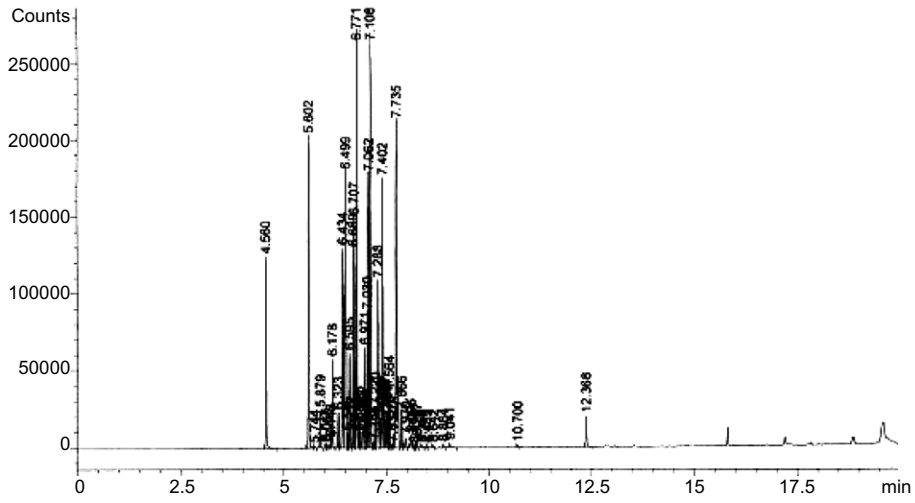

Figure 1 Chromatogram of components of SR-51®. The following retention times (min) for each of the major components was recorded: 4.560 – DMA; 5.602 – TP; 7.108 – TEP; 12.368 – DPDS; and the remaining peaks represent the components of Aro150.

The Ames test

SR-51® (tested up to the cytotoxic concentration of 36 μg/ml) with or without S9 activation showed no evidence of a concentration response or mutagenicity in the Ames II™ test (Table 1). The positive controls used with and without S9 activation gave positive results meeting the required test criteria and confirming the sensitivity of the assay.

The mouse lymphoma assay

SR-51® tested up to 22 μg/ml with or without S9 activation showed no evidence of a concentration

Table 1. Mutagenic assessment of SR-51® in the Ames II™ assay with and without S9 activation

STRAIN – TA98						STRAIN – MIXED					
Concentration SR-51® (μg/ml)	No. of revertants			Mean	Std. dev.	Concentration SR-51® (μg/ml)	No. of revertants			Mean	Std. dev.
	Plate1	Plate2	Plate3				Plate1	Plate2	Plate3		
With S9											
Negative[a]	2	1	0	1.0*	1.0	Negative[a]	0	0	0	0.0*	0.0
1.12	0	2	4	2.0*	2.0	1.12	4	2	1	2.3*	1.5
2.25	3	2	3	2.7*	0.6	2.25	1	1	1	1.0*	0.0
4.5	7	0	1	2.7*	3.8	4.5	1	1	2	1.3*	0.6
9.0	2	3	2	2.3*	0.6	9.0	1	0	0	0.3*	0.6
18.0	1	1	0	0.7*	0.6	18.0	0	1	1	0.7*	0.6
36.0	2	1	0	1.0*	1.0	36.0	0	0	1	0.3*	0.6
Positive[b]	48	48	48	48.0	0.0	Positive[b]	20	23	22	21.7	1.5
Without S9											
Negative[a]	5	3	7	5.0*	2.0	Negative[a]	3	7	6	5.3*	2.1
1.12	6	4	2	4.0*	2.0	1.12	3	1	7	3.7*	3.1
2.25	5	7	1	4.3*	3.1	2.25	7	3	0	3.3*	3.5
4.5	8	5	0	4.3*	4.0	4.5	4	0	2	2.0*	2.0
9.0	6	4	5	5.0*	1.0	9.0	2	4	3	3.0*	1.0
18.0	5	8	0	4.3*	4.0	18.0	0	1	4	1.7*	2.1
36.0	2	2	5	3.0*	1.7	36.0	3	0	1	1.3*	1.5
Positive[c]	13	21	15	16.3	4.2	Positive[c]	23	26	31	26.7	4.0

[a]Negative control: 4% DMSO.
[b]Positive control: 5 μg/ml aminoanthracene.
[c]Positive control: mixture of 4-nitroquinoline-1-oxide and 2-nitrofluorene in DMSO to the final concentrations of 62.4 and 250 ng/ml, respectively.
*Significantly different from positive control ($P < 0.05$).

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Table 2. Mutagenic assessment of SR-51® in the mouse lymphoma assay with and without S9 activation

Concentration SR-51® (μg/ml)	Adjusted relative survival RS (%)	Plating efficiency (PE) survival	Plating efficiency (PE) viability	Mutant frequency (MF)/10^6 viable Cells
With S9				
Negative (DMSO)		65	72	66.8*
2.813	102.4	53	75	67.7*
5.625	59.1	44	93	59.8*
11.25	41.5	30	73	52.6*
22.50	2.3	13	7	Cytotoxic
Positive control[a]	110.1	65	62	167.4
Without S9				
Negative (DMSO)		62	71	69.2*
2.813	55.9	47	54	49.5*
5.625	64.9	38	63	47.2*
11.25	20.4	24	52	73.2*
22.50	0.4	7	6	Cytotoxic
Positive control[b]	109.1	61	68.5	215.5

[a]Cyclophosphamide to the final concentration of 3 μg/ml.
[b]4-nitroquinoline-1-oxide to the final concentration 0.1 μg/ml.
*Significantly different to positive control ($P < 0.05$).

response or mutagenicity in the mouse lymphoma assay (Table 2). The mean mutant frequencies for the various SR-51® concentrations varied between 52.6 and 67.7 (with S9) and 47.2 and 73.2 (without S9). The mean positive control gave mutant frequency values of 167.4 (with S9) and 215.5 (without S9), which was greater than twice the vehicle control values and is within the range expected of the positive controls and indicated that the experiment had sufficient sensitivity (Honma, *et al.*, 1999). Based on preliminary data (not shown), the SR-51® concentration of 22.5 μg/ml was chosen as the maximum concentration for the mouse lymphoma assay with or without S9 activation. Ideally, the maximum concentration should show cytotoxicity (indicated by the adjusted relative survival (RS)) of approximately 10%–20% (Honma, *et al.*, 1999; Clements, 2000). The maximum concentration used for SR-51® (22.5 μg/ml) was less than 10% and showed corresponding low viability plating efficiency and therefore mutant frequency data was not obtained.

The comet assay – single cell gel electrophoresis

SR-51® was tested at three different concentrations. The highest concentration tested (11.2 μg/ml) was the highest concentration in which >85% cells remained viable. There was no evidence of increased DNA damage (%DNA in tail and olive tail moment) compared to the negative control and there was no evidence of a concentration effect (Table 3). The positive control of hydrogen peroxide (200 μM) showed a significant increase in DNA damage.

The mouse micronucleus assay

The effects of orally administered SR-51® on the incidence of MN in bone marrow cells (MN–PCE) of male mice was tested up to the maximum tolerated dose of 360 mg/kg. The results showed no evidence of increased MN formation based on the MN–PCE ratio (Table 4). The positive control showed a significant increase in MN–PCE as expected and confirmed the sensitivity of the assay. Among the SR-51®-treated mice, there was a small dose-dependent increase in the PCE/NCE ratio but this was not statistically significant.

Table 3. The effect of in-vitro exposure to SR-51® on %tail DNA in mouse lymphoma cells using the Comet assay

Concentration of SR-51®	Mean olive tail moment (±SE)	Mean %tail DNA (±SE)
0	6.58 ± 0.26*	21.61 ± 0.75*
2.8 μg/ml	7.52 ± 0.40*	23.66 ± 0.96*
5.6 μg/ml	7.30 ± 0.63*	21.20 ± 1.03*
11.2 μg/ml	5.55 ± 0.34*	19.31 ± 0.89*
200 μM H_2O_2	64.32 ± 1.58	83.72 ± 0.99

The Comet assay detects DNA damage in single cells. Data are mean ± SE values derived from two separate experiments.
*Significantly different to positive control ($P < 0.05$).

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Table 4. Micronucleus assay in male C57Bl6J mice

Chemical formulation	Dose (mg/kg/day)	Ratio PCE/NCE	MN-PCE (%)
Negative control (oil)	0	1.39	0.66
SR-51®	90	1.29	0.65
SR-51®	180	1.42	0.66
SR-51®	360	1.50	0.66
Positive control (cyclophosphamide, single i.p.)	25	1.33	1.77*

SR-51 was administered by oral gavage for 14 days (5 days/week). Bone marrow sampling was made 24 h after the final dosing. Cyclophosphamide was administered (25 mg/kg, ip) as a positive control. There were five animals per dosing group except one mouse was euthanased in the high-dose SR-51®-treatment group before the end of treatment period due to SR-51® toxicity.
*Significantly different to negative control and SR-51®-treated mice at $P < 0.05$.

Necropsy data – organ weights (absolute weights and organ-to-body weight ratios)

Livers, kidneys, and spleens were collected from treated mice after the final dose of SR-51®. There were no significant changes in the absolute or relative kidney or liver weights between the SR-51®-treated and control groups (Table 5). However, spleens collected from the high-dose SR-51®-treated mice showed an increase ($P < 0.05$) in both absolute and relative weights compared to control and low- and middle-dose treatment groups. One of the five high-dose mice was euthanased due to treatment toxicity after day 4. Signs of toxicity were immobility, listlessness, and loss of body weight (>10%). It was also noted that three of the surviving four high-dose animals had pale kidneys when collected at end of treatment period.

Discussion

Despite significant achievements in the risk assessment of chemicals, the toxicological database, particularly for industrial chemicals, remains limited (Bakand, *et al.*, 2005). Studies of chemical formulations/combinations are even less common and often publication is restricted due to proprietary confidentiality of formulation details. This means information on possible interactions between different components within a formulation is not readily available. The current study assessed the genotoxicity potential of the desealant formulation, SR-51®, via a battery of bioassays that included both in-vitro and in-vivo tests.

The Ames II™ test used in this study is based on the same principle as the traditional Ames test, but has different strains and uses a liquid suspension media rather than solid agar plates that are used

Table 5. Summary of absolute organ weights and organ-to-body weight ratios of kidneys, spleens, and livers collected from mice dosed for the micronucleus assay

	Mouse treatment group ($n = 5$)			
Organ	Control (0 mg/kg) Group 1	LD SR-51® (90 mg/kg) Group 2	MD SR-51® (180 mg/kg) Group 3	HD SR-51® (360 mg/kg) Group 4
Absolute weights				
Body (g)	29.68 ± 2.20	27.46 ± 0.89	29.06 ± 1.42	28.32 ± 1.60
Liver (g)	1.455 ± 0.075	1.352 ± 0.173	1.375 ± 0.153	1.436 ± 0.072
Kidney (g)	0.399 ± 0.022	0.367 ± 0.042	0.396 ± 0.039	0.388 ± 0.033
Spleen (g)	0.075 ± 0.007	0.076 ± 0.018	0.082 ± 0.020	0.120 ± 0.026*
Organ-to-body weight ratios				
Liver	4.935 ± 0.552	4.933 ± 0.680	4.721 ± 0.340	5.072 ± 0.146
Kidney	1.347 ± 0.086	1.338 ± 0.144	1.363 ± 0.121	1.370 ± 0.069
Spleen	0.253 ± 0.024	0.274 ± 0.060	0.284 ± 0.075	0.428 ± 0.109*

Organs were collected at necropsy after daily dosing for 2 weeks with either oil (negative control), 90 mg SR-51®/kg (LD = low dose), 180 mg SR-51®/kg (MD = mid dose), and 360 mg SR-51® /kg (HD = high dose). Mice were orally dosed via gavage for 5 days per week for 2 weeks. One HD SR-51® mouse was euthanased after four doses due to signs of treatment toxicity (weight loss and immobility).
*Significantly different to negative control and LD and MD SR-51®-treated mice at $P < 0.05$.

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in the traditional Ames test (Gee, *et al.*, 1994, 1998). Several validation studies have been performed (Gee, *et al.*, 1998; Flückiger-Isler, *et al.*, 2004). The present results show that SR-51® with or without S9 metabolic activation was not mutagenic using this test. Three of the components of SR-51® (TEP, DMA, and TP) have been studied previously using the traditional Ames test and also gave negative results (Lavoie, *et al.*, 1979; Zeiger, *et al.*, 1987, 1988).

When testing for mutagenicity, it is common practice to use the mammalian mouse lymphoma assay in addition to the bacterial-based Ames test. This test can also detect mutagens, which cause chromosomal mutations. There was no evidence of either a mutagenic or clastogenic effect with SR-51® (3 h exposure) using this test. Further experiments at a longer exposure (24 h) could be undertaken to confirm the negative result (Moore, *et al.*, 2007).

The comet assay is a second mammalian-based assay that is particularly sensitive for detecting strand breaks in the DNA (Sasaki, *et al.*, 1997; Annas, *et al.*, 2000; Grant, *et al.*, 2001). The comet assay is used with increasing frequency to determine risk to humans in different exposure situations (Singh, *et al.*, 1990; Hellman, *et al.*, 1997; Møller, *et al.*, 2000). In the present study, the comet assay was performed in the absence of metabolic activation enzymes since the main route of exposure to SR-51® was through the skin and lungs before its passage to the liver where it may be metabolised. The results for the SR-51® in the Comet assay did not show any dose response or induce DNA damage in the mouse lymphoma cells up to a cytotoxic concentration.

The mouse micronucleus test is the only mammalian in-vivo mutagenicity assay in common usage. There was no evidence SR-51® was mutagenic in the mouse micronucleus test. Treatment toxicity was noted at the highest dose resulting in one mouse being euthanased. Surviving mice exposed in the high dose group had significantly larger spleens compared to untreated control mice. This increased spleen weight is suggestive of hemolysis occurring as a consequence of the TP component in SR-51®. *In vivo*, haemolysis may be expected from any disulfide or thiol, which undergoes considerable autooxidation at neutral pH (McBain and Menn, 1969; Munday and Manns, 1985; Munday, 1989; Munday, *et al.*, 1990). DPDS is a significant component of the SR-51® vapour (see GC results). *In vivo*, DPDS can be reduced back to TP by glutathione, which causes a reduction/autooxidation cycle for generation of 'active oxygen' species, superoxide radical and hydrogen peroxide.

The studies in this report were undertaken to examine whether SR-51® had mutagenic properties that may explain a possible increase in cancers among the exposed maintenance personnel (SHOAMP, 2004; D'Este, *et al.*, 2008). The results of these studies show that SR-51® does not have mutagenic properties. Although, most animal carcinogens are mutagens, it is possible for carcinogens to act through nonmutagenic mechanisms and this possibility has not been excluded by these studies.

Much of the human exposure to SR-51® took place at high environmental temperatures. The GC study showed that the oxidation product of TP, DPDS is formed in greater quantities under these conditions. As discussed above, this may have additional toxicological consequences from SR-51® exposure. The TP component of SR-51 has a very strong and unpleasant odour. This meant the maintenance workers were constantly aware of the presence of the chemical and this may have contributed to the workers concern that the SR-51 affected their health (RAAF, 2001). TP, at 62 ppt, has one of the lowest odor thresholds of any chemical. There is evidence suggesting adverse sensory reactions to strong odours and irritants may lead to release of catecholamines and stress hormones, which maybe associated with an adverse effect on physiological and biochemical measurements related to cardiovascular risk (Smith, *et al.*, 1999). A study by Bensafi, *et al.*, (2002) assessed the effect of six odorants (one of which was TP) on the autonomic nervous responses (heart rate and skin conductance) of 12 human subjects. Based on test results, TP was rated as the most intense and most unpleasant odorants. Furthermore research is needed to affirm if changes induced by exposure to an odorous chemical poses a real health risk or are merely reflective of reversible changes within the normal homeostatic range (Smith, *et al.*, 1999). This may be relevant to those DSRS personnel who reported constant exposure to the intensely unpleasant odour of SR51 fumes while working in the confined space of F-111 aircraft fuel tanks.

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Acknowledgments

This research was supported by a grant from the Australian Department of Veterans' Affairs. The authors wish to thank Hege Jeffring and Belinda Hughes for their technical assistance in performing the Comet Assays.

References

Annas, A, Brittbo, E, Hellman, B (2000) Evaluation of benzo(a)pyrene-induced DNA damage in human endothelial cells using alkaline single cell gel electrophoresis. *Mutat Res* 471: 145–155.

Bakand, S, Winder, C, Khalil, C, Hayes, A (2005) Toxicity assessment of industrial chemicals and airbourne contaminants: transition from in vitro and in vivo test methods: a review. *Inhal Toxicol* 23: 299–307.

Bensafi, M, Rouby, C, Farget, V, Bertrand, B, Vigouroux, M, Holley, A (2002) Autonomic nervous system responses to odours: the role of pleasantness and arousal. *Chem Senses* 27: 703–709.

Clements, J (2000) The mouse lymphoma assay. *Mut Res* 455: 97–110.

D'Este, C, Attia, JR, Brown, AM, Gibson, R, Gibberd, R, Tavener, M, *et al*. (2008) Cancer Incidence and Mortality in Aircraft Maintenance Workers. *Am J Indust Med* 51: 16–23.

Donnelly, ET, O'Connell, M, McClure, N, Lewis, SEM (2000) Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. *Hum Reprod* 15: 1552–1561.

Flückiger-Isler, S, Baumeister, M, Braun, K, Gervais, V, Hasler-Nguyen, N, Reimann, R, *et al*. (2004) Assessment of the performance of the Ames II™ assay: a collaborative study with 19 coded compounds. *Mutat Res* 558: 181–197.

Gee, P, Maron, DM, Ames, BN (1994) Detection and classification of mutagens: a set of base-specific Salmonella tester strains. *Proc Natl Acad Sci U S A* 91: 1606–1610.

Gee, P, Sommers, CH, Melick, AS, Gidrol, XM, Todd, MD, Burris, RB, *et al*. (1998) Comparison of responses of base-specific Salmonella tester strains with the traditional strains for identifying mutagens: the results of a validation study. *Mutat Res* 412: 115–130.

Grant, GM, Jackman, SM, Kolanko, CJ, Stenger, DA (2001) JP-8 jet fuel-induced DNA damage in H4IIE rat hepatoma cells. *Mutat Res* 490: 67–75.

Hellman, B, Vaghef, H, Friis, L, Edling, C (1997) Alkaline single cell gel electrophoresis of DNA fragments in biomonitoring for genotoxicity: an introductory study on healthy human volunteers. *Int Arch Occup Environ Health* 69: 185–192.

Honma, M, Hayashi, M, Shimada, H, Tanaka, N, Wakuri, S, Awogi, T, *et al*. (1999) Evaluation of the mouse lymphoma tk assay (microwell method) as an alternative to the in vitro chromosomal aberration test. *Mutagenesis* 14: 5–22.

Konca, K, Lankoff, A, Banasik, A, Lisowska, H, Kuszewski, T, Góźdź, S, *et al*. (2003) A cross-platform public domain PC image –analysies program for the comet assay. *Mutat Res* 534: 15–20.

Lavoie, ET, Tulley, L, Fow, E, Hoffmann, D (1979) Mutagenicity of aminophenyl and nitrophenyl ethers, sulfides, and disulphides. *Mutat Res* 67: 123–131.

McBain, JB, Menn, JJ (1969) S-methylation, oxidation, hydroxylation and conjugation of thiophenol in the rat. *Biochem Pharmacol* 18: 2282–2285.

Møller, P, Knudsen, LE, Loft, S, Wallin, H (2000) The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effects of confounding factors. *Cancer Epidemiol Biomarkers Prev* 9: 1005–1015.

Moore, MM, Honma, M, Clements, J, Awogi, T, Bolcsfoldi, G, Cole, J, *et al*. (2007) Mouse lymphoma thymidine kinase gene mutation assay: meeting of the International Workshop on Genotoxicity Testing, San Francisco, 2005, recommendations for 24-h treatment. *Mutat Res* 627: 36–40.

Munday, R (1989) Toxicity of thiols and disulphides: involvement of free-radical species. *Free Radic Biol Med* 7: 659–673.

Munday, R, Manns, E (1985) Toxicity of aromatic disulphides III. In vivo haemolytic activity of aromatic disulphides. *J Appl Toxicol* 5: 414–417.

Munday, R, Manns, E, Fowke, EA (1990) Steric effects on the haemolytic activity of aromatic disulphides in rats. *Food Chem Toxicol* 28: 561–566.

RAAF (2001) Royal Australian Airforce. Chemical exposure of air force maintenance workers. Report of the Board of Inquiry into F-111 (Fuel Tank) Deseal/Reseal and Spray Seal Programs, Airforce Headquarters, Canberra.

Sasaki, YF, Tsuda, S, Izumiyama, F, Nishidate, E (1997) Detection of chemically induced DNA lesions in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow) using the alkaline single cell gel electrophoresis (Comet) assay. *Mutat Res* 388: 33–44.

SHOAMP (2003) Study of Health Outcomes in Aircraft Maintenance Personnel (SHOAMP) Phase II – Mortality and Cancer Incidence. The University of Newcastle Research Associates (TUNRA).

SHOAMP (2004) Study of Health Outcomes in Aircraft Maintenance Personnel (SHOAMP) Phase III – Report of the General Health and Medical Study. The University of Newcastle Research Associates (TUNRA).

Singh, NP, Danner, DB, Tice, RR, Brant, L, Schneider, EL (1990) DNA damage and repair with age in individual human lymphocytes. *Mutat Res* 237: 123–130.

Smith, CJ, Scott, SM, Ryan, BA (1999) Cardiovascular effects of odours. *Toxicol Ind Health* 15: 595–601.

Zeiger, E, Anderson, B, Haworth, S, Lawlor, T, Mortelmans, K, Speck, W (1987) Salmonella mutagenicity tests: III. Results from the testing of 255 chemicals. *Environ Mutagen* 9(Suppl. 9): 1–110.

Zeiger, E, Anderson, B, Haworth, S, Lawlor, T, Mortelmans, K (1988) Salmonella mutagenicity tests: IV. Results from the testing of 300 chemicals. Environ Mol Mutagen 11(Suppl. 12): 1–158.

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