Bundesinstitut
für gesundheitlichen Verbraucherschutz und Veterinärmedizin

Genotoxicity of 2-Hydroxy-1,4-naphthoquinone (Lawsone, CAS 83-72-7)

Bewertung des BgVV vom 21. Juni 2002

Die Substanz Lawson (2-Hydroxy-1,4-naphthochinon) ist ein Bestandteil von Haarfärbemitteln wie z.B. Henna. Seit einiger Zeit wird diskutiert, ob Lawson mutagen (erbgutverändernd) wirkt. Unter Berücksichtigung aller Daten kommt das Institut zu dem Schluss, dass aus den bisher vorliegenden Studien keine Gefährdung der Gesundheit abgeleitet werden kann.

It is under discussion whether or not Lawsone constitutes a health hazard due to the positive in vivo mutagenicity data. Considering all the available and quite comprehensive genotoxicity data, our conclusion is that no health hazard can be derived.

The main aspect of the discussion is the concern due to positive data from a specific protocol of the in vivo micronucleus test with mice (72 h sampling time). Although a weak positive effect was found at high doses in two independent investigations (of the same lab), the whole amount of data clearly suggests that these positive data are of no relevance for the human situation.

This conclusion is based on the following line of argumentation:

1. The weak positive data are paralleled by a number of negative in vivo findings:

   - negative in the same in vivo micronucleus study for routine sampling times of 24 and 48 h,
   - negative in further in vivo micronucleus tests, including an investigation with 72 h sampling,
   - negative in in vivo chromosomal aberration tests,
   - negative in a rat liver test for induction of DNA repair (UDS).

2. Based on the negative in vivo chromosomal aberration tests [and supported by the unusual time-effect relationship] it is obvious that induction of micronuclei cannot be due to clastogenicity. Thus, there are no direct DNA effects.

3. In principle, micronuclei can also be derived from aneugenic effects (induction of aneuploidies, which does not need direct interaction with DNA [but with tubuline molecules]). However, according to their mechanism of action, aneugen-derived micronuclei occur early after treatment (and not at a late sampling time).

4. In theory, specific induction of micronuclei at late sampling times may be due to extraordinary toxicokinetic properties of the test substance, such as time-consuming transports or stimulation of activating enzymes. With respect to Lawsone, there is no evidence for such a hypothesis.

5. A remaining explanation for the occurence of micronuclei at a late sampling time is given by unspecific "biological stress". Such indirect effects for induction of micronuclei in vivo are well-known, e.g. for variations in body temperature and stimulation of cell proliferati-

on. The haematotoxic properties of Lawsone, leading to stimulation of cell proliferation, represent a sufficient explanation for a weak induction of 'late micronuclei'.

Overview on genotoxicity data for 2-Hydroxy-1,4-naphthoquinone

Potential genotoxicity of 2-hydroxy-1,4-naphthoquinone was tested quite extensively in a number of test systems. In general, test performances were in accordance with the guidelines.

In the following the results of the in vitro and in vivo tests are summarized and mammalian in vivo data are also given in a table.

Bacterial genotoxicity tests: negative

2-Hydroxy-1,4-naphthoquinone was negative with and without S-9 mix in a well-conducted gene mutation test with a selection of bacterial tester strains (ref. 6: CIT rep 11317 MMJ, 1994). Two further investigations with Samonella typhimurium strains do not add relevant information due to methodological insuffiencies and lack of detailed descriptions: a negative test in Salmonella typhimurium TA98 (ref. 17: Stamberg et al, 1979) and a positive test in TA2637 (ref 16: Tikkanen et al., 1983).

Mammalian cell gene mutation test (HPRT test): negative

An HPRT gene mutation test with V79 cells led to a negative result (ref. 19: De Jouffry, CIT rep 13589 MVA, 1996). Doses ranging from 15 to 5000 µg/ml were tested with and without S-9 mix with a 3-h treatment period; adequate negative and positive controls were included; three experiments were performed. Some cytotoxicity was found with and without S-9 mix, especially at 5000 µg/ml.

In the final draft of document SCCNFP/0583/02 it is mentioned that mutation frequencies in treated cells were "higher than 3 times the vehicle control value for some concentrations". Such variations in mutation frequencies are typical for HPRT tests. Since there was no consistency in increased mutation frequencies in relation to doses, between parallel cultures and in the three experiments, it can be concluded that the variations are simply due to statistical reasons and are without biological significance.

Mouse lymphoma assay: positive with S-9 mix

In a mouse lymphoma assay positive effects were obtained in 2 experiments for all tested doses ranging from 25 to 800 µg/ml in the presence of S-9 mix (ref. 7: Safepharm rep 297/3, 1992). The dose-effect relationship for mutation frequencies was surprisingly flat with a ca. 4-fold increase at 25 µg/ml and a ca. 6-fold increase at 800 µg/ml. Cytotoxicity was weak to moderate. Marginal effects were observed in the absence of S-9 mix.

Unfortunately, the test was run without colony-sizing (differentiation between large and small colonies). Therefore, it cannot be deduced from this test whether there was a preferential induction of gene or chromosome mutations. Considering the negative HPRT test and the positive in vitro chromosomal aberration test, preferential induction of chromosomal aberrations is likely.

In vitro chromosomal aberration test: positive with S-9 mix at 5000 µg/ml

In an in vitro chromosomal aberration test with CHO cells, doses of 300, 1000 and 5000 µg/ml were investigated with S-9 mix and doses of 30, 100 and 300 µg/ml without S-9 mix (ref. 8: CIT rep 11318 MIC, 1994). Treatment / sampling times were 3 / 21 h with and 21 / 21 h without S-9 mix. Weak to moderate cytotoxicity was seen at the top doses. A strong positive effect (36 % aberrant cells) was found with S-9 mix at 5000 µg/ml; this dose corresponds to ca. 29 mmol/l and thus exceeds the recommended top dose of 10 mmol/l by far.

In vivo chromosomal aberration test: negative

2-Hydroxy-1,4-naphthoquinone was clearly negative in three investigations on induction of chromosomal aberrations in vivo in bone marrow or peripheral blood cells:

- ref. 10: Freiburger Labor für Mutagenitätsprüfung Proj CHO192H, 1992: negative in Chinese hamster bone marrow cells after single oral administration of 200 mg/kg bodyweight; sampling times 6, 24 and 48 h; 5 male plus 5 female animals per group; vehicle arachis oil; concurrent negative and positive controls; no data on toxicity. In the final draft of document SCCNFP/0583/02 it is criticized that 72 h sampling was not included. However, such a late sampling was never used in routine testing for in vivo clastogenicity and taking the mode-of action for clastogenesis into consideration is absolutely not needed. Therefore, there is no reason to doubt the reliability of the negative result.

- ref. 18: Wright, Safepharm proj 436/8, 1992:
  negative in rat blood lymphocytes after oral doses of 100 mg/kg on 28 consecutive days; 3 male plus 3 female animals per group; vehicle arachis oil; concurrent negative and positive controls; clinical signs of toxicity.
  In general, in vivo chromosomal aberration tests with multiple treatments have a relatively low sensitivity. Nevertheless, the result gives some support to the other negative findings.

- ref. 33: Haddouk, CIT BP 563-27005, study 22244, 2001:
  negative in mouse bone marrow cells after a single oral dose of 250 mg/kg; sampling at 24 and 72 h; 10 males; vehicle methylcellulose; concurrent negative and positive controls; lethal effects; no local cytotoxicity in bone marrow cells (mitotic activity).

In vivo micronucleus test: negative at routine sampling times, weakly positive at 72 h
2-Hydroxy-1,4-naphthoquinone was tested in 4 in vivo bone marrow micronucleus tests with mice.

- ref 9.1: Österreichisches Forschungszentrum study 845/89, August 1989:
  positive after single oral administration of 250 mg/kg at 72 h sampling time, negative at sampling times 24 h and 48 h; only 1 dose tested; vehicle DMSO (5 ml/kg); 5 male plus 5 female animals per group; concurrent negative and positive controls; weak toxic signs, local cytotoxicity in the bone marrow at 24 h sampling time. The positive effect was weak: 0.32 % micronucleated polychromatic erythrocytes (PCE) as compared to 0.11 % in the negative control group.
  In this study the use of, toxic, DMSO as vehicle is not in line with the state of the art.

- ref 9.2: Österreichisches Forschungszentrum study 1880, December 1990:
  positive after single oral administration of 110 mg/kg and 250 mg/kg at 72 h sampling time, negative for 25 mg/kg; only 1 sampling time; vehicle DMSO (5 ml/kg); 5 male plus 5 female animals per group; concurrent negative and positive controls; no local cytotoxicity in the bone marrow. The positive effects were weak: 0.32 % and 0.36 % micronucleated (PCE) as compared to 0.12 % in the negative control group.
  Again the use of DMSO as vehicle is critical.

- ref. 9.3: CIT rep 11319 MAS June 1994:
  negative after single oral administration of 30, 100 or 300 mg/kg at 24 h and 48 h sampling times; vehicle methylcellulose; 5 male plus 5 female animals per group; concurrent negative and positive controls; lethal effects after 300 mg/kg; inconclusive findings on local cytotoxicity in bone marrow.

- ref. 31: Haddouk, CIT BP 563-27005, July 2001:
  negative after single oral administration of 250 mg/kg; sampling only at 72 h; 10 males, vehicle methylcellulose; no clinical signs, no local cytotoxicity; negative control included but no concurrent positive control.
  In the final draft of document SCCNFP/0583/02 it is argued that no conclusion can be drawn due to the absence of a positive control. In fact, although the lab is well-known and routinely performs this type of test, the lack of a positive control is a major drawback which clearly limits the reliability of the result.

A 5th mouse bone marrow micronucleus test was performed with "Henna Rot", a 1 %-solution of Lawsone.

- ref. 37: Jenkinson, Safepharm Proj 436/4, February 1992:
  negative after single intraperitoneal administration of 300 mg/kg; sampling times 24 h, 48 h and 72 h; 5 male plus 5 female animals; vehicle CMC; negative and positive controls included; clinical signs, local cytotoxicity (decreased P/N ratio) at 48 h and 72 h, lethality at a higher dose of 500 mg/kg.
  Since Lawsone is only a 1 % constituent of Henna Rot, this negative result does not add relevant information with respect to Lawsone.

<u>In vitro test on DNA repair (UDS): negative</u>

2-Hydroxy-1,4-naphthoquinone was negative with respect to an induction of DNA excision-repair (UDS) in rat livers after single oral treatment with 150 and 1500 mg/kg (ref. 11: CCR study 432628, 1993). Sampling was done 2 h (both doses) and 16 h (high dose) after treatment; 4 males were used per group; negative and positive controls were included. Signs of toxicity were seen at 1500 mg/kg, in a pre-test lethality occurred after dosing with 2000 mg/kg; no cytotoxic effects were observed in the hepatocytes.

Lawsone: Overview on mammalian in vivo tests

Test system	Species, tissue	animals	Expos. regime	Sampl. times	Dose range	local cytotox.?	Genetic effect	LOED (mg/kg)	max. eff. (neg.co)	GLP?	Ref
test substance: 2-Hydroxy-1,4-naphthoquinone [FC 200488, Lawsone]											
CAbviv	Ch.H., b.m.	5m+5f	1x po	6, 24, 48 h	200 mg/kg	n.d.	neg	-	-	yes	10
CAbviv	R, PBL	3m+3f	28x po		100 mg/kg	n.d.	neg	-	-	yes	18
CAbviv	M, b.m.	10m	1x po	24, 72	250 mg/kg	no	neg	-	-	yes	33
MNTviv	M, b.m.	5m+5f	1x po	24, 48h	30 - 100 - 300 mg/kg	inconcl	neg	-	-	yes	9.3
MNTviv	M, b.m.	5m+5f	1x po	24, 48h	250 mg/kg	slight	neg	-	-	yes	9.1
				72 h		no	weak	250 mg/kg	0.32% (0.11)		
MNTviv	M, b.m.	5m+5f	1x po	72 h	25 - 250 mg/kg	no	weak	110 mg/kg	0.36% (0.12)	yes	9.2
MNTviv	M, b.m.	10m	1x po	72 h	250 mg/kg	no	neg	-	-	yes	31
UDSviv	R, hpc	4m	1x po	2, 16h	150 - 1500 mg/kg	no	neg	-	-	yes	11
test substance: Henna Rot, batch 830.72											
MNTviv	M, b.m.	5m+5f	1x ip	24, 48, 72 h	300 mg/kg	yes	neg	-	-	yes	37

References

[Nos. according to SCCNFP/0583/02, final draft; http://europa.eu.int/comm/food/fs/sc/sccp/out177_en.pdf.]:

(9.1) Österreichisches Forschungszentrum, Austria. Study No: 845/89 (Aug 1989)

(9.2) Österreichisches Forschungszentrum, Austria. Study No: 1880 (Dec1990)

(9.3) CIT, France. Report No: 11319 MAS (Jun1994)

(10) Freiburger Labor für Mutagenitätsforschung, Germany. Project No: CHO192H (6/1992)

(11) Cytotest Cell Research GmbH and Co., Germany. Study No: 432628 (Sep 1993)

(18) Wright N.P. 2-Hydroxynaphthoquinone: metaphase analysis of lymphocyte chromosomes of rats from a28-day toxicity range finding study (Project no.436/7) in vivo (Project no. 436/8); Safepharm Lab. Ltd. P.O. Box n^{0}45 Derby, UK, 1992

(19) De Jouffry S. In vitro mammalian cell gene mutation tests (HPRT/V79 system). Centre International de Toxicologie, Evreux, France. Report n^0 13589 MVA, 26.11.1996

(31) Haddouk H., Bone marrow micronucleus test by oral route in mice. CIT BP 563-27005 Evreux France, Laboratory Study no. 21786 MAS, 6July 2001

(33) Haddouk H., In vivo mammalian bone marrow cytogenetic test by oral route in mice chromosomal analysis. CIT BP 563-27005 Evreux France, Laboratory Study n^{0}22244 MOS, 6December 2001

(37) Jenkinson, PC. Henna Rot: Micronucleus Test in the Mouse. Safepharm Laboratories Ltd. Project n^0 436/4, February 10, 1992