Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

barium bis[2-chloro-5-[(2-hydroxy-1-naphthyl)azo]toluene-4-sulphonate]; C.I. Pigment Red 53:1

EC Number: 225-935-3
CAS Number: 5160-02-1

CLH-O-0000007323-79-01/F

Adopted

8 June 2023

8 June 2023

CLH-O-0000007323-79-01/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **barium bis[2-chloro-5-[(2-hydroxy-1-naphthyl)azo]toluene-4-sulphonate]; C.I. Pigment Red 53:1**

EC Number: **225-935-3**

CAS Number: **5160-02-1**

The proposal was submitted by **Germany** and received by RAC on **1 August 2022.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Germany has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **19 September 2022**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **18 November 2022**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Anca Oana Docea**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **8 June 2023** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	TBD	barium bis[2-chloro-5-[(2-hydroxy-1-naphthyl)azo]toluene-4-sulphonate]; C.I. Pigment Red 53:1	225-935-3	5160-02-1	Carc. 2	H351	GHS08, Wng	H351			
RAC opinion	TBD	barium bis[2-chloro-5-[(2-hydroxy-1-naphthyl)azo]toluene-4-sulphonate]; C.I. Pigment Red 53:1	225-935-3	5160-02-1	Carc. 2	H351	GHS08, Wng	H351			
Resulting Annex VI entry if agreed by COM	607-RST-VW-Y	barium bis[2-chloro-5-[(2-hydroxy-1-naphthyl)azo]toluene-4-sulphonate]; C.I. Pigment Red 53:1	225-935-3	5160-02-1	Carc. 2	H351	GHS08, Wng	H351			

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Barium bis[2-chloro-5-[(2-hydroxy-1-naphthyl)azo]toluene-4-sulfonate]; C.I. Pigment Red 53:1 (PR 53:1) belongs to the group of β-naphthol azo lake pigments with widespread uses, especially in the imparting of colour to printing inks and plastic products, but also for coating and masterbatches. The substance has no current entry in Annex VI to the CLP Regulation. The dossier submitter (DS) proposed classification as Carc. 2, H351. The DS did not assess other hazard classes in the CLH report.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS reported the following *in vivo* carcinogenicity studies (cf. Table 12 and 13 of the CLH report):

Reference, Method, guideline, deviations if any	**Test substance**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Results**	**Historical control data (HCD)**
Studies on rats				
NTP, 1982a 2-year feeding study in rats Non-GLP According to OECD TG 451 Reliability without restriction according to the DS	PR 53:1 Purity 89.8 % Impurities sodium and barium sulfates	Species: rats Strain: F344 Route: oral (feed) Number: 50/dose group/sex Treatment time: 103 weeks, daily Post exposure period: 1 week Dose levels: 0, 1 000, 3 000 ppm[1] Food conversion factor: 20 (for older rats) Calculated doses[2]: 0, 50, 150 mg/kg bw/d	Carcinogenicity: - Increased incidence of sarcoma of the spleen and dose-related increase in neoplastic nodules of the liver in male rats; no effects in female rats Spleen neoplastic lesions in male rats: - Combined types of splenic sarcoma (0, 1 000, 3 000 ppm): 0/50 (0 %); 0/50 (0 %), 26/48 (54 %)[*,#] - Fibrosarcoma (17/48) (35 %)[*,#] arising from red pulp or capsule of the spleen, leiomyosarcoma (1/48), splenic osteosarcoma (5/48), sarcoma (1/48), fibrosarcoma of the splenic capsule (1/48), fibrosarcoma of the splenic red pulp (1/48). 11 of splenic tumors metastasized to peritoneal tissues, 2 sarcoma of multiple organs originated in the spleen.	Splenic neoplastic lesions NTP data (1984-1994)[4]: - All types of splenic sarcoma[5]: 7/1 003 (0.7 %) in males and 0/1 003 in females - Fibrosarcoma: 4/1 003 (0.4 %), range 0-6 % in males; 0/1 003 in females NTP report (1982): - Fibrosarcoma in males: 0/140 (same lab), 3/2 960 (0.1 %) (same lab, entire bioassay program) Liver neoplastic lesions NTP data (1984-1994)[4]: - Hepatic neoplastic nodules: no data - Hepatocellular carcinoma: 7/1 002 (0.7 %), range 0-6 % in males;

Reference, Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Historical control data (HCD)
			Liver neoplastic lesions (0, 1 000, 3 000 ppm): - Hepatocellular carcinoma 1/50 (2 %), 0/50, 0/49 in males and 0/50, 0/50, 0/50 in females - Neoplastic nodules of the liver 0/50 (0 %), 6/50 (12 %), 7/49 (14%)[*,#] (hepatocytes with basophilic or eosinophilic cytoplasm) in males and 1/50 (2 %), 1/50 (2 %), 5/50 (10 %)[*] in females Non-neoplastic lesions (3 000 ppm): - 14/48: congestion of splenic parenchyma; 23/48 focal or multifocal area of fibrosis; 3/48 diffuse fibrosis; 13/48 areas of fatty metamorphosis in the spleen in males - Areas of fibrosis in 2 control males - 25/50 multifocal, diffuse or focal fibrosis of the spleen in females Survival: - No effects on mortality, body weight (BW) and food consumption; 6 % weight depression in high-dose female rats	1/1 000 (0.1 %), range 0-2 % in females NTP report (1982): - Neoplastic nodules in liver (same lab, no time range specified): M/F: 5/140 (3.6 %)
CTFA, 1982a; CTFA, 1982b Both studies cited in FDA, 1986 26-30 months dietary study (F0 and F1 dosed) including in-utero exposure Non-GLP According to FDA guidelines including in utero treatment and F1 generation Reliable with restriction [&]uncertainty regarding the number of tissues examined from each group, incidence	PR 53:1 Purity: 76 %	Species: rats Strain: Charles-River CD Sprague-Dawley Route: oral (feed) Number: 70/dose group/sex Treatment time: 30 months, daily Dose levels: Part I: 0, 100, 200, 500 ppm Part II: 0, 10 000 ppm Corrected doses[3]: Part I F0: 8, 17, 43 mg/kg bw/d in males; 9, 17, 42 mg/kg bw/d in females F1: 5, 10, 26 mg/kg bw/d in males; 6, 13,	Carcinogenicity: - Increased incidence of splenic sarcoma in rats Neoplastic lesions: - Haemangiosarcomas in 2/70[&] (3 %) male control animals involving spleen and/or liver - Splenic sarcoma in 4/70[&] (6 %) males and 1/70[&] (1 %) female at 10 000 ppm in F1 animals (not statistically significant) Survival: - no effects on mortality and food consumption in parental animals or offspring - slight BW decrease from day 21 postpartum in male and female pups at 10 000	No data

Reference, Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Historical control data (HCD)
percentages calculated by the DS based on the group size of 70 animals/group bear uncertainties		32 mg/kg bw/d in females Part II F0: 790 mg/kg bw/d for males, 894 mg/kg bw/d for females F1: no data available for males (calculated: 500 mg/kg bw/d); 521 mg/kg bw/d for females	ppm; lower BW through chronic phase (< 10 %) Clinical findings at 10 000 ppm: - Sings of anemia in males and females - Increase in spleen and heart weight in males and females; increased kidney weight in females and increased testicular weight in males Splenic lesions at 10 000 ppm in males: - Splenic congestion, fibrosis, mesothelial hyperplasia, mesothelial cysts, haemosiderosis and splenic hematopoiesis	
Davis and Fitzhugh, 1962, publication 2-year feeding study Non-guideline study Non-GLP Limited reliability due to limited reporting, no data on individual animals, only 6 animals from each group examined histopathologically, incidences only on a limited number of findings, no body weight information, according to the DS	PR 53:1 Purity: 86 % Vehicle: ethanol	Species: rats Strain: Osborne-Mendel Route: oral (feed) Number: 25/dose group/sex Treatment time: 103 weeks, daily Dose levels: 0, 100, 500, 2 500, 10 000 ppm Food conversion factor: 20 (for older rats) Calculated doses2: 0, 5, 25, 125, 500 mg/kg bw/d Post exposure period: 10 days	Carcinogenicity: - No increased evidence for carcinogenicity but severe splenic effects Survival: - No effects on mortality 10 000 ppm: - Moderate splenomegaly, splenic infarcts, haematomas or scars (6 rats), splenic haemosiderosis ≥ 2 500 ppm: - Slight bone marrow hyperplasia, decreased hemoglobin, abnormal red blood cells ≥ 500 ppm: - Slight to moderate splenomegaly (7/12 at 500 ppm, 4/12 at 2 500 ppm, 2/12 at 10 000 ppm)	No data
Studies on mice				
NTP, 1982b 2-year feeding study OECD TG 451 (NTP guideline including single dose, 2-week and 13-week studies) Non-GLP Reliable without restriction according to the	PR 53:1 Purity: 89.8 % Impurities: sodium and barium sulfates	Species: mice Strain: B6C3F1 Number: 50/dose group/sex Route: oral (feed) Treatment time: 103 weeks, daily Dose levels: 0, 1 000, 2 000 ppm	Carcinogenicity: - No carcinogenicity Hepatic neoplastic lesions (0, 1 000, 2 000 ppm): - Hepatocellular carcinoma 4/50 (8 %), 9/50 (18 %), 11/50 (22 %)[*,#] (not above HCD in the same laboratory) in males; 4/50 (8 %), 2/50 (4 %), 2/49 (4 %) in females	Liver neoplastic lesions NTP report (1982): - Hepatocellular carcinoma (same lab, no time range specified): 65/297 (22 %) in males NTP data (1984-1994)[4]: - Hepatocellular carcinoma:

Reference, Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Historical control data (HCD)
DS		Food conversion factor: 7 (for mice) Calculated doses[2]: 0, 142, 285 mg/kg bw/d Post exposure period: 1 week Dose level selected based on effects observed in 91 day study	Haematopoietic system neoplasias (0, 1 000, 2 000 ppm): - Malignant lymphomas, mixed type: 0/50, 1/50 (2 %), 0/50 in males and 2/50 (4 %), 2/50 (4 %), 7/49 (14 %)* in females - All malignant lymphomas 5/50 (10 %), 4/50 (8 %), 4/50 (8 %) in males; 11/50 (22 %), 17/50 (34 %), 12/49 (24 %) in females Survival: - No effect on mortality, body weight and food consumption, except mean body weight of treated females slightly lower in 2nd year (< 10 %)	194/950 (20.4 %), range 10-40 % in males; 104/951 (10.9 %), range 4-20 % in females Haematopoietic system neoplasias NTP data (1984-1994): - No data for malignant lymphomas, mixed type - All malignant lymphomas: 71/952 (7.5 %), range 2-14 % in males; 167/953 (17.5 %), range 6-30 % in females
CTFA, 1982c Cited in FDA, 1986 Combined repeated dose and carcinogenicity test Similar to OECD TG 453 Non-GLP Reliable without restriction according to the DS	PR 53:1 Purity: 76 % (according to FDA report)	Species: mice Strain: Charles-River CD1 Number: 60/dose group/sex Route: oral (feed) Treatment time: 24 months/105 weeks, daily Dose levels: 0. 50, 250, 1 000 ppm Corrected dose using food consumption: 7, 38, 147 mg/kg bw/d in males; 12, 56, 237 mg/kg bw/d in females	Carcinogenicity: - No carcinogenicity Survival: - No effects on mortality, body weight, food consumption Clinical findings of toxicity: - Signs of anemia at 1 000 ppm in females (decreased red blood cells, increased reticulocytes, decreased hemoglobin and hematocrit), anemia not evident at 250 ppm - Decreased absolute kidney weight in males (1 000 ppm), but relative kidney weight similar to control	No data
Carson, 1984, publication 18-month skin painting study Non-guideline study Non-GLP Limited reliability according to the DS: Limited reporting, no data on individual animals, limited number of organs analysed, only selected animals from solvent and positive control,	PR 53:1; Purity: 90 %; Vehicle: distilled water Positive control: 3,4-benzpyrene in acetone	Species: mice Strain: 100 ICR Number: 50/dose group/sex, 150 in control group Route: dermal, application to dorsal area Treatment time: 483 days Dose levels: dermal application to dorsal area; 0.1 mL of 1 % solution of dye (6 cm^2) twice a week for 18 months (mean total	Carcinogenicity: - No carcinogenicity - Single incidences of mammary gland adenosarcoma (2 females); hepatic cell carcinoma (1 male/1 male in control), reticulum cell sarcoma (1 male) Survival: - No effect on survival compared to control Full histopathology of a low number of randomly chosen animals did not give an indication of	No data

Reference, Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Historical control data (HCD)
study period 18 month, dermal application twice a week with very low dose, incidences only on a limited number of findings, no body weight information, no HCD		dose of applied material 134.7 mg)	systemic toxicity or carcinogenicity	

*statistically significant positive trend; #statistically significant increase compared to control; [1]Dose level selected based on effects observed in 91 day study; [2]Doses calculated by the DS; [3]Doses calculated based on food consumption; [4]No other time range available; source: https://ntp.niehs.nih.gov/data/controls/index.html; [5]including fibrosarcoma, splenic osteosarcoma, leiomyosarcoma, sarcoma

There were seven studies available that investigate carcinogenic potential of PR 53;1 (4 in rats and 3 in mice). No human data were available.

Studies in rats

A 2-year feeding study in rats, conducted according to NTP guidelines and equivalent to OECD TG 451 was performed by NTP using 50 male and 50 female F344 rats (NTP, 1982a). Doses of 0, 1 000 and 3 000 ppm PR 53:1 were administered in feed daily for 103 weeks (calculated doses based on a general conversion factor of 20 for older rats are as follows: 0, 50, 150 mg/kg bw/d). No effects on survival, body weight, or food consumption were reported except for high-dose females that showed a 6 % lower body weight compared with the control. In the male high-dose group, 26/48 (54 %) combined types of splenic sarcomas were reported, including fibrosarcoma (17/48 (35 %)) arising from red pulp or capsule of the spleen, leiomyosarcoma (1/48) and splenic osteosarcoma (5/48). Eleven of the splenic tumours metastasised to peritoneal tissues. Non-neoplastic spleen lesions were observed in the high-dose group in both sexes. In males, 14/48 animals showed congestion of splenic parenchyma, 23/48 focal or multifocal area of fibrosis, 3/48 diffuse fibrosis and 13/48 areas of fatty metamorphosis in the spleen. In females, in the high-dose group 25/50 animals reported multifocal, diffuse or focal fibrosis of the spleen. The increased incidence of fibrosarcoma in males was significantly above the HCD from the laboratory (0/140) and above the historical records for the entire bioassay program of the laboratory (3/2 960, 0.1 %). Males and females reported a dose-related increase of neoplastic nodules in liver above the HCD (M/F: 5/140 (3.6 %)). The increase was more significant in males (6/50 and 7/49 males in the mid-dose and high-dose groups) than in females (only 5/50 animals affected in the high-dose group). The nodules consisted of hepatocytes with basophilic or eosinophilic cytoplasm and were of relatively small size.

A report from United States Food and Drug Administration (FDA, 1986) discussed some limitations of this study regarding the use of solid-bottom cages that can lead to coprophagy. This can determine the ingestion of higher doses of chemical and its metabolites and the presence of other carcinogens in the room, as other substances were also tested at the same time. These limitations were considered to be not relevant as also in the CTFA studies where wire cages were used, the same type of tumours were observed and due to the fact that no splenic neoplasms were observed with other test substances or in control animals in other studies (according to the HCD).

Long-term feeding studies with lifetime exposure in rats were performed according to the FDA guidelines (CTFA, 1982b; CTFA, 1982a). In the first study, 70 Sprague-Dawley rats/dose/sex were treated with 0, 100, 200, 500 ppm of PR 53:1 in the diet (corrected doses: F0: 8/9, 17/17,

43/42 mg/kg bw/d M/F, F1: 5/6, 10/13, 26/32 mg/kg bw/d M/F; calculated doses: 0, 5, 10 and 25 mg/kg bw/d, based on a general conversion factor of 20 for older rats according to the CLP guidance) for 60 days before mating, during mating, gestation, lactation and rearing (CTFA, 1982a). Seventy F1 pups/dose/sex were selected for the long-term feeding study dosing for 30 months (CTFA, 1982b). The dose levels of the first study were found too low and a new experiment was required by the FDA with higher concentrations (0 and 10 000 ppm PR 53:1 in the diet; corrected dose: F0: 790/894 mg/kg bw/d M/F, F1: no data/521 mg/kg bw/d M/F; calculated dose for M: 0 and 500 mg/kg bw/d) using the same method (CTFA, 1982b). No effects on survival and food consumption were observed in both studies, while a slight decrease in body weight was observed in male and female pups from day 21 postpartum. At 10 000 ppm, splenic sarcoma was observed in 4 males and 1 female. Details on the number of examined tissues from each group is not reported in the study report and it is stated that in 'minor cases' some tissues could not be investigated due to autolysis, sectioning difficulties or laboratory errors. These uncertainties could lead to underestimation of the effects. The evaluation of clinical signs of toxicity reveals anaemia and increase in spleen and heart weight in both sexes at 10 000 ppm. In males, splenic lesions such as splenic congestion, fibrosis, mesothelial hyperplasia, mesothelial cysts, haemosiderosis and splenic haematopoiesis were also reported.

In the study by David and Fitzhugh (1962), 25 Osborne-Mendel rats/sex/dose were administered 0, 100, 500, 2 500 and 10 000 ppm PR 53:1 by feed for 103 weeks (calculated doses: 0, 5, 25, 125, 500 mg/kg bw/d, based on a general conversion factor of 20 for older rats according to CLP guidance). The study report has several limitations as only 6 animals from each group were examined histopathologically and a limited number of findings were reported. Carcinogenicity was not observed, but the evaluated animals showed slight to moderate splenomegaly (7/12 at 500 ppm, 4/12 at 2 500 ppm, 2/12 at 10 000 ppm), slight bone marrow hyperplasia, decreased haemoglobin, abnormal red blood cells at 2 500 ppm and above, and moderate splenomegaly, splenic infarcts, haematomas or scars (6 rats), splenic haemosiderosis at 10 000 ppm.

Studies in mice

In the NTP 2-year feeding study (1982b) equivalent to OECD TG 451 (NTP guidelines) 50 B6C3F1 mice/sex/dose group were administered 0, 1 000 and 2 000 ppm, PR 53:1 in feed for 103 weeks (calculated doses: 0, 142, 285 mg/kg bw/d, based on a general conversion factor of 7 for mice according to the CLP guidance). The exposure determined no effects on survival, body weight or food consumption. A positive trend of hepatocellular carcinoma incidence was reported, 4/50 (8 %), 9/50 (18 %) and 11/50 (22 %) in 0, 1 000 and 2 000 ppm males. Statistically significant increase compared with the control was reported only in the high-dose group males and in all dose groups the incidence did not exceed the HCD. In female mice at 2 000 ppm, an increased incidence in mixed-type malignant lymphomas of the haematopoietic system was reported without statistical difference compared with the control, although a positive dose-response trend was established. No HCD were available.

In another study conducted according to FDA guidelines (CTFA, 1982c) 60 Charles-River CD1 mice/sex/dose were administered 0, 50, 250 and 1 000 ppm PR 53:1 (corrected doses: 7/12, 38/56, 147/237 mg/kg bw/d M/F; calculated doses: 0, 7, 35 and 142 mg/kg bw/d, based on a general conversion factor of 7 for mice according to the CLP guidance) for 18 months. No effects on survival, body weight or food consumption and no neoplastic lesions were reported.

An 18-month skin painting study investigated the toxicity of PR 53:1 in dose levels based on lipstick use assessments via dermal administration (Carson, 1984). A skin area of 6 cm^2 from the animal dorsal region was treated twice weekly with 0.1 mL suspension; the mean total dose applied was 134.7 mg. The study has several limitations as complete pathology was performed only on a limited number of animals. No effects on survival or increase of neoplasia compared with the control group were reported. Only single incidences of any gross lesions were reported.

A chronic drinking water study in both rats and mice investigating barium chloride dihydrate carcinogenic potential is available (NTP, 1994). The study author stated that "PR53:1 is a barium-containing pigment" and that "barium and its salts are known to be toxic to muscle and nervous tissue. Although the toxicity of this metal is limited due to the insolubility of barium salts, a potential for barium toxicity must be recognized." The study reported no evidence of carcinogenic activity of barium chloride dehydrate and the author's conclusion was that barium cation in PR 53:1 presumably does not contribute to the carcinogenic effects of PR 53:1.

The outcome of the overall evaluation of animal studies regarding the carcinogenic potential of PR 53:1 is that there is evidence of carcinogenic potential based on an increased incidence of splenic sarcomas in male rats, a rare type of tumours in this organ (CTFA, 1982b; CTFA, 1982a; NTP, 1982a). This incidence was statistically significant and above the HCD in the NTP study (1982a), but not in the CTFA study. The results of CTFA study are considered only supportive evidence as a similar pattern of non-neoplastic splenic lesions were observed in both studies (CTFA, 1982b; CTFA, 1982a; NTP, 1982a). FDA report (1986) also stated a common pattern of splenic lesions in NTP and CTFA studies that include fatty metamorphosis, focal or diffuse splenic fibrosis, unusually severe forms of splenic congestion with or without haemorrhages or infarcts, capsular fibrosis and hyperplasia and the association of these splenic lesions with the occurrence of fibrosarcoma in male rats (CTFA, 1982b; CTFA, 1982a; Davis and Fitzhugh, 1962; NTP, 1982). It is considered that Sprague-Dawley rats are less sensitive than F344 rats to aniline-related compounds, but still show the pre-neoplastic trend observed in the F344 rats.

The studies on mice reported no carcinogenic effects (CTFA, 1982c; NTP, 1982b). The NTP report for rat and mouse studies however states that "with the possible exception of female mice, all other dosed groups of rats or mice might have tolerated higher doses" and that "thus a clear maximum tolerated dose may not have been utilized in this study."

Supporting information from specific target organ toxicity – repeated exposure studies

Specific target organ toxicity-repeated exposure was not evaluated in the CLH report for classification but data were provided to support the assessment of carcinogenicity.

Reference, Method, guideline, deviations if any	**Test substance**	**Relevant information about the study including rationale for dose selection (as applicable)**	**Results**	**Guidance Value for STOT RE**
Studies on rats				
NTP, 1982a 2-year feeding study in rats No GLP According to OECD TG 451 Reliability without restriction according to the DS	PR53:1, Purity 89.8 % Impurities sodium and barium sulfates	Species: rats Strain: F344 Route: oral (feed) Number: 50/dose group/sex Treatment time: 103 weeks, daily Post exposure period: 1 week Dose levels: 0, 1 000, 3 000 ppm[1] Food conversion factor: 20 (for older rats) Calculated doses[2]: 0. 50, 150 mg/kg bw/d	Spleen lesions in male (150 mg/kg bw/d): - Congestion of the splenic parenchyma (14/48), focal or multifocal areas of fibrosis (23/48), diffuse fibrosis (3/48), areas of fatty metamorphosis in the spleen (13/48) Spleen lesions in female (150 mg/kg bw/d): - Multifocal, diffuse, or focal fibrosis (25/50) Areas of fibrosis present in 2/50 control male rats Increased incidence of testis/tubule degeneration: - 10 % (5/50), 23 % (11/48) at 50 and 150 mg/kg bw/d compared to 6 % (3/50) in controls	GV STOT RE 2 ≤ 12.6 mg/kg bw/d for 103-week exposure

Reference, Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Guidance Value for STOT RE
NTP, 1982c Range finding study for carcinogenicity - feeding study in rats (13-week study) No GLP Reliability with restriction according to the DS (no data on haematology, clinical biochemistry, urine analysis)	PR53:1 Purity 89.8 % Impurities sodium and barium sulfates	Species: rats Strain: F344 Route: oral (feed) Number: 10/dose group/sex Treatment time: 91 days, daily Dose levels: 0, 3 000, 6 000, 12 500, 25 000, 50 000 ppm Food conversion factor: 20 (for older rats) Calculated doses: 0, 150, 300, 625, 1 250, 2 500 mg/kg bw/d	Haemosiderosis of the liver in all dosed female rats, and in 3/10, 6/10, 9/10 males at 150, 300, 625 mg/kg bw/d Pigment deposition in kidney tubular epithelium in all dosed rats Enlarged spleen (2-5 fold) in all dosed rats; congestion and lymphoreticular hyperplasia in spleens of all dosed female rats, and in 8/10 male rats at 150 mg/kg bw/d and in all male rats ≥ 300 mg/kg bw/d Lymphoreticular hyperplasia of thymic lymph nodes: - 75-100 % of female rats in each dosed group except 0/10 at 150 mg/kg bw/d - 70-100 % of male rats in each dosed group except 3/7 at 2 500 mg/kg bw/d	GV STOT RE 2 ≤ 100 mg/kg bw/d for 13-week exposure
CTFA, 1982a and CTFA, 1982b 30-months chronic toxicity and potential carcinogenicity study with in utero and lifetime exposure According to FAD guidelines Pre-GLP Reliable with restrictions (individual data e.g. clinical signs missing)	PR 53:1, Purity: 86 % Vehicle: ethanol	Species: rats Strain: CD [CRL:COBS CD (SD) BR] Route: oral (feed) Number: F0/F1:70/dose group/sex Treatment time: 8 weeks prior to mating (part I), 9 weeks prior to mating (part II), continued during mating, gestation, and lactation; females were allowed to litter and raise their pups until weaning; F1 generation rats exposed for 30 months after weaning Dose levels: Part I: 0, 100, 500, ppm Part II: 0, 10 000 ppm Doses calculated based on food consumption: Part I 8, 17, 43 mg/kg bw/d in F0 males; 9, 17, 42 mg/kg bw/d in F0 females; 5, 10, 26 mg/kg bw/d in F1 males; 6, 13, 32 mg/kg bw/d F1 females Part II	Haemoglobin decrease Part I: - Statistically significant (-8 %) in F1 high dose females (12 months) - Statistically significant (-6 %) in F1 mid dose males (18 months) Part II: - Decrease in all treated groups - Statistically significant in treated F1 males at month 3, 12, 18, and 24 (-9, -18, -10, -9 %) - Statistically significant in treated F1 females at 3, 12, 18, and 24 month (-14, 18 %, no further data available) Haematocrit decrease Part I: - Statistically significant in F1 high dose females at 3 and 12 months (-6 %) Part II: - Statistically significant in F1 males at 3, 12, 18, and 24 month (-8, -10, -8, -9 %) - Statistically significant in treated F1 females at 3, 6, 12, 18 and 24 months (-8, -14, -15 %, no further data) Red blood cell count decrease Part I:	GV STOT RE 2 ≤ 11 mg/kg bw/d for 30-month exposure

Reference, Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Guidance Value for STOT RE
		10 000 ppm corresponds to 790 and 894 mg/kg bw/d for F0 males and females No data available for F1 males (calculated: 500 mg/kg bw/d); 521 mg/kg bw/d for F1 females	- Statistically significant (-10 %) in F1 high dose females (12 months) Part II: - Statistically significant in treated F1 males at month 3, 6, 12, 18, and 24 (-31, -21, -24, -10, -18 %) - Statistically significant in treated F1 females at 3, 12, 18, and 24 months (-32, -24 %, no further data available) Reticulocyte count increase Part I: - Statistically significant (92 and 100 %) in F1 mid and high dose females at month 18 Part II: - Statistically significant in treated F1 males (468, 223, 142, 60, 139 %) and females (526, 127, 99 %, no further data) after 3, 6, 12, 18, and 24 months Spleen weight and spleen weight-body weight ratio increase Part I: - Statistically significant (20.9 %, respectively 22.5 %) in F1 high dose females at month 12 - Spleen weight of high dose males increased (not statistically significant) Spleen weight and spleen weight-body weight ratio values for F1 high dose of both sexes were increased compared to combined control (control 1 plus control 2), but not statistically significant at 30 month; high mean spleen weight and spleen weight-body weight percentages for control 1 males at 30 months terminal kill were due to an extremely enlarged spleen in one individual; Part II: - Statistically significant in treated F1 males (318 %, respectively 372 %) and females (210 %, respectively 246 %) at 12 months, and F1 males (183 %, respectively 175 %) and females (349 %, respectively 382 %) at terminal kill (month 30)	

Reference, Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Guidance Value for STOT RE
			Haemosiderosis of the spleen Part I: - F1 high dose females after 12 months Hemosiderin accumulation in liver Part II: - dosed females (unknown incidence) Hemosiderin accumulation in kidneys Part II: - dosed females and males (unknown incidence) Further observations (part II) - Splenomegaly, splenic extramedullary haematopoiesis, splenic congestion, fibrosis, haemosiderosis, mesothelial hyperplasia, mesothelial cyst formation, capsular fibrosis, and multifocal cellular proliferations in the spleenic capsule of dosed rats - Testis weight and testis weight/bw: Statistically significant decrease in F1 mean weight (-24 %) and weight/bw percentage (-26.1 %), compared to controls at terminal kill (month 30)	
Hoechst AG, 1973 32-days feeding study Similar to OECD TG 407 Pre-GLP Not reliable (insufficient characterisation of test material, no data on clinical biochemistry, main description of test conditions missing)	Mixture of two azo dyes Purity: unknown	Species: rats Strain: SPF-Wistar Route: oral (feed) Number: 10/dose group/sex Treatment time: 32 days Dose levels: 5 %, 1 % and 0.2 % Calculated doses: 0, 10, 50, 250 mg/kg bw/d	Erythrocytes: - Dose-dependent decrease in all treated groups Leucocytes: - Dose-dependent increase Heinz bodies in erythrocytes: - increase in high, mid and low dose groups (100 %, 30 % and 10 %) Spleen weight: - Statistically significant and dose-dependent increase; enlarged and blackish coloured spleen and brownish coloured kidneys in mid and high dose animals Iron storage: - Dose-dependent increase in liver, kidney tubular epithelium (except for low dose group), moderate to strong increase in iron levels in spleen in all treatment groups	GV STOT RE 2 ≤ 281 mg/kg bw/d for 32-day exposure

Reference, Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Guidance Value for STOT RE
Davis and Fitzhugh, 1962, publication 2-year feeding study Non-guideline study Reliability: Not assignable (no full study report available)	PR 53:1, Purity: 86 %	Species: rats Strain: Osborne-Mendel Route: oral (feed) Number: 25/dose group/sex Treatment time: 103 weeks Dose levels: 0, 0.01, 0.05, 0.25, 1 %. Calculated doses: 0, 5, 25, 125 and 500 mg/kg bw/d	Slight to moderate splenomegaly: - In rats of the 0.01 % (2/12 rats), 0.05 % (4/12), 0.25 % and 1.0 % (both 7/12) dose groups - significant increase in spleen weight/body weight ratio in the 0.25 % and 1.0 % dose groups Splenic infarcts, scars, haemosiderosis, or cysts in high dose group (1 %) Slight hematologic effects (slight decrease of haemoglobin, presence of abnormal circulating red blood cells) noted early in the test, did not increase in severity (no raw data) Bone marrow of 0.25 and 1.0 % dose groups was slightly hyperplastic compared to controls Significantly less chronic nephritis in the 0.25 and 1.0 % dose groups Light yellow, non-ferrous, granular pigment in the renal tubular epithelium in the kidneys of 0.25 % dose group animals (2/12) and of all 1 % level rats 12/12)	GV STOT RE 2 ≤ 12.6 mg/kg bw/d for 2-year exposure
Studies on mice				
NTP, 1982b 2-year feeding study in mice Non-GLP According to OECD TG 451 Reliability without restriction according to the DS	PR53:1, Purity 89.8 % Impurities sodium and barium sulfates	Species: mice Strain: B6C3F1 Route: oral (feed) Number: 50/dose group/sex Treatment time: 103 weeks, daily Post exposure period: 1 week Dose levels: 0, 1 000, 2 000 ppm Food conversion factor: 7 Calculated doses: 0, 142, 285 mg/kg bw/d	No non-neoplastic findings in treated mice	GV STOT RE 2 ≤ 12.6 mg/kg bw/d for 2-year exposure
NTP, 1982 Range finding study for carcinogenicity - feeding study in rats (13-week study) Non-GLP Reliability with restriction according	PR53:1 Purity 89.8 % Impurities sodium and	Species: mice Strain: B6C3F1 Route: oral (feed) Number: 10/dose group/sex	Congestion of the spleen in 55/60 mice at ≥ 357 mg/kg bw/d Deposits of haemosiderin were present to a greater extent in all dosed animals than in controls with exception of females at 86	GV STOT RE 2 ≤ 100 mg/kg bw/d for 13-week exposure

Reference, Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Guidance Value for STOT RE
to the DS (no data on haematology, clinical biochemistry, urine analysis)	barium sulfates	Treatment time: 91 day, daily Dose levels: 0, 600, 1 250, 2 500, 5 000 or 10 000 Food conversion factor: 7 Calculated doses: 0, 86, 179, 357, 714, and 1 429 mg/kg bw/d	or 179 mg/kg bw/d and males at 86 mg/kg bw/d	
CTFA, 1982c Combined repeated dose and carcinogenicity (daily for 24 months/105 weeks) Similar to OECD TG 453 Non-GLP Reliable with restrictions according to the DS: no data on clinical biochemistry of plasma or serum, no data collected for oestrus cycle or sperm parameters, no urinalysis	PR 53:1; Purity: ≥ 76 %	Species: mice Strain: CD-1 Number: 60/dose group/sex, Route: oral, diet Treatment time: 105 weeks Dose levels: 0, 50, 250, 1 000 ppm Calculated doses: 0, 7, 38, 147 mg/kg bw/d in males; 0, 12, 56, 237 mg/kg bw/d in females	Haemoglobin: - Statistically significant decrease in high-dose females (-11.4 % vs. control) at 18 months, in high-dose males at 6 months (-7.2 %) Haematocrit: - Statistically significant decrease in low-dose (-8.1 % vs. control) and high dose females (-9.9 %) at 18 months - decrease (but not significant) at the mid-dose, (-5.1 %) Red blood cell count: - Statistically significant increase for high dose females (14.8 % vs control) at 3 months - Statistically significant decrease (-10.7 %) after 18 months Gross and histopathologic evaluation did not reveal any compound related findings.	GV STOT RE 2 ≤ 12.6 mg/kg bw/d for 103 week exposure)

[1]Dose level selected based on effects observed in 91 day study; [2]Doses calculated by the DS

Six studies in rats were assessed as supporting information to carcinogenicity for specific target organ toxicity and non-neoplastic effects produced by PR 53:1. The first study performed according to OECD TG 451 on F344 rats exposed over a period of 2 years (NTP, 1982) showed that non-neoplastic lesions in the spleen including focal, multifocal and diffuse fibrosis were significantly increase in both sexes at high dose group compared to control. The second study was the 91-day study used for dose selection for the two-years feeding study (NTP, 1982c). At all the tested doses (3 000, 6 000, 12 500 and 50 000 ppm corresponding to 150, 300, 625, 1 250 and 2 500 mg/kg bw/d) showed enlarged spleen and pigment deposition in the renal tubular epithelium. In a 30-month chronic toxicity study with in utero and lifetime exposure levels of 5, 10, 26 mg/kg bw/d in males and 6, 13, 32 mg/kg bw/d in females (CTFA, 1982a), a decrease in haemoglobin (< 10 %), haematocrit (< 10 %), and red blood cell count (-10 %) at interim withdrawal, and an increase in reticulocyte count (100 %) at the final investigations (32 months) were reported. A similar study design using higher doses (500 mg/kg bw/d in males and 521 mg/kg bw/d in females) reported that the effects observed at lower doses were increased (CTFA, 1982b). Red blood cell parameters were significantly decreased in dosed males and females (haemoglobin ≥ 10 %, haematocrit ≤ 10 %, and red blood cell count ≥ 10 %) at several time points investigated during the study. Treated rats of both sexes showed an increased spleen weight and spleen weight-body weight ratio (> 100 %), splenomegaly, splenic extramedullary haematopoiesis, splenic congestion, fibrosis, haemosiderosis, mesothelial hyperplasia,

mesothelial cyst formation, capsular fibrosis, and multifocal cellular proliferations in the splenic capsule. Haemosiderosis accumulation in liver and kidney were found in rats fed with PR 53:1 (no data on incidence available) (CTFA, 1982a; CTFA, 1982b). A dose-dependent decrease in erythrocytes and an increase in leucocytes in treated animals were reported in a 32-days feeding study (Hoechst AG, 1973) but the study is not reliable as it does not provide information on the composition and purity of test material. Furthermore, Heinz bodies (formed by irreversible precipitation of oxidative denatured haemoglobin) in erythrocytes were increased in a dose-dependent manner in the study, and there was a significant and dose-dependent increase in spleen weight accompanied by blackish-coloured and enlarged spleens and brownish-coloured kidneys in treated rats. Histological evaluation revealed a dose-dependent increase in iron storage in the liver, kidney tubular epithelium, and spleen which could be interpreted as indicative of haemosiderin (iron-positive) deposition as a consequence of (intravascular) haemolysis.

The study by Davis and Fitzhugh (1962) showed a slight to moderate splenomegaly at 125 and 500 mg/kg bw/d. High dosed rats (500 mg/kg bw/d) showed splenic haemosiderosis and splenic infarcts.

Three studies in mice were assessed as supporting information to carcinogenicity for specific target organ toxicity and non-neoplastic effects produced by PR 53:1. A 2-year feeding study reported no non-neoplastic findings at doses up to 285 mg/kg bw/d (NTP, 1982b). This study was conducted based on a 91-day study results reporting congestion of the spleen at doses above 375 mg/kg bw/d. Deposits of haemosiderin were present to a greater extent in the spleen of all dosed mice compared to controls, with exception of females at 86 or 179 mg/kg bw/d and 86 mg/kg bw/d males. There were no data collected on haematology, clinical biochemistry, or urinalysis. A combined repeated dose and carcinogenicity study similar to OECD TG 453 on CD-1 mice treated for 18 months with PR 53:1 (doses 7, 38, 147 mg/kg bw/d in males and 12, 56, 237 mg/kg bw/d in females) (CTFA, 1982c) reported a significant decrease in haemoglobin (≥ -10 %), haematocrit, and red blood cell count in high dose females at 18 months. Furthermore, the red blood cell count was statistically increased (≥ 10 %) for high-dose females at 3 months.

The reported data on haematology and pathology/histopathology reveal adverse effects on animals treated with PR 53:1. There is consistency between the different studies in rats and mice that PR 53:1 induced haematolytic anaemia, including a decrease in blood parameters (e.g. haemoglobin, haematocrit, and red blood cell count) accompanied by haemosiderosis of the spleen. Effects appeared less severe in mice compared to rats. Chronic exposure of PR 53:1 in rats resulted in neoplastic lesions of the spleen accompanied by increased incidences of diffuse/multifocal splenic and capsular fibroses, and haemosiderin deposition in spleen, liver and kidneys.

Germ cell mutagenicity studies

Germ cell mutagenicity was not evaluated in the CLH report but data from mutagenicity studies were provided to support the assessment of carcinogenicity.

The DS reported the following *in vitro* and *in vivo* mutagenicity/genotoxicity studies (cf. Table 10 and 11 of the CLH report and information regarding study design in Annex I):

Reference, method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Results	Reliability
***In vitro* studies**				
Hoechst AG, 1989, unpublished study report Bacterial Reverse Mutation Test GLP study Similar to OECD TG 471 (Ames test) Deviations: 5th strain missing	PR 53:1 Purity: technically pure Vehicle: DMSO Positive controls: yes Negative controls: yes	Supporting study according to the DS Strains: *S. typhimurium* TA100, TA98, TA1537, TA1535 Metabolic activation system: S9 mix-hamster liver S9, untreated and rat liver S9 Aroclor induced Test concentrations (± metabolic activation (S9 mix)): 4, 20, 100, 500, 2 500, 5 000 µg/plate	Genotoxicity: negative with (hamster and rat S9) and without metabolic activation. No significant increase in the number of revertants in any bacterial strains with and without Prival with and without metabolic activation. Cytotoxicity: no Precipitations: ≥ 500 µg/plate Neg. control: valid Pos. control: valid	Reliable with restriction according to the DS (5th strain missing, results for TA100, TA98, TA1537, TA1535 are reliable without restrictions)
Hoechst AG, 1985a Bacterial Reverse Mutation Test GLP study Similar to OECD TG 471 (Ames test) Deviations: 5th strain missing	PR 53:1 Purity: technically pure Vehicle: DMSO Positive controls: yes Negative controls: yes	Supporting study according to the DS Strains: *S. typhimurium* TA100, TA98, TA1537, TA1535 Metabolic activation system: S9: hamster liver (Prival activation) S9: untreated and rat liver S9 Aroclor-induced (classical test protocol) Test concentrations (± metabolic activation (S9 mix)): 4, 20, 100, 500, 2 500, 5 000/10 000 µg/plate	Genotoxicity: negative with (hamster and rat S9) and without metabolic activation. Cytotoxicity: no Precipitations: ≥ 100 µg/plate Neg. control: valid Pos. control: valid	Reliable with restrictions according to the DS (5th strain missing results for TA100, TA98, TA1537, TA1535 are reliable without restrictions)
Hoechst AG, 1985b, unpublished study report Bacterial Reverse Mutation Test GLP study Similar to OECD TG 471 (Ames test) Deviations: none	PR 53:1 Purity: technically pure Vehicle: DMSO Positive controls: yes Negative controls: yes	Key study according to the DS Strains: *S. typhimurium* TA100, TA98, TA1537, TA1535, TA 1538, Escherichia coli WP2uvrA Metabolic activation system: S9: Aroclor 1254 induced rat liver Test concentrations (± metabolic activation (S9 mix)): 4, 20, 100, 500, 2 500, 10 000 µg/plate	Genotoxicity: negative with and without metabolic activation. Cytotoxicity: no Precipitations: ≥ 100 µg/plate Neg. control: valid Pos. control: valid	Reliable without restrictions according to the DS
CIBA-GEIGY Limited, 1985 Bacterial Reverse Mutation Test Non-GLP	PR 53:1 Purity: no data Vehicle: DMSO Positive controls: yes	Supporting study according to the DS Strains: *S. typhimurium* TA100, TA98, TA1537	Genotoxicity: negative with and without metabolic activation. Cytotoxicity: no Precipitations: ≥ 100 µg/plate	Reliable with restrictions according to the DS (only three strains tested, no verification of negative results)

Similar to OECD TG 471 (Ames test) (without Prival activation) Deviations: No verification of negative result Only three strains tested (e.g. no TA 1535, *E.coli* WP2 missing) No data on purity	Negative controls: yes	Metabolic activation system: S9: rat liver, Aroclor-induced Test concentrations (± metabolic activation (S9 mix)): 20, 78, 313, 1 250, 5 000 µg/plate	Neg. control: valid Pos. control: valid	
Brown *et al*., 1979 Bacterial Reverse Mutation Test Non-GLP Similar to OECD TG 471 (Ames test) Deviations: •Documentation insufficient •Purity insufficient •Data on 5th strain missing •Low max. concentration •Only 3 concentrations tested •No detailed data on results (data table)	PR 53:1 Purity: 33-73 % Vehicle: DMSO Positive controls: yes Negative controls: yes	Disregarded study according to the DS Strains: S*. typhimurium* TA100, TA98, TA1537, TA1535, TA1538 Metabolic activation system: S9: rat liver, Aroclor-induced Test concentrations (± metabolic activation (S9 mix)): 50, 100, 500 µg/plate	Genotoxicity: negative with and without metabolic activation. Cytotoxicity: no data Precipitations: no data Neg. control: valid Pos. control: valid	Not assignable (insufficient documentation and methodical deficiencies)
Zeiger *et al*., 1988 Bacterial Reverse Mutation Test GLP not specified Similar to OECD TG 471 (Ames test) (Prival activation and without Prival) Deviations: No detailed data on results No information on purity Data on 5th strain missing	PR 53:1 Purity: unknown Vehicle: DMSO Positive controls: yes Negative controls: yes	Disregarded study according to the DS Strains : *S. typhimurium* TA100, TA98, TA1537, TA1535, TA97 Metabolic activation system: S9: hamster liver S9, untreated and rat liver S9 Aroclor-induced Test concentrations (± metabolic activation (S9 mix)): 100, 333, 1 000, 3 333, 10 000 µg/plate	Genotoxicity: ambiguous with and without metabolic activation: ambiguous for TA97 without S9 and for TA98 with and without S9 Cytotoxicity: not determined Precipitations: ≥ 100 µg/plate Neg. control: valid Pos. control: valid	Not assignable (detailed result data missing to evaluate relevance of ambiguous results)

Cytotoxicity not determined				
Myhr *et al.*, 1991 *In vitro* mammalian cell gene mutation test using the thymidine kinase gene GLP not specified Similar to OECD TG 490 Deviation: No data on purity	PR 53:1 Purity: no data Vehicle: DMSO Positive controls: yes Negative controls: yes	Key study according to the DS Cell culture: mouse lymphoma L5178Y cells Metabolic activation system: S9: rat liver S9 Aroclor-induced Test concentrations: without metabolic activation (S9 mix)): 1.25, 2.5, 5, 7.5, 15 µg/mL With metabolic activation: 2, 3, 4, 5, 6 µg/mL Treatment time(s): 4 h Sampling time(s): after 2 days	Genotoxicity: negative with and without metabolic activation: Cytotoxicity: no Precipitations: ≥ 7.5 µg/mL Neg. control: valid Pos. control: valid	Reliable with restrictions
Hoechst AG, 1989b *In vitro* mammalian chromosomal aberration test GLP study Similar to OECD TG 473 Deviation: Only 100 metaphases scored per concentration No data on purity	PR 53:1 Purity: no data Vehicle: DMSO Positive controls: yes Negative controls: yes	Key study according to the DS Cell culture: Chinese hamster lung fibroblasts (V79) Metabolic activation system: S9: rat liver S9 Aroclor-induced Test concentrations: ± metabolic activation (S9 mix)): 30, 150, 300 µg/mL Treatment time(s): 4 and 18 h Sampling time(s): 4.5, 15.5, 25.5 h after beginning of treatment Justification for top concentration: significant cytotoxicity ≥ 400 µg/mL	Genotoxicity: negative with and without metabolic activation Cytotoxicity: significant cytotoxic effect ≥ 400 µg/mL Precipitations: ≥ 500 µg/mL Neg. control: valid Pos. control: valid	Reliable with restrictions (only 100 metaphases scored per concentration)
Ivett *et al.*, 1989 *In vitro* mammalian chromosomal aberration test GLP: not specified Not similar to OECD TG 473 Deviation: Continuous exposure of about 12-14 h without metabolic activation missing Short-term treatment with	PR 53:1 Purity: 89.8 % Vehicle: DMSO Positive controls: yes Negative controls: yes	Disregarded study according to the DS Cell culture: CHO Metabolic activation system: S9: rat liver S9 Aroclor-induced Test concentrations: without metabolic activation (S9 mix)): 37.1, 50, 123.8 µg/mL with metabolic activation (S9 mix)): 5, 16.7, 50 µg/mL Treatment time(s): Without metabolic activation: 8 h With metabolic activation: 2 h and 8 h	Genotoxicity: negative with and without metabolic activation: Cytotoxicity: no detailed data Precipitations: ≥ 250 µg/mL Neg. control: valid Pos. control: valid	Not reliable (exposure and sampling times are not according to OECD TG, too few cells analysed)

and without metabolic activation not adequate (8 h and 2 h instead of 3-6 h) Sampling time too short (2-2.5 h instead of 1.5 times the normal cell cycle length) Only 200 (instead of 300) metaphases evaluated No specific data on justification for top dose		Sampling time(s): 2-2.5 h Justification for top concentration: no specific data		
***In vivo* studies**				
Westmoreland and Gatehouse, 1992 Unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo* GLP: not specified Similar to OECD TG 486	PR 53:1 Purity: no data Vehicle: Corn oil Positive controls: yes (2-acetylaminofluorene for 16 h) Negative controls: yes	Supporting study according to the DS Species: rats Piebald Virol Glaxo Number of animals per group: 7 males Target organs: liver Administration route: oral (gavage), single dosage Dose level: 1 000 and 2 000 mg/kg bw/d Justification for top dose: limit test Sampling: 16 h	Genotoxicity: No marked increase in incidence of cells in repair at 16 h sampling time Toxicity: no toxicity observed. Controls were valid.	Reliable without restrictions
Westmoreland and Gatehouse, 1992 Mammalian erythrocyte micronucleus test GLP: not specified Similar to OECD TG 474 Deviation: No evidence of exposure of bone marrow	PR 53:1 Purity: no data Vehicle: Corn oil Positive controls: yes (cyclophosphamide, 24 h) Negative controls: yes	Supporting study according to the DS Species: rats Piebald Virol Glaxo Number of animals per group: 7 males Target organs: bone marrow Administration route: oral (gavage), single dosage Dose level: 500, 1 000 and 2 000 mg/kg bw/d Justification for top dose: limit test Sampling: 24 or 48 h	Genotoxicity: negative, no increase in the frequency of micronuclei Toxicity: no toxicity observed Evidence of exposure of bone marrow: no, as ratio PCE/NCE not decreased, no other evidence Controls were valid.	Not reliable (no evidence of exposure of bone marrow shown)

There are six *in vitro* bacterial reverse mutation tests performed using PR 53:1, four of them being considered reliable (CIBA-GEIGY Limited, 1985; Hoechst AG, 1985a; Hoechst AG, 1985b; Hoechst AG, 1989a). All the reliable studies reported negative results for the strains *S. typhimurium* TA100, TA98, TA1537 and TA1535 with and without metabolic activation (all six studies used S9 obtained from Aroclor-induced rat liver, and two studies additionally with

Prival activation). PR 53:1 is an azo dye and according to the OECD TG 471 the use of reductive metabolic activation system (Prival activation) is considered more appropriate than the classical test protocol using S9 obtained from Aroclor-induced rat liver. For the 5th strain (*E.coli* WP2 *uvrA*), negative results were obtained with and without metabolic activation using the classical test protocol. Test results for the 5th strain using Prival activation are not present in any available bacterial reverse mutation test conducted with PR 53:1. These results are supported by one reliable *in vitro* mammalian gene mutation test similar to OECD TG 490 (Myhr *et al*., 1991). There are also two *in vitro* cytogenicity tests. One of the cytogenicity studies is an *in vitro* mammalian chromosomal aberration test (Hoechst AG, 1989b) similar to OECD TG 473, and considered reliable by the DS supporting the negative results. In conclusions, *in vitro* data indicates no mutagenic effects for PR 53:1 with some limitations (no information for the 5th strain in a bacterial reverse mutation test using Prival activation).

There are two *in vivo* somatic cells genotoxicity tests performed with PR 53:1. The unscheduled DNA Synthesis (UDS) test with mammalian liver cells (Westmoreland and Gatehouse, 1992) and the mammalian erythrocyte micronucleus test (MN) (Westmoreland and Gatehouse, 1992) both reported negative results. The UDS test is similar to OECD TG 486 and is considered reliable without restrictions by the DS. As specified in the REACH endpoint-specific guidance (Chapter R.7a, Version 6.0), not all gene mutagens are positive in the UDS test and a negative result in a UDS assay alone is not proof that the substance does not induce gene mutations. The mammalian erythrocyte micronucleus test is used only as supportive study as the exposure of bone marrow was not demonstrated.

According to the classification criteria, even if germ cell mutagenicity is not assessed in this dossier, the information supports the conclusion that the carcinogenic effects of PR 53:1 occurs through a non-genotoxic mode of action.

The possible non-genotoxic mode of action of PR 35:1 was described by Goodman *et al*. (1984) and Weinberger *et al*. (1985). Both authors consider that splenic lesions are the starting point for tumour formation. Goodman *et* at. (1984) suggested splenic haemosiderosis secondary to methaemoglobinemia led to tumour formation, whereas Weinberger *et al*. (1985) suggested acute vascular congestion as the initial alteration in the spleen leading to haemorrhage, fibrosis, and transformed cells. The data from chronic and sub-chronic studies revealed the induction of haemolytic anaemia in mice and rats, including a decrease in blood parameters (e.g. haemoglobin, haematocrit, and red blood cell count) accompanied by haemosiderosis of the spleen. The effects were less severe in mice compared to rats. Methaemoglobin formation was not evaluated in any of the studies.

For the structurally related compound aniline, data suggests that erythrocyte toxicity (indicated by methaemoglobin formation) leads to splenic lesions due to overload with cell debris, haemoglobin and redox-active iron released from damaged erythrocytes and induced oxidative stress resulting in fibrosis, fatty metamorphosis, severe splenic congestion, mesothelial hyperplasia and cyst formation, capsular fibrosis, and multifocal cellular proliferations in the splenic capsule. This is a major cause for pre-neoplastic splenic lesions related to aniline, which can result in tumour formation in rats upon chronic exposure (MAK-Collection for Occupational Health and Safety, 2007). The lower sensitivity of mice in comparison to rats can be explained by species differences in methaemoglobin reductase activity, which is responsible for regeneration of functional haeme from methaemoglobin.

The non-genotoxic mode of action of aniline that explain the tumour formation can be plausible also for PR 53:1, but a final conclusion on the mode of action cannot be drawn due to lack of available data.

Comments received during consultation

Six comments were received during public consultation, five from industry and 1 from a Member State Competent Authority (MSCA). The comment received from the MSCA was in agreement with the DS conclusion that PR 53:1 should be classified as Carc. 2, H351. The other five comments received from the industry are all against PR 53:1 classification as Carc.2, H351 pointing out that the chemical has no genotoxic potential and does not act as a primary carcinogen, and that the adverse effects seen in liver and spleen are more likely to appear due to metabolites leading to hemosiderosis in both target organs and to fibrosis and promotion of tumour formation in spleen. The comments by industry noted that the incidence of tumours was seen only in one study, one species (rat) and one sex (males). The industry comments emphasised that PR 53:1 is handled safely by workers and professionals. Personal precautions, protective equipment as well as protective clothing, have been established to minimize the risk of exposure in manufacturing and processing by inhalation or dermal contact, and by accidental oral exposure. Additionally, PR 53:1 is not handled by the general population. In consumer articles the material is included at very low concentrations, embedded in a matrix, e.g. a polymer matrix or binders matrix. Uptake of the substance at dose level relevant for adverse toxic effects is not expected.

The DS responded that the classification of a chemical as a carcinogen is based on its potential to induce tumours, to increase tumour incidence and/or malignancy, or shorten the time to tumour occurrence, and that the mode of action (as genotoxic carcinogen or not) is not relevant for classification. The studies included in the CLH report provide evidence of carcinogenic potential of PR 53:1 based on the increased incidence of splenic sarcomas in male rats, a rare type of tumour in this organ (CTFA, 1982b; CTFA, 1982a; NTP, 1982a). In the NTP study there is a statistically significant increase in splenic sarcoma incidence in male rats of the high dose group that is above the HCD and is reported with frequently observed metastases that indicate a high malignancy. The results of the CTFA studies are considered supportive, as similar patterns of non-neoplastic splenic lesions were observed in both studies. The study by Davis and Fitzhugh (1962) showed no tumour formation, but severe splenic effects. According to the DS, the study has limited reliability due to limited reporting, no data were provided for individual animals, only six animals from each group were examined histopathologically and no body weight information or HCD were provided. Similar limitations were also identified for the dermal study published by Carson (1984). The DS proposed classification of Pigment Red 53:1 as carcinogen, Category 2. According to Regulation (EC)1272/2008 category 2 is fulfilled, when there is limited evidence of carcinogenicity.

Assessment and comparison with the classification criteria

For PR 53:1, no human evidence for carcinogenicity is available to support the classification in category 1A for carcinogenicity.

A high incidence of splenic sarcoma (statistically significant) in F344 male rats and increased incidence (not statistically significant) in Charles-River CD Sprague-Dawley male rats was reported for PR 53:1. The study in F344 rats showed high incidence, high malignancy and metastasis, with low spontaneous incidence according to HCD and also proofs of progression of lesions to malignancy. These data can be supportive for classification in category 1B, but are not definitive based on the total weight of evidence, effects being observed only in one experiment that according to Annex I: 3.6.2.2.3 represents a limited evidence of carcinogenicity. The criteria for classification as Carc.1B are therefore not fulfilled.

The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category

1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies.

One animal study on F344 rats reported a statistically significant increase in splenic sarcomas in males above the HCD, with high malignancy and metastasis potential and low spontaneous incidence. The effects are supported by another study on Charles-River CD Sprague-Dawley rats reporting a similar trend without statistical significance. There is no possibility of confounding effects of excessive toxicity at the tested doses. The effects were observed only in males. In females, an increasing trend of non-neoplastic spleen lesions was reported, the lesions being also reported in studies with mice. Splenic lesions are considered as a starting point for tumour formation. The possibility of a genotoxic mode of action is dismissed based on the available negative *in vitro* and *in vivo* genotoxicity studies. The non-genotoxic mode of action starting from splenic lesions is plausible and its relevance to humans cannot be excluded. Similar findings and mode of action is described also for aniline and other aromatic amines and aromatic azo compounds that are structurally related with PR 53:1. Taking all the above into consideration, the increasing incidence of splenic sarcoma in male rats is considered as limited evidence of carcinogenicity, and the criteria for classification in Category 2 are met. The generic concentration limit (GCL) of ≥ 1.0 % shall apply. RAC agrees with the DS classification proposal and with the non-genotoxic mode of action.

RAC agrees with the DS proposal that **classification of PR 53:1 in Category 2 (H351) for carcinogenicity is warranted.**

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).