Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

1,4-Benzenediamine, *N,N'*-mixed Ph and tolyl derivs.; Reaction mass of *N*-phenyl,*N'*-o-tolyl-phenylene diamine, *N,N'*-diphenyl-p-phenylene diamine and *N,N'*-di-o-tolyl-phenylene diamine

EC Number: 273-227-8

CAS Number: 68953-84-4

CLH-O-0000007054-80-01/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

26 November 2021

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

1,4-Benzenediamine, N,N'-mixed Ph and tolyl derivs. ;

Reaction mass of N-phenyl,N'-o-tolyl-phenylene diamine, N,N'-diphenyl-p-phenylene diamine and N,N'-di-o-tolyl-phenylene diamine

EC Number: **273-227-8**

CAS Number: **68953-84-4**

Index Number: **-**

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Version number: **2.0**

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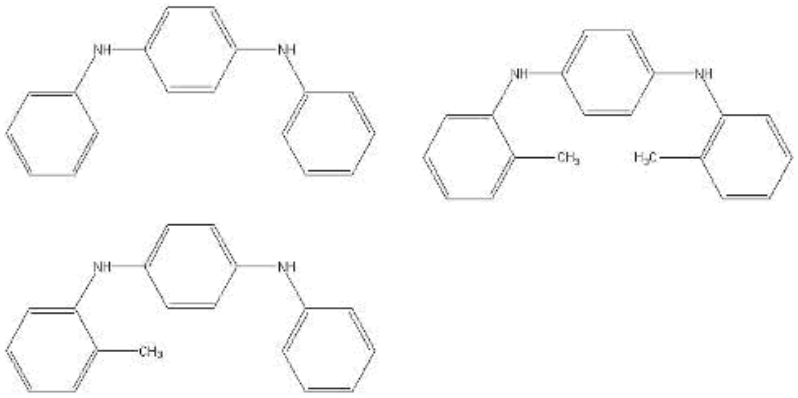
ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 1,4-BENZENEDIAMINE, *N*,*N'*-MIXED PH AND TOLYL DERIVS.; REACTION MASS OF *N*-PHENYL,*N'*-*O*-TOLYL-PHENYLENE DIAMINE, *N*,*N'*-DIPHENYL-P-PHENYLENE DIAMINE AND *N*,*N'*-DI-*O*-TOLYL-PHENYLENE DIAMINE

CONTENTS

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	Reaction mass of N-phenyl,N'-o-tolyl-phenylene diamine, N,N'-diphenyl-p-phenylene diamine and N,N'-di-o-tolyl-phenylene diamine
Other names (usual name, trade name, abbreviation)	BENPAT Wingstay 100, DAPD
ISO common name (if available and appropriate)	-
EC number (if available and appropriate)	273-227-8
EC name (if available and appropriate)	1,4-Benzenediamine, N,N'-mixed ph and tolyl derivs.
CAS number (if available)	68953-84-4
Molecular formula	Non available Constituents: C18H16N2 (N,N'-diphenylbenzene-1,4-diamine) C20H20N2 ((2-methylphenyl)benzene-1,4-diamine) C19H18N2 (N,N'-bis, N-(2-methylphenyl)-N'-phenylbenzene-1,4-diamine)
Structural formula	
Molecular weight or molecular weight range	260.3 – 288.39 g/mol

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)
N,N'-diphenylbenzene-1,4-diamine (CAS: 74-31-7, EC: 200-806-4)	See confidential annex	Skin Sens. 1 – H317 Aquatic Chronic 3 – H412	Skin Sens. 1 – H317 Muta. 2 – H341 Repr. 2 – H361 (fertility) Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410 Aquatic Chronic 3 – H412
N,N'-bis(2-methylphenyl)benzene-1,4-diamine (CAS: 15017-02-4, EC: 239-102-7)	See confidential annex	-	-
N-(2-methylphenyl)-N'-phenylbenzene-1,4-diamine (CAS: 27173-16-6)	See confidential annex	-	-

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
2-methyl-N-phenylaniline CAS: 1205-39-6	See confidential annex I	-	-	-
Diphenylamine CAS: 122-39-4; EC: 204-539-4	See confidential annex I	Acute Tox. 3* – H301/H311/H331 STOT RE 2*– H373** Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410	Acute Tox. 3 – H301/H311/H331 Eye Irrit. 2 – H319 Skin Irrit. 2 – H315 Eye Dam. 1 – H318 STOT RE 2 – H373 (several) STOT SE 3 – H335 (Respiratory tract) STOT SE 1- H370 (central nervous system) Aquatic Acute 1 – H400 Aquatic Chronic 1 – H410 Flam. Sol. 2 – H228 Repr. 2 – H361	-
Phenylenediamine oligomers	See confidential annex I	-	-	-
Other low molecular weight diphenylamine derivatives	See confidential annex I	-	-	-

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 4: Proposed harmonised classification and labelling according to the CLP criteria

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	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 entry										
Dossier submitters proposal	tbd	1,4-Benzenediamine, N,N'-mixed Ph and tolyl derivs. ; Reaction mass of N-phenyl,N'-o-tolyl-phenylene diamine, N,N'-diphenyl-p-phenylene diamine and N,N'-di-o-tolyl-phenylene diamine	273-227-8	68953-84-4	Skin Sens. 1 Repr. 1B	H317 H360FD	GHS07 GHS08 Dgr	H317 H360FD			
Resulting Annex VI entry if agreed by RAC and COM					Skin Sens. 1 Repr. 1B	H317 H360FD	GHS07 GHS08 Dgr	H317 H360FD			

Table 5: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	Hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)		
Oxidising gases		
Gases under pressure		
Flammable liquids		
Flammable solids		
Self-reactive substances		
Pyrophoric liquids		
Pyrophoric solids		
Self-heating substances		
Substances which in contact with water emit flammable gases		
Oxidising liquids		
Oxidising solids		
Organic peroxides		
Corrosive to metals		
Acute toxicity via oral route		
Acute toxicity via dermal route		
Acute toxicity via inhalation route		
Skin corrosion/irritation		
Serious eye damage/eye irritation		
Respiratory sensitisation		
Skin sensitisation	**Harmonised classification proposed**	**Yes**
Germ cell mutagenicity	Hazard class not assessed in this dossier	No
Carcinogenicity		
Reproductive toxicity	**Harmonised classification proposed**	**Yes**
Specific target organ toxicity-single exposure	Hazard class not assessed in this dossier	No
Specific target organ toxicity-repeated exposure		
Aspiration hazard		
Hazardous to the aquatic environment		
Hazardous to the ozone layer		

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

BENPAT does not have an existing entry in Annex VI of CLP and has not been considered for harmonised classification and labelling previously in the EU. BENPAT has been listed on the Community rolling action plan (CoRAP) in 2013. Concerns for substance evaluation were that BENPAT is suspected to be PBT/vPvB, has consumer and wide-dispersive uses, and the substance is produced/ imported in a high (aggregated) tonnage in the EU (> 1000 t/a). There are still open information requests concerning environmental fate.

RAC general comment

1,4-Benzenediamine, *N,N'*-mixed phenyl and tolyl derivates (DAPD or BENPAT; the latter is used throughout) is a reaction product consisting of three main structures and some impurities. The three main constituents are:

- *N,N'*-diphenylbenzene-1,4-diamine (DPPD; CAS: 74-31-7, EC: 200-806-4)
- *N,N'*-bis(2-methylphenyl)benzene-1,4-diamine (CAS: 15017-02-4, EC: 239-102-7)
- *N*-(2-methylphenyl)-*N*'-phenylbenzene-1,4-diamine (CAS: 27173-16-6)

BENPAT is used as an anti-degradant in tires and industrial rubber products, and include widespread uses by professional workers (in formulation, re-packing, manufacturing), and the substance is used by consumers in articles (polymers). This substance is used in synthetic materials and likely released (high or low) from various long-life outdoor and indoor materials.

BENPAT does not have an existing entry in Annex VI of CLP and has not been considered for harmonised classification and labelling previously in the EU. BENPAT has been listed on the Community rolling action plan (CoRAP) in 2013. Concerns for substance evaluation were that BENPAT is suspected to be PBT/vPvB, has consumer and wide-dispersive uses, and the substance is produced/ imported in a high (aggregated) tonnage in the EU (> 1000 t/a). There are still open information requests concerning environmental fate.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Animal studies present in this dossier provide strong evidence that BENPAT causes dystocia (obstructed labour) and high incidences of polycystic kidneys in the offspring. There is no robust data on the Mode of Action (MoA) to conclude that the effects of BENPAT are not relevant to humans or to raise doubt about the human relevance. Therefore, BENPAT acts as a presumed human reproductive toxicant and classification and labelling as Repr.1B (H360FD, May damage fertility. May damage the unborn child) is proposed and the DS disagrees with the current self-classification. According to Article 36 paragraph 1 (d), reproductive toxicity, category 1A, 1B or 2 (Annex I, section 3.7) shall normally be subject to harmonised classification and labelling.

Furthermore, most, but not all of the notifiers to the C&L Inventory self-classified BENPAT as a skin sensitiser, without sub-categorisation, while fewer registrants notified BENPAT as Skin Sens. 1B. In light of its high market volume and consumer/wide-spread uses, the sensitising properties of BENPAT should be acknowledged throughout the Community, and therefore harmonised classification is required.

5 IDENTIFIED USES

According to the ECHA dissemination site (last accessed 19 November 2019), BENPAT is not naturally found in the environment; it is used in synthetic materials such as polymers. Release to the environment of this substance is likely to occur from: outdoor use in long-life materials with low release rate (e.g. metal, wooden

and plastic construction and building materials), outdoor use in long-life materials with high release rate (e.g. tyres, treated wooden products, treated textile and fabric, brake pads in trucks or cars, sanding of buildings (bridges, facades) or vehicles (ships)) and indoor use in long-life materials with low release rate (e.g. flooring, furniture, toys, construction materials, curtains, footwear, leather products, paper and cardboard products, electronic equipment).

6 DATA SOURCES

Data for BENPAT were taken from the publically disseminated REACH Registration Dossier (last accessed 19 November 2019), from study reports on toxicity to reproduction made available by the Registrants in the REACH lead registration dossier, and from the results of a systematic literature screening.

7 PHYSICOCHEMICAL PROPERTIES

Table 6: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Blue-brown flakes or pastilles with an amine-like odour.	MSDS Data	
Melting/freezing point	93-101°C	(Taylor, 2010)	Experimental data
Boiling point	Decomposition before boiling at 350 °C	(Taylor, 2010)	Experimental data
Relative density	1.2 at 20 °C	(Skrok, 2010)	Experimental data
Vapour pressure	Estimated vapour pressures for the 3 main constituents of 1,4-benzenediamine, N,N'-mixed phenyl and tolyl derivatives were in the range of 10^{-7} to 10^{-8} hPa at 25 °C.	(EPA's Office of Pollution Prevention Toxics and Syracuse Research Corporation, 2000)	QSAR estimation
Surface tension			The multi-constituent substance 1,4-Benzenediamine, N,N'-mixed Ph and tolyl derivs. does not contain any amphiphilic constituents and as a consequence there is no structural alert for surface activity. In addition, surface activity is not a desired property of the material.

Property	Value	Reference	Comment (e.g. measured or estimated)
Water solubility	(1) N,N'-diphenyl-p-phenylenediamine; CAS: 74-31-7: 0.13 mg/L; (3) N-(2-methylphenyl)-N'-phenylbenzene-1,4-diamine; CAS: 27173-16-6: 0.11 mg/L; (2) N,N'-bis(2-methylphenylbenzene)-1,4-diamine CAS: 15017-02-4: 0.045 mg/L	(Douglas and Kogovsek, 2004)	The water solubility was determined of the 3 main constituents. Experimental data
Partition coefficient n-octanol/water	N,N'-diphenyl-p-phenylenediamine; CAS: 74-31-7: log Kow = 3.3 N-(2-methylphenyl)-N'-phenylbenzene-1,4-diamine; CAS: 27173-16-6: log Kow = 3.9 N,N'-bis(2-methylphenylbenzene)-1,4-diamine; CAS: 15017-02-4: log Kow = 4.6	*(Dix, 2000)*	The partition coefficient was determined of the 3 main constituents. Experimental data
Flash point			The study does not need to be conducted because the flashpoint is only relevant to liquids and low melting points solids
Flammability	DAPD is a non-flammable solid.	(Krzysiak-Warzała, 2010)	The sample melted after applying the burner's flame. On the surface the burner's flame was applied to, a small sparking was observed, but when the flame was withdrawn, the sparking stopped.
Explosive properties			There are no chemical groups associated with explosive properties present in the molecule.
Self-ignition temperature			Testing should not be conducted for substances that melt at <160°C.
Oxidising properties			Test is not needed for organic substances that do not contain oxygen, fluorine or chlorine.

Property	Value	Reference	Comment (e.g. measured or estimated)
Granulometry	The test substance in its final form (i.e. as supplied to customers) consists of pastilles with a minimum size of approximately 3.9 mm. In a sieving experiment using a 100 micrometer mesh sieve, no particles < 100 micrometer were detected.	(DJCHEM CHEMICALS POLAND SA, 2010)	Experimental data
Stability in organic solvents and identity of relevant degradation products	Recovery data for test chemical in octanol showed all components were > 97 % recovered after 7 days.	(Douglas and Kogovsek, 2004)	Experimental data
Dissociation constant	pKa = 1.47 at 20 °C	(Hambrick, 1994)	Value for N,N'-diphenyl-p-phenylenediamine
Viscosity			Viscosity is relevant only to liquids.

8 EVALUATION OF PHYSICAL HAZARDS

Not addressed in this dossier.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 7: Summary table of toxicokinetic studies – non-human information

Method	Results	Remarks	Reference
Rat, Sprague-Dawley, male Oral exposure: Gavage Exposure regime: One oral gavage dose administered to each rat. Dose/conc.: ~20 mg DAPD/kg that included ~8 mg R-1679/kg Radioactivity doses Phase 1: ~15 µCi/rat Radioactivity doses Phase 2: ~33 µCi/rat Test material was synthesised with radioactivity (^{14}C) positioned in the centre ring. This radiotracer was added to the test substance to allow quantification of excreted material. Three sampling sites were included in the study - urine, faeces, and bile Samples were analysed for radioactivity using liquid scintillation counting and HPLC	Identification of metabolites was not conducted. However, HPLC profiles demonstrate (a) in bile, the parent compound R-1679 is a minor fraction (< 5 %) of that administered, and (b) > 90 % of the radiolabel elutes in the chromatographic region associated with metabolites exhibiting higher polarity than the parent compound. 40 % of that radioactivity was located in one peak (B4). Evaluation of results: Bioaccumulation potential cannot be judged based on these study results.	2 (reliable with restrictions) Supporting study Test material: (1) Component: N-phenyl-N'-o-tolyl-p-phenylene-diamine (R-1679), typically 40 % of the registration substance DAPD; CAS No: 27173-16-6 (2) Component: N,N'-di (o-tolyl)-p-phenylenediamine (R-898), typically ~20 % of the registration	(RTI, 1998a; RTI, 1998b)

Method	Results	Remarks	Reference
with both UV and flow-through scintillation counting.		substance DAPD; CAS No: 15017-02-4	
Rat, Fischer 344, female (n=6/exposure group) Dermal exposure Solid test material was radiolabelled (^{14}C with 94 % chemical and radiochemical purities), making possible accurate quantitation of absorption kinetics using beta counting assay. Specific activity was 11.7 Ci/mol. The location of the ^{14}C label was the centre ring. Dermal doses: 350 µg ^{14}C R-898 (10 µCi radioactivity) added to 1660 µg of DAPD in solution of acetone; total volume = 50 µL. Dosage for intravenous administration: 35 µg ^{14}C R-898 (1 µCi radioactivity) added to 1660 µg of DAPD in solution of acetone; total volume = 20 µL. These levels of exposure were estimated to lack toxic activity based upon acute toxicity test results.	Comparison of excreted radioactivity following dermal vs. i.v. administration of radioactive R-898 to groups of rats indicated that 60 % of the dermally applied test chemical was absorbed during the 7-day study. The bulk (> 95 %) of absorbed test chemical was excreted via the faeces.	Reliability: 2 (reliable with restriction) GLP-compliant Supporting study Test material: Component: N,N'-di (o-tolyl)-p-phenyl-enediamine (R-898), ~20 % of DAPD (CAS 15017-02-4)	(University of California, 1997)

The information on oral toxicokinetic (and also on oral absorption percentages) stems from two oral toxicokinetic studies in which two components of BENPAT, N-phenyl-N'-(o-tolyl) p-phenylenediamine (CAS No. 27173-16-6) and N,N'-di-(o-tolyl)-p-phenylenediamine (CAS No. 15017-02-4), have been investigated, respectively as radiolabelled admixture to BENPAT. Both oral studies were not performed according to an acknowledged test guideline. However, the results are useful to assess the extent of oral absorption. Both studies also investigated the extent of biliary excretion and metabolism. Urine, faecal and bile samples were collected over a period of 48 hours following oral dosing to bile duct-cannulated animals. Results showed that 76 to 78 % of the administered radioactivity was excreted within this 48 hour observation period. The main part, about 75 % of the dosed radioactivity was excreted in the faeces. Urinary excretion consisted of (unidentified) metabolites and accounted for maximally 2.5 % of the total amount of radioactivity. Analysis of the biliary fraction showed that 30 to 35 % of the administered radioactivity entered into the gastro-intestinal tract via the biliary route. HPLC analysis of bile demonstrated that > 95 % of the metabolites excreted by this route exhibited greater polarity than the parent compound, suggesting metabolic formation of oxidation and conjugation products of these components.

In both studies, reference is given to a study performed with a further main constituent of BENPAT, DPPD (N,N'-diphenyl-p-phenylenediamine; CAS No.: 74-31-7). However, the study performed with CAS number 74-31-7 is not presented in the CSR or in the IUCLID file. The registrant stated that "the majority (55 %) of orally administered DPPD is excreted in faeces of rats with < 0.1 % excreted in urine" (ECHA dissemination site, last accessed 19 November 2019).

Dermal absorption has been investigated with N,N'-bis(2-methylphenyl) benzene-1,4-diamine (CAS No. 15017-02-4), one of the main constituents of BENPAT. The radiolabelled ([^{14}C]-label) substance had been administered by the dermal and intravenous route to female rats (University of California, 1997). Urinary and faecal excretion was monitored over a period of 168 h after dosing. Recoveries of radioactivity were 70 % and 88 % after intravenous and dermal dosing, respectively. After dermal administration, skin site rinses, tape stripping and skin digestion samples to recover unabsorbed residues were also analysed. Comparison of excreted radioactivity from intravenously and dermally treated animals indicated that 60 % of the dermally

applied test chemical was absorbed during the study period (7 days). Most of the absorbed test compound (> 95 %) had been excreted via the faeces.

The study report states that the study has been performed according to EPA FIFRA 40 CFR, part 160 guideline. However, that guideline describes a GLP statement rather than a test guideline and does not address how to conduct *in vivo* dermal absorption studies. Thus, the assignment of Klimisch code 1 (reliable without restriction) is not supported by the DS. Further, important measurements considered essential in OECD TG 427 (*in vivo* dermal absorption study) apparently were not addressed in this study, such as determination of quantities in blood and quantities in the remaining carcass.

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

Data for oral and dermal toxicokinetics for the registered substance or components thereof are available from animal studies. Human information is not available at present.

Study results show that one main component of BENPAT (CAS No.: 15017-02-4) is dermally absorbable and most amounts of BENPAT components (CAS No. 15017-02-4, 27173-16-6) are excreted in the faeces after oral and dermal administration. Furthermore, bile analysis in oral toxicokinetic studies demonstrated that the major fraction of the administered substances were present as metabolites, however, metabolites had not been identified.

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

Hazard class not assessed in this dossier.

10.2 Acute toxicity - dermal route

Hazard class not assessed in this dossier.

10.3 Acute toxicity - inhalation route

Hazard class not assessed in this dossier.

10.4 Skin corrosion/irritation

Hazard class not assessed in this dossier.

10.5 Serious eye damage/eye irritation

Hazard class not assessed in this dossier.

10.6 Respiratory sensitisation

Hazard class not assessed in this dossier.

10.7 Skin sensitisation

Skin sensitisation is an immunological process consisting of two phases. At first, during induction, the chemical forms a hapten-protein-complex in the skin of naive individuals. A sequential set of events follows, leading to the production of allergen-specific memory T-cells. In the second phase, the elicitation, exposure of the sensitised individual to the allergen leads to proliferation and activation of these T-cells, secretion of

cytokines and mobilisation of other inflammatory cells resulting in a clinical outcome of allergic contact dermatitis (ECHA, 2017).

There is one animal study available addressing the sensitising potential of BENPAT and performed according to OECD TG 406.

Table 8: Summary table of animal studies on skin sensitisation

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance,**	**Dose levels duration of exposure**	**Results[1]**	**Reference**
According to OECD TG 406 (GPMT) Reliability: 1, reliable without restriction GLP-compliant	Guinea pig, Hartley, male/ female n=20/test group n=10/ control group	BENPAT, test substance, reference 1 Purity: no information	Intradermal induction: (1) 5.0 % test chemical in acetone/propylene glycol), (2) 5.0 % test chemical in acetone/propylene glycol/ FCA emulsion, and (3) FCA emulsion; Topical induction: 100 % test chemical (0.35 g); Epicutaneous challenge: 25 % or 100 % test substance (vehicle: acetone/mineral oil); Reading after 24 and 48h: 24h (25 %): 5/20 positive reactions 48h (25 %): 3/20 positive reactions 24h (100 %): 0/20 positive reactions 48h (100 %): 5/20 positive reactions Re-challenge (day 28): 24h (25 %): 15/20 positive reactions 48h (25 %): 11/20 positive reactions 24h (100 %): 15/20 positive reactions 48h (100 %): 15/20 positive reactions Positive control (0.1 % DNCB[2]): valid, 6/6 positive reactions	**Positive** **No sub-categorisation possible due to high intradermal induction dose**	

The sensitising potential of BENPAT was investigated in a GLP-compliant guinea-pig maximisation test (GPMT) performed according to OECD TG 406 (Charles River, 1995). The induction phase included both intradermal and epidermal exposure to solutions of 5 % test substance in Freund's adjuvant or acetone/propylene glycol. Animals received two epicutaneous challenge administrations on day 21 (1st challenge) and day 28 (2nd challenge), respectively. The dermal response following 48h after the first challenge to 25 % or 100 % of chemical resulted in clinical effects (oedema or desquamation) for 15 % or 25 % of test animals, respectively. Forty-eight hours after the second challenge, 55 % of the animals exposed to 25 % of test chemical showed signs of desquamation, oedema or blanching. In the test group exposed to 100 % of test chemical, 75 % of the animals showed signs of a sensitisation reaction after 48 hours. This study indicates that BENPAT acts as a sensitiser. Since the concentration selected for intradermal induction (5 %) is higher than those required for classification as Skin Sens. 1A, this study does not allow for a reliable sub-categorisation.

There are no human data available, addressing the sensitising potential of BENPAT.

Notably, several studies showed that the BENPAT constituent, N, N'-diphenyl-p-phenylene-diamine (DPPD; EC: 200-806-4, CAS: 74-31-7), produced sensitisation reactions in animal models (LLNA BrdU-ELISA, GPMT) and elicits skin sensitisation in humans (human patch test studies, case reports). DPPD has a

[1] According to the Guidance on the Application of the CLP Criteria, Version 5.0

[2] 2,4-Dinitrochlorobenzene, CAS: 97-00-7

harmonised classification and labelling in Annex VI to the CLP regulation as Skin Sens. 1, supporting the skin sensitising potential of BENPAT.

10.7.1 Short summary and overall relevance of the provided information on skin sensitisation

A GLP-compliant GPMT conducted according to OECD TG 406 shows that BENPAT should be classified as a sensitiser. The study does not provide a reliable basis for sub-categorisation.

Data addressing the skin sensitisation potential of BENPAT in humans are not available to the DS.

Furthermore, DPPD, one of the main constituents of BENPAT, already has a harmonised classification as Skin Sens. 1 in Annex VI to the CLP regulation.

10.7.2 Comparison with the CLP criteria

In Table 9, animal studies on skin sensitisation are compared with CLP criteria, as laid down in ECHA's Guidance on the Application of the CLP criteria.

Table 9: Comparison of available data (animal studies) for skin sensitisation of BENPAT or DPPD with the CLP criteria

<table>
<tr><th>Reference(s)</th><th>Criteria acc. to CLP regulation, as laid out in (ECHA, 2017)</th><th>Results</th><th>Resulting Classification</th></tr>
<tr><td colspan="4">Animal data</td></tr>
<tr><td>GPMT

(Charles River, 1995)</td><td><u>Skin Sens. 1A:</u>
≥ 30 % responding at ≤ 0.1 % intradermal induction dose or
≥ 60 % responding at > 0.1 % to ≤ 0.1 % intradermal induction dose intradermal induction dose

<u>Skin Sens. 1B:</u>
≥ 30 % to < 60 % responding at > 0.1 % ≤ 1 % intradermal induction dose or
≥ 30 % responding at > 1 % intradermal induction dose</td><td>Following intradermal induction with a test substance concentration of 5 %, 55-75 % of the guinea pigs showed a positive reaction at re-challenge.</td><td>Skin Sens. 1

No sub-categorisation</td></tr>
<tr><td>Annex VI to the CLP regulation, index number 612-132-00-1: N,N'-diphenyl-p-phenylene-diamine
(DPPD; EC 200-806-4, CAS 74-31-7) classified as skin sensitiser, Category 1</td><td>Generic concentration limits (GCL) of components classified as skin sensitiser that trigger classification of the mixture as Skin Sens. 1

<table><tr><th>Component classified as</th><th>Conc. that trigger classification of mixture</th></tr><tr><td>Skin Sens. 1</td><td>≥ 1.0 %</td></tr><tr><td>Skin Sens. 1A</td><td>≥ 0.1 %</td></tr><tr><td>Skin Sens. 1B</td><td>≥ 1.0 %</td></tr></table></td><td>BENPAT component DPPD (≥ 1.0 %), classified as Skin Sens 1
→ BENPAT classified as Skin Sens 1</td><td>Skin Sens. 1

No sub-categorisation</td></tr>
</table>

A GLP-compliant GPMT performed according to OECD TG 406 (Charles River, 1995) reveals that BENPAT is a skin sensitiser (55-75 % of the guinea pigs showed a positive reaction upon re-challenge, following intradermal induction with a test substance concentration of 5 %). Category 1 is appropriate since a lower concentration to show absence of effects at lower doses than 1 % was not tested.

In addition, one of the main BENPAT constituents, DPPD, has a harmonised classification as Skin Sens. 1 in Annex VI to the CLP regulation, without sub-categorisation. In line with Art. 11 (1) and Table 3.4.5 in Annex I of the CLP Regulation, therefore, a substance containing DPPD as a constituent at concentrations ≥ 1.0 % should also be classified as Skin Sens. 1.

In summary, the available data provide conclusive evidence that BENPAT acts as skin sensitiser, but do not allow for sub-categorisation.

10.7.3 Conclusion on classification and labelling for skin sensitisation

The DS proposes to classify BENPAT as a moderate skin sensitiser, i.e. **Skin Sens. 1 (H317 - May cause an allergic skin reaction)**.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

One GLP compliant guinea-pig maximisation test (GPMT; OECD TG 406) is available for BENPAT. According to this test, BENPAT is a skin sensitizer but the data does not allow for sub-categorisation.

No human studies regarding skin sensitisation are available for BENPAT. The Dossier Submitter (DS) noted that *N,N'*-diphenyl-*p*-phenylene-diamine (DPPD; CAS 74-31-7), a constituent of BENPAT (> 1 %), is a known skin sensitizer in animals and humans. DPPD has a harmonised classification for Skin Sens 1.

The DS proposed classification for effects on skin sensitization in category 1 (H317 – May cause an allergic skin reaction).

Comments received during consultation

One industry representative commented on the proposal of Skin Sens. 1 by the DS, the only comment received regarding this health hazard, and supported the proposed harmonised classification of Skin Sens. 1 without further sub-categorisation.

Assessment and comparison with the classification criteria

In the GPMT study (Klimisch score 1) animals (n = 20 per group, n = 10 in control) were induced to BENPAT by intradermal and epidermal exposure to a concentration of 5 %. RAC notes that the purity of the test substance is not known. However, the purity of a multi-constituent substance is defined by its quantitative composition. Therefore, it is assumed that BENPAT used in this study (and also studies assessed for other toxicological endpoints) fulfils the criteria of the main identifiers, according to Chapter 4.2.2. of the REACH Guidance for identification and naming of substances under REACH and CLP[3], for multi-constituent substances. Upon re-challenge to 25 or 100 % of BENPAT on day 21 (first challenge) and day 28 (second challenge), clinical signs (oedema, blanching or desquamation) were described in 15-25 % (first challenge) and 55-75 % (second challenge) of animals 24 h or 48 h after administration of BENPAT. From these results it is concluded that BENPAT is a skin sensitizer. A high response was observed in the GPMT test upon exposure to a high concentration of BENPAT. However, there is no data available for lower concentrations of BENPAT, sub-categorisation is therefore not possible. In addition, one constituent of BENPAT, DPPD, has a harmonised classification for Skin Sens. 1 and is present in BENPAT at ≥ 1 %.

[3] https://echa.europa.eu/documents/10162/23036412/substance_id_en.pdf/ee696bad-49f6-4fec-b8b7-2c3706113c7d

Conclusion

Based on one positive GPMT study with BENPAT, RAC considers that **classification as Skin Sens. 1 is warranted**. In addition, the presence of DPPD (≥ 1 %), which has a harmonised classification for Skin Sens. 1, supports a harmonised classification of BENPAT for Skin Sens. 1.

10.8 Germ cell mutagenicity

Hazard class not assessed in this dossier.

10.9 Carcinogenicity

Hazard class not assessed in this dossier.

10.10 Reproductive toxicity

Table 10: Summary table of animal studies on (statistically significant) adverse effects on sexual function and fertility, and development (Data include changes in organ weight, with an at least 10 % effect level compared to controls.)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group, reference</th><th>Test substance, dose levels</th><th>Results</th></tr>
<tr><td>Two-Generation Reproductive Toxicity Study, according to OECD TG 416

<u>Key study for adverse effects on sexual function and fertility, and development</u>

Reliability: 1, reliable without restriction
GLP-compliant

Rats, CD® Sprague-Dawley, N=30/sex/dose group (F0 & F1 parents)

Exposure F0 and F1-generation: Pre-breeding (10 weeks), mating (two weeks), gestation (three weeks), lactation (three weeks)

Exposure F2-generation: Mating (two weeks), gestation (three weeks), lactation (three weeks)

Necropsy:
F0 males after delivery (histologic evaluation of reproductive & target</td><td>Dietary doses of <u>BENPAT,</u> 1,4-Benzene-diamine, N,N'-mixed Ph and tolyl derivs., <u>CAS: 68953-84-4</u>, test substance reference 1 (in 2 % corn oil) at 120, 400, and 1500 ppm, corresponding to 0, 7.5, 25 and 100 mg/kg bw/d

Purity: No data</td><td>
<table>
<tr><th>F0 adults</th><th>F1 adults</th></tr>
<tr><td colspan="2">Effects on sexual function and fertility[4]</td></tr>
<tr><td colspan="2">100 mg/kg bw/ d</td></tr>
<tr><td>↑ gestational length (5.9 %; 23.5 d vs. control: 22.2 d);
↑ % post-implantation loss (41.6 %; 52.3 % vs. control: 10.7 %);
↓ no. total pups/ litter (12.1 vs. control: 15.7);
↓ no. live pups/ litter (7.6 vs. control: 15.6);
↑ no. of dead pups/ litter (4.1 vs. control: 0.1)</td><td>↑ gestational length (4.5 %; 23.2 d vs. control: 22.2 d);
↑ % post-implantation loss (16.8 %, 23.6 % vs. control: 6.8 %);
↓ no. live pups/ litter (10.8 vs. control: 15.6);
↑ no. of dead pups/ litter (2.5 vs. control: 0.1);

↑ no. with abnormal stage of oestrous cycle (43.3 % (13/30), control: 6.7 % (2/30)); ↑ ♀ in metestrus (31.0 % (9/29), control 10 % (3/30)); ↑ mean cycle length (7.2 d, compared to control: 5.1 d; not statistically significant)</td></tr>
<tr><td colspan="2">25 mg/kg bw/d</td></tr>
<tr><td>↑ gestational length (2.7 %; 22.8 d vs. control: 22.2 d);
↑ % post-implantation loss (15.4 %; 26.1 % vs. control: 10.7 %);
↓ no. total pups/ litter (12.3 vs. control: 15.7);
↓ no. live pups/ litter (11.9 vs. control: 15.6)</td><td>↑ gestational length (4.1 %; 23.1 d vs. control: 22.2 d);

↑ no. with abnormal stage of oestrous cycle (28.6 %, control: 6.7 %);

↑ abnormal cycles (8/30 abnormal cycling and 2/30 not cycling, control 2/30 with abnormal cycle and 0/30 not cycling); ↑ ♀ in metestrus (7/29, compared to control 3/30); cycle length comparable to control</td></tr>
<tr><td colspan="2">7.5 mg/kg bw/d</td></tr>
</table>
</td></tr>
</table>

[4] Values for effects on sexual function and fertility are included in Table 11.

Method, guideline, deviations if any, species, strain, sex, no/group, reference	Test substance, dose levels	Results	
tissues for high dose and control males). F1/ F2 litters at weaning (three weanlings/sex/litter) F0 females after weaning of F1 litters (histopathology of reproductive & target organs for high dose and control animals) F1 males were necropsied after the delivery period Parental F1 females after weaning of the F2 litters **(RTI, 2001a)**			↑ gestational length (2.7 %; 22.8 d vs. control: 22.2 d); ↑ ♀ in metestrus (8/30; control 3/30); cycle length comparable to control
		General toxicity[5], [6]	
		100 mg/kg bw/ d	
		↓ BW (ca. -7 %) in ♂ for pre-breeding and mating period; ↓ BW (-7 %) in ♀ for SD 7 to SD 63 of pre-breeding period; ↓ BW (-10 %) in ♀ during gestation, ↓ BWG in ♀ during gestation (-18 %) ↓ BW in ♀ for lactation, PND 0, 4, 7 (-11 %); ↑ BWG in ♀ during lactation (PND 0-21) 4 ♀ dead during lactation period (died in process of delivering (1 ♀), euthanised in process of delivering, PND 0 (1 ♀), euthanised moribund, PND 0 (1 ♀), found dead, PND 1 (1 ♀); 3 ♀ died and 1 ♀ euthanised moribund during holding period until scheduled sacrifice, all ♀ had pups that died during delivery; ↑ gestation length (23d -25d for 6 ♀, 2 ♀ died/euthanised in process of delivering); piloerection (6 ♀); vaginal bleeding, GD23/24 (5 ♀); litter with all-dead pups on PND 0 (7 ♀); *Unscheduled necropsy of dams that died during lactation:* Liver: necrosis (7 ♀), inflammation (1 ♀), haemorrhage (1 ♀); kidney: necrosis (5 ♀), inflammation (1 ♀); uterus: haemorrhage (2 ♀), inflammation (3 ♀), retained dead foetuses *in utero* (3 ♀), retained placenta (2 ♀), resorbing implants (1 ♀), uterus was too autolyzed to evaluate (1 ♀); vagina: retained foetus (2 ♀), haemorrhage (1 ♀); adrenal	↓ BW (-7 %) in ♂ for last three weeks of pre-breeding; ↓ BW (-7.0 %) in ♂ for mating period; ↓ BWG (-11 %) in ♀ during gestation; ↓ BW (-9 %) in ♀ for lactation PND 0, 4, 7; ↓ BW (-7 %) in ♂ at scheduled sacrifice One ♀ died (PND 0) while delivering; uterus inflammation, haemorrhage, thrombosis; kidney polycystic, mineralisation, inflammation; liver necrosis; bladder hyperplasia, inflammation; lungs oedema, alveolus; five foetuses retained in left horn of uterus, five foetuses and numerous blood clots in right horn of uterus, and one placenta retained in vagina *Scheduled necropsy*: ↑ relative paired kidney weight (10.1 %) in ♂; ↓ % progressively motile sperm (21.4 %, control: 27.7 %); ↑ polycystic kidneys in ♂ (21/30) & ♀ (18/30); ↑ tubule dilatation in the renal papilla in ♂ (3/30) & ♀ (4/30); ↑ kidney chronic inflammation in ♀ (9/30); ↑ renal tubule regeneration in ♂ (16/30) & ♀ (14/30)

[5] Absolute body weight assumed, not specified by study author

[6] Incidence of polycystic kidneys in F0 and F1 animals is summarised in Table 13.

Method, guideline, deviations if any, species, strain, sex, no/group, reference	Test substance, dose levels	Results	
		cortex: degeneration (haemorrhage) (2 ♀); ovary: cysts (follicle) (1 ♀); lung: thrombosis (2 ♀), inflammation (2 ♀), congestion (1 ♀) *Scheduled necropsy:* ↑ relative paired kidney weight (10.7 %) in ♂ & (12.0 %) in ♀; ↑ absolute liver weight (14.0 %) in ♀, ↑ relative liver weight (19.8 %) in ♀; ↓ absolute (-23.0 %) and relative (-19.6 %) uterine weight; polycystic kidneys (3/30 ♀), kidney cortical necrosis (5/30 ♀); liver hematopoietic cell proliferation (4/30 ♀), hepatocellular centrilobular necrosis (7/30 ♀); lung inflammation (2/30 ♀), thrombosis (2/30 ♀); adrenal cortex degeneration (2/30 ♀)	
		25 mg/kg bw/d	
		1 ♀ found dead (GD 17), implantation sites not consistent with gestational age; amniotic sacs blood filled; 16 resorbing foetuses *in utero*; ovary mineralisation, oviduct; uterus haemorrhage; mammary gland adenocarcinoma neoplasm (malignant without metastasis) 1 ♀ died in process of delivering (PND 0); vagina: one retained foetus; uterus left horn: three retained foetuses, blood in amniotic sacs; uterus right horn: six retained foetuses; thymus with red foci (haemorrhage); lungs all lobes reddened; liver all lobes pale; kidney cortex pale; adrenals enlarged 1 ♀ found dead (PND 2), prolonged gestation (23 d), delivered six living pups; uterus right horn: two retained foetuses; vagina: one retained foetus; liver necrosis; lungs – congestion; kidney necrosis cortex; uterus haemorrhage, inflammation *Scheduled necropsy:* ↓ absolute (-20.4 %) and relative uterine weight (-20.7 %)	1 ♀ died on PND 3, delivered 10 live pups and 4 dead pups; liver necrosis, all lung lobes congested with irregular patchy consolidation, and one foetus retained in the vagina. *Scheduled necropsy:* ↓ relative paired ovary weight (-17.61%) ↑ polycystic kidneys in ♂ (10/30) & ♀ (1/30); ↑ tubule dilatation in the renal papilla in ♂ (1/30) & ♀ (1/30); ↑ kidney chronic inflammation in ♀ (4/30); ↑ renal tubule regeneration in ♂ (1/30) & ♀ (1/30)

Method, guideline, deviations if any, species, strain, sex, no/group, reference	Test substance, dose levels	Results	
		kidney cortical necrosis (1/11 ♀); hepatocellular centrilobular necrosis (1/11 ♀)	
		7.5 mg/kg bw/d	
		↓ absolute (-11.5 %) and relative paired ovary weight (-12.0 %); two ♂ with kidneys exhibiting "pits"	↓ % progressively motile sperm (21.7 % vs control: 21.4 %) ↑ polycystic kidneys in ♂ (5/30) & ♀ (2/30); ↑ kidney chronic inflammation in ♀ (2/30); ↑ renal tubule regeneration in ♂ (1/30)
		F1 offspring	**F2 offspring**
		Offspring toxicity (PND 0)	
		100 mg/kg bw/ d	
		F1 pup necropsy findings in dead pups: Ductus (arteriosus) open (patent) and *in utero*; dead pups with closed or open (patent) ductus; cannibalised, autolysed, unable to evaluate	↑ BW (5 %); F2 pup necropsy findings in dead pups: dead pups with closed or open (patent) ductus; autolysed, unable to evaluate; abdominal organs autolyzed, unable to evaluate
		25 mg/kg bw/d	
		↑ BW (7 %); F1 pup necropsy findings in dead pups: Ductus (arteriosus) open (patent) and *in utero*; dead pups with closed or open (patent) ductus; cannibalised, autolysed, unable to evaluate	↑ BW (11 %); F2 pup necropsy findings in dead pups: dead pups with closed or open (patent) ductus; abdominal organs autolyzed, unable to evaluate
		7.5 mg/kg bw/d	
		↑ BW (6 %); F1 pup necropsy findings in dead pups: dead pups with closed or open (patent) ductus; cannibalised, unable to evaluate	↑ BW (9 %); F2 pup necropsy findings in dead pups: dead pups with closed or open (patent) ductus; autolysed, unable to evaluate
		Weanlings toxicity (PND 21)	
		100 mg/kg bw/ d	
		↓ absolute (-11.2 %) thymus weight in ♂, ↑ relative spleen weight (16.6 %) in ♀, ↓ absolute (-10.8%) & relative brain weight (-10.2 %) in ♀; ↑ polycystic kidney in ♂ (10/11) & ♀ (11/11),	↑ polycystic kidney in ♂ (15/16) & ♀ (15/15)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group, reference</th><th>Test substance, dose levels</th><th>Results</th></tr>
<tr><td></td><td></td><td>
<table>
<tr><td>↑ renal tubule regeneration in ♂ (9/11) & ♀ (10/11)</td><td></td></tr>
<tr><td colspan="2">25 mg/kg bw/ d</td></tr>
<tr><td>↑ BW in ♂ (9.2 %) & (9.1 %) in ♀;
↑ absolute thymus weight (11.6 %) in ♂ & (12.7 %) in ♀;
↑ absolute spleen weight (34.7 %) in ♂ & (23.2 %) in ♀;
↑ relative spleen weight (23.8 %) in ♂ & (13.8 %) in ♀;
↓ relative brain weight in ♀ (-11.2 %);
↑ polycystic kidneys in ♂ (8/20) & ♀ (7/18)</td><td>↑ BW (12.3) in ♂ & (8.6 %) in ♀,
↑ absolute thymus weight (10.9 %) in ♂; ↑ absolute spleen weight (22.7 %) in ♂;
↓ relative brain weight (-13 %) in ♂
↑ polycystic kidney in ♂ (6/19) & ♀ (8/19)</td></tr>
<tr><td colspan="2">7.5 mg/kg bw/ d</td></tr>
<tr><td>↑ absolute spleen weight (20.0 %) in ♂ & (17.3 %) in ♀;
↑ relative spleen weight (13.7 %) in ♂ & (12.7 %) in ♀;
↑ polycystic kidney in ♂ & ♀, chronic inflammation, nephropathy
↑ polycystic kidneys in ♂ (1/25) & ♀ (5/26)</td><td>↑ BW (8.1 %) in ♂;
↑ absolute thymus weight (13.6 %) in ♂;
↑ absolute spleen weight (15.1 %) in ♂;
↑ polycystic kidney in ♂ (3/64) & ♀ (5/64)</td></tr>
</table>
One F0 female at 0 mg/kg bw/d was euthanised on SD 17, probably due to a broken left hind leg; one male at 0 mg/kg bw/d was sacrificed moribund on SD 85; one F1 female at 0 mg/kg bw/d died on SD 97

A maternal NOAEL of 25 mg/kg bw/d is derived based on lethality of dams during lactation. It was not possible to identify a NOAEL for sexual function and fertility, a LOAEL of 7.5 mg/kg/day is identified, the lowest dose tested, based on prolonged gestation and dystocia. A developmental NOAEL was not established and a developmental LOAEL of 7.5 mg/kg bw/d is derived, based on polycystic kidneys in F1 and F2 generations.
</td></tr>
<tr><td>Prenatal Developmental Toxicity Study, according to OECD TG 414

<u>Key study for adverse effects on development</u>

Reliability: 1, reliable without restriction(defined by the registrant); however the DS assess this study with Reliability 2: reliable with restriction, because evaluation of effects of BENPAT</td><td>Oral gavage of BENPAT, test substance reference 2 (dissolved in corn oil) at doses of 0, 20.0, 70.0 and 200.0 mg/kg bw/d</td><td><u>General toxicity F0 generation</u>
<u>200 mg/kg bw/d</u>
↓ maternal BW at GD 12 (-5.4 %); ↓ maternal weight gain during dosing period, for GD 6-9 (-87.8 % weight gain, compared to control), and 6-15 (-30.5 % weight gain, compared to control) ; no statistical significance for maternal body weight gain, when corrected (minus gravid uterine weight); data for maternal body weight corrected (minus gravid uterine weight) not available

<u>Effect on Development</u>
No treatment-related statistically or biologically significant changes in the incidence of individual or pooled external, visceral (including craniofacial), skeletal or total foetal malformations or variations</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group, reference	Test substance, dose levels	Results
during late pregnancy were not possible (no treatment from GD 16) GLP-compliant Rats, CD® (Sprague-Dawley), N=25/dose group Exposure of F0 ♀ on gestation days (GD) 6-15; Scheduled sacrifice on GD 20 Evaluation of dams body, liver, & gravid uterine weights; ovarian corpora lutea; uterine implantation sites (resorptions, dead foetuses, live foetuses) Foetuses were dissected from uterus, counted, weighed, sexed, & examined for external abnormalities; examination for visceral malformations & variations, soft tissue craniofacial malformations and variations, and skeletal malformations and variations **Deviations:** No treatment from GD 16 until GD 20, time window too short to evaluate adverse effects of BENPAT during late pregnancy **(RTI, 1995)**		There were no adverse effects seen during this study, the maternal and developmental NOAEL is 200 mg/kg bw/d, the highest dose tested. A NOEL of 70 mg/kg bw/d was established in maternal animals, due to decreased body weight and body weight gain in high dose animals.
Range-finding study, for Prenatal Developmental Toxicity Study (OECD TG 414) **Supporting study**	Oral gavage of BENPAT, test substance reference 2	**600 mg/kg bw/d** ↓ Mean maternal body weights at GD 9 (-11.6 %), 12 (-16.8 %), and 15 (-11.6 %); significant dose-related downward trends for maternal body weights on GD 18 (-8.0%) and 20 (-6.5 %); no data available for body weight corrected (minus gravid uterine weight);

Method, guideline, deviations if any, species, strain, sex, no/group, reference	Test substance, dose levels	Results
Rats, CD® (Sprague-Dawley) N=8/ dose group Exposure: GD 6-15 Observation: dams body weight and food consumption (GD 0,6, 9, 12, 15, 18, and 20); clinical signs (once daily through gestation, at least twice daily during dosing period) Necropsy on GD 20: gravid uterus and liver weight, ovarian corpora lutea count, status of uterine implantation sites; total, non-live (early or late resorptions, dead foetuses), live foetuses. All live foetuses were weighed, sexed externally (except tor two litters which were inadvertently not sexed), examined for external malformations (including cleft palate) and variations **(RTI, 1995)** – Appendix V	(dissolved in corn oil) at doses of 0, 20.0, 70.0, 200.0, and 600 mg/kg bw/d	↓ maternal weight gain tor GD 6-9 (-22.8 g vs control: 11.7 g), 6-15 (dosing period; 15.3 g vs control: 51.7 g); ↓ maternal weight gain corrected to weight of the gravid uterus, GD 0-20 (41.8 g vs control: 72.3 g) ↓ maternal feed consumption (g/kg/d) for GD 6-9; -60.0 % (GD 6-15: the lack of statistical significance due to the fewer dams alive at this dose tor this interval); ↑ maternal feed consumption for GD 15-18 (22.6 %) and 15-20 (19.7 %; post-dosing period), consistent with weight gain for this period 4/8 dams died: One dam died on GD 14, evidence of vaginal bleeding on GD 12 and 13; one dam found dead on GD 14, with blood still evident around vaginal area; Two other dams, who died or were sacrificed moribund on GD 12, with resorbing conceptuses; At necropsy: Dead dams exhibited pale organs (e.g. kidneys, lungs, liver, ovaries, spleen) and extremities (ears, eyes, tail, and skin) Evidence of vaginal bleeding in one third dam at GD13 (15 implants and 15 live foetuses at scheduled sacrifice) ↓ Foetal body weights/ litter (-12.6 %; all foetuses, and males and females separately) **200 mg/kg bw/d** ↓ Mean maternal body weights at GD 9 (-7.8 %) and 15 (-8.6 %) ↓ maternal weight gain for GD 6-9 (-7.1 g vs control: 11.7 g), 6-15 (27.1 g vs control: 51.7 g), 0-20 (gestation; (127.8 g vs control: 154.0 g)) ↓ maternal weight gain corrected to weight of the gravid uterus, GD 0-20 (gestation; 48.1 g vs control: 72.3 g) ↓ maternal feed consumption (g/kg/d) for GD 6-15 (58.2 g vs control: 67.3 g) ↓ Foetal body weights/ litter (-6.3 %; all foetuses, and females; value for male foetuses was also reduced but not statistically significantly) **70 mg/kg bw/d** Evidence of vaginal bleeding in one dam on GD 13 (15 implants and 15 live foetuses at scheduled sacrifice) **20 mg/kg bw/d** Evidence of vaginal bleeding in one dam on GD 13 (13 implants and 13 live foetuses at scheduled sacrifice) Maternal gravid uterine weights, and absolute and relative maternal liver weights were equivalent across all groups at scheduled sacrifice

Method, guideline, deviations if any, species, strain, sex, no/group, reference	Test substance, dose levels	Results
		No statistically significant or biologically important treatment- or dose-related changes in pre- or post-implantation loss/litter, incidence of non-live implants (including resorptions and dead foetuses)/litter, incidence of adversely affected implants (non-live plus externally malformed), of the number at live foetuses/litter, or of sex ratio (% male foetuses)/litter. External examination of all live foetuses indicated no external malformations or variations detected in this study.
One Generation Mechanistic Study, non-guideline study **Supporting study** Reliability: 2, reliable with restriction None GLP-compliant Rats, CD® Sprague-Dawley, N=20/dose group Exposure: Group 1: Negative control, Group 2: Prebreed and mating Group 3: Gestation and lactation Group 4: Prebreed, mating, gestation, and lactation Group 5: Prebreed, mating, gestation, and lactation, plus 600 mg iron gluconate in drinking water (prebreed through lactation) Corresponding to 180-185 mg/kg bw/day of BENPAT during prebreed and mating, 157-167 mg/kg bw/day (gestation), and 347-436 mg/kg bw/day (lactation) Analysis of maternal blood (GD 21 and PND 21), F1 offspring blood	Dietary dose of **BENPAT,** 1,4-Benzene-diamine, N,N'-mixed Ph and tolyl derivs., test substance reference 1 (in corn oil) at 2500 ppm Purity: No data	**F0 generation - effects on sexual function and fertility** **Group 2 (prebreed & mating)** No significant effects One ♀ delivering on GD 21 (no longer exposed) **Group 3 (gestation & lactation)** ↑ gestational length (23.6 d vs control: 22.2 d), ↓ gestational index (no. females with live litters/no. females pregnant; 64.7) ↑ dams delivering litters with all-dead pups, followed by dams euthanasia (5 dams), ↓ live birth index (54.6 vs control: 96.9), ↑ stillbirth index (45.4 vs control: 3.1), ↓ total & average pups/ litter (PND 0; 9.9 vs control: 13.0 and 7.9 vs control: 12.6, respectively) **Group 4 (pre-breeding, mating, gestation, & lactation)** ↑ gestational length (23.8 d vs control: 22.2 d, ↓ gestational index (no. females with live litters/no. females pregnant; 71.4) ↑ dams delivering litters with all-dead pups, followed by dams euthanasia (5 dams), ↓ live birth index (54.0 vs control: 96.9), ↑ stillbirth index (46.0 vs control: 3.1), ↓ average pups/ litter (PND 0; 8.8 vs control: 12.6) **Group 5 (prebreed, mating, gestation, & lactation plus iron)** ↑ gestational length (23.5 d vs control: 22.2 d), ↓ No. implantation sites/ litter (10.64 vs control: 14.5), ↓ total & average pups/ litter (PND 0; 9.5 vs control: 13.0 and 8.2 vs control: 12.6, respectively) **F0 generation - general toxicity** **Group 2 (prebreed & mating)** ↓ ♂ BW gain for study day 0-7 (43.8 g vs. control: 53.7 g); ↓ ♂ feed consumption (g/kg/day) for study day 21-28 (exposed; 5.1 %); ↓ ♀ BW at study days 7 (-6.2 %), 14 (-7.7 %), 21 (-9.0 %), and 28 (-8.1 %) ↓ ♀ BW gain for study days 0-7 (16.0 g vs. control: 27.8 g), 7-14 (11.7 g vs. control: 16.1 g), 14-21 (11.2 g vs. control: 15.9 g), and 0-28 (42.4 g vs. control: 61.2 g) ↓ ♀ feed consumption (g/kg/day) from study day 0-7 (exposed; -12.8 %); ↑ ♀ feed consumption (g/kg/day) from gestation day 0-7 (no longer exposed; 12.1 %) **Group 3 (gestation & lactation)** ↑ ♂ BW gain for study day 0-28 (not exposed; 179.8 g vs. control: 158.1 g); ↓ ♀ BW at gestation day 7 (-8.0 %) and 21 (-12.3 %); ↓ ♀ BW gain from gestation day 0-21 (97.0 g vs control: 140 g);

Method, guideline, deviations if any, species, strain, sex, no/group, reference	Test substance, dose levels	Results
(PND 21) for cellular components and methemoglobinemia F0 females and pups (one pup/sex/litter, for a maximum of ten/sex/group) were necropsied at PND 21. Maternal spleens, livers, kidneys were weighed and retained in fixative; and spleen, liver, kidneys, and heart of pups were weighed and retained fixative. Kidneys of control group & group 5 and offspring kidneys from all groups were examined histopathologically **(RTI, 2000)**		↓ ♀ BW at PND 0 (-12.7 %), 4 (-13.2) and 7 (-12.6 %); ↑ BW gain PND 0-21 (32.6 g vs control: 12.2 g) ↓ ♀ feed consumption (g/kg/day) for gestation day 0-7 (-5 %), and PND 7-14 (-17.5 %) One ♀ found dead (GD 19), 16 retained dead foetuses in utero, blood clots in amniotic sacs, no other findings Five dams "euthanized, entire litter dead" on PND 0/3, prolonged gestation: 24-25d; Findings in uterus: dead foetuses present; vagina: dead foetus present; liver: pale/ white foci present on ventral surface of distal portion of median lobe; kidney: pale, Tail: tip necrotic *Scheduled necropsy:* Pale spleen with white foci on surface in one ♀, kidneys not histopathologically examined **Group 4 (prebreed, mating, gestation, & lactation)** ↓ ♂ BW at study day 7 (-4.7 %), 14 (-5.4 %), and 21 (-5.7 %); ↓ ♂ BW gain for study days 0-28 (137.3 g vs. control: 158.1 g) ↓ ♂ feed consumption (g/kg/day) for study day 21-28 (exposed; 5.6 %) ↓ ♀ BW at study days 7 (-5.5), 14 (-7.3 %), 21 (-9.8 %), and 28 (-8.6 %); ↓ ♀ BW gain for study days 0-7 (16.3 g vs. control: 27.8 g), 7-14 (11.2 g vs. control: 16.1 g), 14-21 (8.3 g vs. control: 15.9 g), and 0-28 (40.1 g vs. control: 61.2 g); ↓ ♀ BW at PND 0 (-12.8 %), 4 (-11.7) and 7 (-11.3 %); ↑ BW gain PND 0-21 (39.3 g vs control: 12.2 g) Five dams "euthanized, entire litter dead" on PND 0/3, prolonged gestation: 24-25 d; Findings in liver: pale; kidney: pale, bilateral, not histopathologically examined; uterus: dead foetuses present *Scheduled necropsy:* ↑ absolute and relative liver weight in ♀, ↑ relative paired kidney weight in ♀; kidneys not histopathologically examined **Group 5 (prebreed, mating, gestation, & lactation plus iron)** ↓ ♂ BW at study day 7 (-4.5 %), 14 (-5.1 %), and 21 (-5.1 %); ↓ ♂ BW gain for study day 0-7 (42.3 g vs. control: 53.7 g) ↓ ♂ feed consumption (g/kg/day) for study day 21-28 (exposed; 4.9 %) ↓ ♀ BW at study days 7 (-5.3 %), 14 (-5.7 %), 21 (-7.2 %), and 28 (-7.2 %), gestation day 21 (-9.4 %); ↓ ♀ BW gain for study days 0-7 (16.2 g vs. control: 27.8 g), 14-21 (11.3 g vs. control: 15.9 g), and 0-28 (43.0 g vs. control: 61.2 g); gestation day 0-21 (112.1 g vs control: 140.0 g); ↓ ♀ BW at PND 0 (-8.8 %), 4 (-8.8) and 7 (-8.8 %); ↑ BW gain PND 0-21 (32.5 g vs control: 12.2 g) One ♀ unscheduled sacrifice post-mating; with foetal/placental remains; kidney: foci; adrenals: congestion; lungs: congestion; spleen: enlarged; thymus: reduced *Scheduled necropsy:* ↑ absolute and relative liver weight in ♀, ↑ relative paired kidney weight in ♀; ↑ polycystic kidneys (15 %) in ♀ **F1 generation - offspring toxicity (PND 0)** **Group 2 (prebreed & mating)** No significant effects

Method, guideline, deviations if any, species, strain, sex, no/group, reference	Test substance, dose levels	Results
		Group 3 (gestation & lactation) ↑ post-implantation (prenatal) loss (57.5 % vs control 12.2%), ↓ live litter size (10 vs control: 15); **Group 4 (prebreed, mating, gestation, & lactation)** ↑ post-implantation (prenatal) loss (55.8 % vs control 12.2%), ↓ live litter size (9 vs control: 15); **Group 5 (prebreed, mating, gestation, & lactation plus iron)** ↑ Average pup BW/litter (PND 0; 14.1 %) **F1 generation – general toxicity weanlings (PND 21)** **Group 2 (prebreed & mating)** No indications of polycystic kidneys **Group 3 (gestation & lactation** ↑ polycystic kidney in ♂ (97 %) & ♀ (96 %), ↑ dilation of collecting tubules in renal papilla (♂: 31/38 vs control: 0/80; ♀: 20/25 vs control: 0/67); ↑ haemoglobin concentration in ♂ (15.2 %); ↑ mean corpuscular haemoglobin concentration in ♂ (1.9 %) & ♀ (2.4 %); ↑ platelets in ♂ (29.5 %) ↑ relative liver weights in ♂ (22.6 %) & ♀ (18.4 %) **Group 4 (prebreed, mating, gestation, & lactation)** ↑ polycystic kidney in ♂ (95 %) & ♀ (91 %), ↑ dilation of collecting tubules in renal papilla (♂: 28/34 vs control: 0/80 ♀: 30/32 vs control: 0/67); ↑ mean corpuscular haemoglobin concentration in ♂(2.1 %) & ♀ (1.8 %); ↑ platelets in ♂ (53.1 %) & ♀ (29.8 %) ↑ relative liver weights in ♂ (26.3 %) & ♀ (19,6 %); ↑ relative heart weights in ♂ (26.1 %) **Group 5 (prebreed, mating, gestation, & lactation plus iron)** ↑ polycystic kidney in ♂ (91 %) & ♀ (100 %), ↑ dilation of collecting tubules in renal papilla (♂: 36/40 vs control: 0/80; ♀: 33/48 vs control: 0/67); ↑ haemoglobin concentration in ♂(15.2 %); ↑ mean corpuscular haemoglobin in ♂ (12.5 %) & ♀ (13.7 %); ↑ mean corpuscular haemoglobin concentration in ♂(2.7 %) & ♀ (2.8 %) ↑ absolute and relative liver weights in ♂ (23.6 % absolute, 25.6 % relative) & ♀ (25.9 % absolute, 25.1 % relative)

*p<0.05
**p<0.0
BW – body weight; BWG – body weight gain; GD – gestation day; PND – postnatal day; SD – study day

Reproductive toxicity of BENPAT was investigated in a GLP-compliant two-generation reproductive toxicity study, performed according to OECD TG 416 (RTI, 2001a). Rats received dietary doses of BENPAT at 0, 120, 400, and 1500 ppm (corresponding to approx. 0, 7.5, 25, and 100 mg/kg bw/d of BENPAT), based on a range-finding study. The DS considers this study as a key study for adverse effects on sexual function and fertility, and on development.

The developmental toxicity of BENPAT was investigated in a GLP-compliant study in line with OECD TG 414 (RTI, 1995). Doses were selected based on the outcome of a range finding study. For the main study, pregnant rats received daily doses of 0, 20, 70, and 200 mg/kg bw/d BENPAT by oral gavage on gestation days (GD) 6-15 (there was no treatment after GD 15 until scheduled caesarean section on GD 20). The DS considers this study as a key study for adverse effects on development.

A one-generation mechanistic, non-guideline, study was conducted to determine the necessary and sufficient exposure of BENPAT to maternal females to produce dystocia, prolonged gestation, and offspring polycystic kidneys, effects previously described in the two-generation reproductive toxicity study (RTI, 2001a). Furthermore, the goal was to determine whether test substance administration results in macrocytic anaemia in maternal animals, and if F0 maternal females exhibit polycystic kidneys after exposure to BENPAT for up to 12 weeks. During this study, also a possible effect of iron supplementation on the above effects was investigated. Rats received a dietary dose of 2500 ppm BENPAT in corn oil, corresponding to approx. 250 mg/kg bw/d. Exposure groups were defined as follows: (1) no exposure (negative control), (2) exposure during pre-breeding (four weeks) and mating (up to two weeks), (3) exposure during gestation (three weeks) and lactation (three weeks), (4) exposure during pre-breeding, mating, gestation, and lactation, and (5) exposure during pre-breeding, mating, gestation, and lactation plus supplementation of 600 ppm iron gluconate in the drinking water (for more details see Annex I). This study provides supportive evidence for adverse effects of BENPAT on sexual function and fertility, and on development.

10.10.1 Adverse effects on sexual function and fertility

(a) Effects of BENPAT on sexual function and fertility

Two-Generation Reproductive Toxicity Study (OECD TG 416)

During the two-generation reproductive toxicity study, effects on gestation and parturition were observed at all dose levels of BENPAT. Gestational length was significantly prolonged at 25 and 100 mg/kg bw/d for F0 dams with F1 litters and at 7.5, 25, and 100 mg/kg bw/d for F1 dams with F2 litters. Both, mid and high dose F0 and F1 females exhibited dystocia (obstructed labour) and animals showed an increased incidence of pallor, piloerection, and vaginal bleeding (mainly F0 dams) during late gestation (days 23-24). Increased delivery length was accompanied by perinatal mortality of F1 and F2 offspring, including litters with all-dead pups. Furthermore, percentages of post-implantation losses per litter were significantly increased for F1 litters, at 25 and 100 mg/kg bw/d, and F2 litters at 100 mg/kg bw/d, and clearly, but not significantly increased for F2 litters at 25 and 7.5 mg/kg bw/d. At the highest dose tested, there was a significant increase in the numbers of dead pups per litter and reduction in live birth index for F0 and F1 dams. The numbers of total pups and live pups per litter were significantly reduced at 25 and 100 mg/kg bw/d for F1 litters and at 100 mg/kg bw/d for F2 litters (statistically significantly reduced for live pups, but not significant for total pups). Additionally, prolonged gestation was associated with increased pup body weights. The effects of BENPAT on reproductive parameters are given in Table 11.

Findings of F1 pups that died during lactation indicate that most deaths occurred mainly on postnatal day (PND) 0, with some deaths on PND 1 to 4. Observations revealed many dead pups with patent (open) ductus arteriosus and no air in lungs, indicating primary atelectasis (defective expansion of the pulmonary alveoli at birth), and no milk in stomach. Many pups however died with closed ductus and air in lungs. At 100 mg/kg bw/d, there was an increased incidence of distended ureter/hydroureter and hydronephrosis, compared to the incidence at low doses. However, due to high number of perinatal deaths at 100 mg/kg bw/d relative to other dose groups, and the low number of pups able to evaluate in all dose groups and controls, data should be considered with care. Clinical observation of F2 pups during lactation revealed treatment related incidence of pups found dead, euthanised moribund, and missing and presumed dead at all dose groups of BENPAT, mainly at PND 0. Prominent findings in all dose groups showed hypothermic pups, no milk in stomach. Examinations

of F2 pups that died during lactation show many pups with patent (open) ductus arteriosus and no air in lungs, indicating primary atelectasis (defective expansion of pulmonary alveoli at birth), many pups, however, died with closed ductus and air in lungs. Kidney lesions, including hydronephrosis were observed at 7.5 and 100 mg/kg bw/d and in controls. However, many pups had autolysed abdominal organs and data should be taken with care due to low animal numbers.

Furthermore, exposure of BENPAT significantly increased the incidence of F1 females (but not F0 females) with abnormal stage of oestrous cycle (25 and 100 mg/kg bw/d) at necropsy. The percentage of females in metestrus was statistically significantly increased at all dose groups, due to non-significant reductions in the percentages of females in dioestrus at all dose levels, and of females in oestrus at 25 and 100 mg/kg bw/d. Cycle length in days exhibited a significant upward trend ($p < 0.05$), but no significant pairwise comparisons. The mean cycle length at highest dose was increased (7.15 days), relative to the control group (5.11 days; and 4.84 days at 7.5 mg/kg bw/d and 5.51 days at 25 mg/kg bw/d).

However, dietary doses of BENPAT in F0 and F1 female parental animals did not result in treatment-related effects on mating or pregnancy rates or fertility indices. There were no histopathologic findings in F0 or F1 female reproductive organs, no difference between high dose and control F0 and F1 females in paired ovarian follicle counts. There were no effects of treatment on implantation sites/litter or sex ratio of pups at birth (or during lactation).

For male reproductive toxicity, there were no effects on preputial separation in F1 males, no effects on mating or fertility indices, no histopathologic findings in male reproductive organs, and no effects on seminal parameters (epididymal sperm number, motility, morphology) or on calculated testicular daily sperm production.

Table 11: Effects of BENPAT on reproductive parameters (OECD TG 416; (RTI, 2001a))

	F0 animals for F1 litters				**F1 animals for F2 litters**			
Dose (mg/kg bw/d)	0	7.5	25	100	0	7.5	25	100
Abnormal cycles (%)	17.2	6.7	6.7	13.3	6.7	10	**28.6****	**43.3****
Mean cycle length (d)	4.7	4.5	4.7	4.7	5.1	4.8	5.5	7.2
No. of mating pairs	29	30	30	30	30	30	30	30
No. of ♂ sperm positive	26	30	26	27	26	26	24	26
No. of ♀ pregnant	24	27	24	25	22	23	22	24
No. of ♀ with live litters, PND 0	24	26	23	**15****	22	22	20	21
No. of dams with live & dead pups, PND 0	2	4	3	6	3/22	5/23	4/22	11/24
No. of dams with no live litters, PND0	0	1/27[7]	1/24	10/25[8]	0	1/23	2/22[7]	3/24
Gestational length (d)	22.2	22.4	**22.8****	**23.5****	22.2	**22.8****	**23.1****	**23.2****
No. of implantation sites/litter	16.9	15.9	15.6	14.5	16.5	16.4	15.1	15.0
Post-implantation loss /litter (%)	10.7	14.9	**26.1****	**52.3****	6.8	18.5	20.2	**32.6****
No. of total pups/litter, PND 0	15.7	14.9	**12.3****	**12.1****	15.7	14.5	15.2	13.3
No. of live pups/litter, PND 0	15.6	14.1	**11.9***	**7.6****	15.6	13.7	13.4	**10.8****
No. of dead pups/litter on PND 0	0.1	0.3	0.4	**4.1****	0.1	0.7	0.4	**2.5****
Live birth index (%)	99.2	98.0	97.0	**57.5****	99.2	91.9	97.2	**77.8****
Stillbirth index (%)	0.8	2.0	3.0	**42.5****	0.8	7.6	2.8	**22.2****
Sex ratio (% males)	50.4	54.1	55.2	44.1	44.4	47.1	46.2	48.6
Pup body weights (g) on PND 0	6.4	**6.8****	**6.9****	6.6	6.3	**6.9****	**7.0****	**6.6***

* = $p < 0.05$ versus control group value; ** = $p < 0.01$ versus control group value

[7] Implantation sites only

[8] One female with implantation sites only

Mechanistic study with BENPAT (non-guideline)

During the one-generation mechanistic study with BENPAT a significant increase in gestational length associated with dystocia was found in parental females in groups 3 (exposed during gestation and lactation), 4 (exposed from pre-breeding until lactation), and 5 (exposed from pre-breeding until lactation, plus iron), compared to control group and group 2 (only exposed during pre-breeding and mating). There was no difference between group 4 and 5 in the incidence or severity of dystocia. Furthermore, the percentage of post-implantation loss (prenatal) was significantly increased in groups 3 and 4, while in females that received iron (group 5) the number of implantation sites per litter was significantly reduced. On PND 0, live F1 pups per litter were significantly reduced in groups 3, 4, and 5, and numbers of dead pups per litter were significantly increased in groups 3 and 4. The total number of pups per litter were significantly reduced in groups 3 and 5. Additionally, the live birth index was significantly reduced (and stillbirth index significantly increased) in groups 3 and 4, on PND 0. Survival index for the rest of lactation (PND 4, 7, 14, and 21) was high and equivalent among all groups. Body weights on PND 0 of F1 Pups (groups 2, 3, and 4) that were born alive were comparable to control values, except for pups of group 5 (dams received iron supplementation), which showed a significantly increased body weight in comparison to controls. Effect of BENPAT on reproductive parameters are summarised in Table 12.

During the one-generation mechanistic diet study with BENPAT, there were no significant effects on female mating and fertility indices. F0 male mating, fertility, and pregnancy indices were equivalent among all groups.

Table 12: Effects of BENPAT on reproductive parameters from the one-generation mechanistic study (RTI, 2000)

Dose groups and effects	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**
Pre-breeding and mating, BENPAT (mg/kg bw/d)	0	250	0	250	250[9]
Gestation and lactation, BENPAT (mg/kg bw/d)	0	0	250	250	250[9]
No. of animals started	20	20	20	20	20
Mating index (%)	95.0	95.0	100	95.0	85.0
Fertility index (%)	78.9	73.7	90.0	78.9	70.6
Gestational index (%)	100	92.9	**64.7**[10]	**71.4**[11]	100
Gestational length (d), (No. animals)	22.2 ± 0.1 (13)	22.3 ± 0.1 (13)	**23.6 ± 0.2 (14)**[12]	**23.8 ± 0.2 (13)**[12]	**23.5 ± 0.2 (11)**[12]
No. of live litters, PND 0	15	13	11	10	11
No. of implantation sites/ litter	14.7± 0.8	13.9± 0.6	11.6± 1.2	13.5± 0.8	**10.6 ± 1.4**[13]
Post-implantation loss/litter (%)	12.2 ± 2.8	16.9 ± 6.8	**57.5 ± 10.4**[12]	**55.8± 10.6**[12]	18.0 ± 5.9
No. of live pups/litters, PND 0	12.6 ± 0.6	12.8 ± 0.6	**5.8 ± 1.5**[14]	**6.8± 1.6**[14]	**8.2 ± 1.0**[14]
No. of dead pups, PND 0	0.4 ± 0.2	0.2 ± 0.2	**4.1 ± 1.3**[15]	**5.3± 1.4**[14]	**1.3 ± 0.7**
Live birth index (%)	96.9 ± 1.3	98.9 ± 1.1	**54.6 ± 12.0**[15]	**54.0 ± 12.0**[14]	90.0 ± 5.2
Sex ratio (% males) (PND 0)	57.0 ± 4.3	47.6 ± 3.5	64.9 ± 6.3	44.9 ± 9.4	39.4± 4.6

[9] Iron Supplementation

[10] P<0.01, Fisher's Exact Test

[11] P<0.05, Fisher's Exact Test

[12] P< 0.01; Dunnett's TEST

[13] P< 0.05; Dunnett's TEST

[14] p<0.01; Mann-Whitney U Test

[15] p<0.05; Mann-Whitney U Test

(b) General toxicity maternal animals

Two-Generation Reproductive Toxicity Study with BENPAT (OECD TG 416)

During this study, there was an increased number of (mainly F0) females that either died or were euthanised associated with extended parturition and dystocia (at 25 and mainly at 100 mg/kg bw/d). Maternal mortality during lactation included two mid dose F0 dams: one female delivered three live pups but died in the process of delivering, and the second female delivered six living pups but was found dead on PND 2. In both females, retained dead pups were found *in utero* and in the vagina. Eight high dose F0 dams were euthanised in the process of delivering/ died in the process of delivering/ were euthanised moribund, or were found dead during the holding period until scheduled sacrifice. Maternal mortality was highly associated with the delivery of litters with all-dead pups. There were foetuses retained *in utero* (in three dams) and/or *in vagina* (in two dams), and retained placentae were evident in two females. For F1 maternal animals, mortality was relatively low, including one F1 dam, which died at 100 mg/kg bw/d (while delivering). This female delivered one live pup and five dead pups, while foetuses were retained *in utero*, and one placenta in the vagina. One F1 dam at 25 mg/kg bw/d delivered 10 live and four dead pups. This female was found dead on PND 3 with one foetus retained in the vagina. However, there were F0 and F1 females that delivered dead pups per litter or a litter of only dead pups (one female at 100 mg/kg bw/d) and survived until scheduled sacrifice. Unscheduled necropsy of females that died during the lactation and holding period until scheduled sacrifice revealed possible treatment-related effects on the kidneys (necrosis and inflammation); liver (necrosis, inflammation, and haemorrhage); uterus (haemorrhage, inflammation, and retained foetuses, retained placenta) and vagina (retained foetuses and bleeding**;** lungs (thrombosis, inflammation, and congestion); and adrenal cortex (degeneration and haemorrhage). Deposition of a fibrin-like material in renal glomeruli of the kidney associated with cortical necrosis was present in a few animals. Findings support the premise for the presence of disseminated intravascular coagulation (DIC), which can be activated by endotoxaemia and/or septicaemia (RTI, 2001b).

During the two-generation reproductive toxicity study, at 100 mg/kg bw/d F0 maternal body weight was significantly reduced during the last nine weeks of the ten-week pre-breeding phase (-7 %) and during the three-week gestation period (-10 %). Gestational body weight gain was significantly reduced for high dose females (-18 % compared to controls). Body weight of F0 females was statistically significantly reduced during lactation on PND 0, 4, and 7 at 100 mg/kg bw/d, however, high dose F0 female gained more weight, compared to other dose groups and control group, and lactational weight gain was significantly increased, in comparison to controls. Gestational body weight of F1 females was not significantly different between dose groups and controls, however, high dose F0 dams gained significantly less body weight (-11 %) compared to controls. F0 maternal body weight was significantly reduced during lactation on PND 0, 4, and 7. For F0 and F1 maternal animals of all dose groups, body weight at the end of the lactational period (scheduled sacrifice) was not significantly different from controls (for more information on organ weights and feed consumption, see Annex I).

There was a dose-dependent increase in relative F0 organ weights of the liver (20 %, at 100 mg/kg bw/d) and paired kidneys (9 % at 25 and 12 % at 100 mg/kg bw/d). F1 maternal relative liver and kidney weights were statistically equivalent across all groups. Dose-dependent statistically significant differences in F1 maternal organ weights (compared to controls) were restricted to the brain; absolute female brain weight was significantly reduced at 100 mg/kg bw/d (-6 % vs. controls). Dose-dependent and treatment-related histopathological findings in maternal F0 and F1 animals were limited to the kidneys, indicating a high incidence of polycystic kidneys at scheduled sacrifice. Furthermore, there were statistically significant differences in the weight of reproductive organs (absolute and relative paired ovary, absolute and relative uterine weight) in F0 and F1 maternal animals, however not in a dose-dependent manner (detailed values for F0 and F1 female organ weights are summarised in Annex I).

Mechanistic study with BENPAT (non-guideline)

During the four-week pre-breeding period, body weights of F0 females were significantly reduced in groups 2, 4, and 5 (all exposed), in comparison to controls. Furthermore, F0 female gestational body weights were significantly reduced in group 3 (exposed) on GD 7 and 21, and in group 5 (exposed) on GD 21. Body weight gain during gestation was significantly lower in groups 3 and 5, relative to controls. Examination during

lactation revealed significantly reduced F0 maternal body weights in groups 3, 4, and 5 (all exposed), on PND 0, 4, and 7. However, body weight gain throughout lactation was significantly increased in groups 3, 4 and 5.

Clinical observation of F0 females during gestation included one female each found dead in group 3 (GD 19) and 4 (GD 24). F0 females observed during lactation revealed "dam euthanised, entire litter dead" on PND 0 in groups 3 (two dams) and 4 (three dams), on PND 3 in groups 3 (two dams) and 4 (one dam), and on PND 4 in group 3 (one dam). During gestation and lactation there were findings in groups 2, 3, 4 and 5, including dams with alopecia; pale eyes and tail, pallor; piloerection (including females in process of delivering); and chromodacryorrhea.

F0 maternal absolute and relative liver weights were significantly increased in groups 4 and 5. Furthermore, relative (but not absolute) paired kidney weights were significantly increased in both groups. Examination of F0 females of groups 1 (controls) and 5 (iron supplementation) did not provide gross evidence of polycystic kidneys, but there were findings of polycystic kidneys in group 5 (2/20 (15 %) of F0 females), but no findings in group 1 (control) after microscopy. The haematological profile indicated no demonstrable macrocytic anaemia on GD 21 in F0 dams in any treatment group. However, at PND 21, there was evidence of macrocytic anaemia (increased MCV, measure of red blood cell size; evidence of release of larger, immature erythrocytes into the peripheral circulation) in F0 maternal animals in groups 4 (exposure during mating, gestation, and lactation) and 5 (exposure during mating, gestation, and lactation plus iron), without any differences between group 4 and 5. In conclusion, iron supplementation did not affect PND 21 maternal anaemia or dystocia.

(**c) Relevance for humans**

There were no data available to the DS, including e.g. epidemiological studies or case reports, addressing an effect of BENPAT on sexual function, fertility or development in humans. Valid animal data discussed in the sections above are considered as relevant in humans.

Furthermore, several studies are available from animal models which suggest that BENPAT constituent DPPD (a) causes similar effects as BENPAT (dystocia, prolonged parturition followed by maternal mortality) and (b) acts as a prostaglandin inhibitor (Fujimoto *et al.*, 1984; Marois, 1998). Studies are summarised in Annex I.

Prostaglandins are involved in several physiological processes, including ovulation, luteolysis, pregnancy, birth, inflammation, gastric secretion, and blood flow in humans (Bakker *et al.*, 2017; Bennegard *et al.*, 1991; Hahlin *et al.*, 1988; Wiltbank and Ottobre, 2003). There is a large body of evidence that human birth originates from processes leading to elevated levels of prostaglandins (Bakker *et al.*, 2017; Mitchell *et al.*, 1978; Reece *et al.*, 1996; Romero *et al.*, 1994). They play critical roles in cervical ripening, functional progesterone withdrawal, contraction modulation of the human myometrium, and stimulation of proteins responsible for uterine activation for labour (Astle *et al.*, 2005; Madsen *et al.*, 2004; Parkington *et al.*, 1999; Xu *et al.*, 2015).

Studies with the BENPAT constituent DPPD as described above, give supportive information for a possible mode of action. Furthermore, there are no robust data on the MoA to conclude that the effects of BENPAT are not relevant to humans or to raise doubt about the human relevance. These findings are therefore considered relevant to humans.

10.10.1 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Reliable animal data give strong evidence that BENPAT causes adverse effects on fertility. Studies show that BENPAT causes dystocia (obstructed labour), including a significantly prolonged pregnancy in F0 dams ((RTI, 2000; RTI, 2001a), ≥ 25 mg/kg bw/d), and F1 dams ((RTI, 2001a), ≥ 7.5 mg/kg bw/d), associated with an increased incidence of pallor, piloerection, and vaginal bleeding, in high dose females. The mechanistic study performed with BENPAT (approx. 250 mg/kg bw/d) revealed that treatment during gestation (groups 3, 4, and 5) produces dystocia and prolonged gestation and further indicated that dystocia was not caused by iron deficiency. The increased delivery length was accompanied with a statistically significant increase in the post-implantation loss in F1 (≥ 25 mg/kg bw/d) and F2 litters (100 mg/kg bw/d), pups that retained in vagina, vaginal bleeding, and uterus haemorrhage. Furthermore, there was a BENPAT treatment-related increased offspring mortality, represented by an increased stillbirth index of F1 and F2 pups at 7.5, 25 (not significant),

and 100 mg/kg bw/d (statistically significant). Death of F1 and F2 pups occurred mainly on PND 0, with patent (open) ductus arteriosus and no air in lungs, indicating primary atelectasis (defective expansion of pulmonary alveoli at birth). The available data strongly support that pup mortality occurred perinatal, due to prolonged parturition/ dystocia. However, there were also pups that died with closed ductus and air in lungs.

Dystocia resulted in severe consequences for (mainly F0) maternal animals. There was maternal mortality during the lactation and holding periods (until scheduled sacrifice) for F0 dams at ≥ 25 mg/kg bw/d. However, maternal mortality was relatively low in F1 dams (at 25 and 100 mg/kg bw/d). Dams that died or were euthanised moribund during the process of delivery, lactation, and holding periods revealed treatment-related effects on the uterus and vagina**,** lungs, liver, and kidneys, characterised by retained foetuses and bleeding, vascular congestion or haemorrhage, degeneration or necrosis and inflammatory lesions including vascular thrombosis. Kidney and lung alterations are most likely changes associated with dystocia and maternal infection consequent to resorbing foetuses *in utero*.

Furthermore, BENPAT exposure starting possibly *in utero* until the end of lactation affected oestrous cycling of F1 females (25 and 100 mg/kg bw/d), including a significant increase in the percentage of F1 females in metestrus at necropsy. However, there were no obvious effects on mating or fertility of F1 females. Oestrous cycling of F2 females was not evaluated.

F0 mothers exposed prior to and during mating, gestation, and lactation showed macrocytic anaemia at PND 21. During a repeated dose 28-day oral toxicity study (similar to OECD TG 407) macrocytic anaemia was the primary change in both genders of rats after dietary exposure to BENPAT (120 mg/kg bw/d). Furthermore, macrocytic anaemia was identified in male and female rats during a one-year dietary toxicity study (none-guideline) with BENPAT (120 mg /kg bw/d; repeated dose toxicity studies are summarised in the Annex I). Therefore, maternal anaemia might not be a consequence of dystocia. As shown in the mechanistic study, maternal anaemia (PND 21) is not induced by iron deficiency.

Statistically significant maternal effects were observed at 100 mg/kg bw/d and included decreased body weights during pre-breeding and gestation (less than 10 % reduction compared to controls), and a reduced gestational body weight gain in F0 and F1 animals (18 % and 11 % decrease compared to controls, respectively). Body weight of F0 and F1 dams was reduced during lactation, but only for PND 0, 4, and 7, and in contrast to the other groups, lactational weight gain was increased for high dose F0 animals. Investigations of maternal organ weights revealed a significant increase of F0 liver weight (absolute and relative) at 100 mg/kg bw/d (20 % increase compared to relative control weight). Absolute and relative paired kidney weights were significantly increased in F0 dams at 25 (9 % increase, compared to relative control weight) and 100 mg/kg bw/d (12 %, increase, compared to relative control weight). Polycystic kidneys were observed with a low incidence in F0 maternal animals (only kidneys with gross lesions were histologically examined) and with a much higher incidence in F1 maternal animals.

Any effect of a substance that has the potential to interfere with parturition should be considered an adverse effect on sexual function and fertility. A large body of evidence from animal studies indicates that BENPAT causes adverse effects on fertility, namely dystocia, post-implantation losses, and increased stillbirth (for RAC opinions considering dystocia as an adverse effect on fertility, please see (RAC, 2012; RAC, 2015)). Moreover, the DS concludes that data on body and organ weights do not represent severe maternal toxicity and do not explain the adverse effects on fertility. Therefore, effects on fertility are not considered to be a secondary non-specific consequence of maternal toxicity. Furthermore, there is no robust data on the MoA to conclude that the effects of BENPAT are not relevant for humans or to raise doubt about their human relevance.

10.10.2 Adverse effects on development

From the two-generation reproductive toxicity study (OECD TG 416) and from the one-generation mechanistic study conducted with BENPAT, there are meaningful data on a treatment-related and dose-dependent incidence of polycystic kidneys (for characterisation see Annex I) in the F1 and F2 generation in both sexes. Polycystic kidneys were found in some high-dosed F0 dams as well (for F0 adults, only kidneys with gross lesions were examined histologically in any group). From macroscopic examination, there was no evidence of polycystic kidneys in pups, which died during parturition or lactation. However, microscopic examination of pups kidneys was not conducted. Polycystic kidneys were observed in F1 weanlings (PND 21), F1 adults, and

F2 weanlings at all dose groups (Table 13). Controls had neither macroscopic nor microscopic indications of polycystic kidneys.

Table 13: Incidence of polycystic kidneys in F0 and F1 animals from OECD TG 416 with BENPAT (RTI, 2000)

	Polycystic kidneys in male animals				**Polycystic kidneys female animals**			
[mg/kg bw/d]	0	7.5	25	100	0	7.5	25	100
F0 adults[16]	0 %	0 %	0 %	0 % 0/1	0 %	0 %	0 % 0/2	33 % 3/9
F1 weanlings[17]	0 % 0/23	4 % 1/25	40 % 8/20	91 % 10/11	0 % 0/22	19 % 5/26	39 % 7/18	100 % 11/11
F1 adults[18]	0 % 0/30	17 % 5/30	33 % 10/30	70 % 21/30	0 % 0/30	7 % 2/30	3 % 1/30	60 % 18/30
F2 weanlings[17]	0 % 0/60	5 % 3/64	32 % 6/19	94 % 15/16	0 % 0/60	8 % 5/64	42 % 8/19	100 % 15/15

The one-generation mechanistic study with BENPAT was performed to determine whether F0 parental females exhibit polycystic kidneys after exposure to dietary doses of BENPAT for up to 12 weeks. Therefore, all kidneys from females of group 5 (treated during pre-breeding, mating, gestation, and lactation with iron supplementation) were examined and compared to controls. There were no macroscopic indications of polycystic kidneys. Histopathological examination revealed evidence of polycystic kidneys in F0 dams of group 5 (15 % of dams), but no incidence of polycystic kidney in controls. Other treated groups of F0 females (treatment during pre-breeding, mating; or pre-breeding, mating, gestation, and lactation without iron supplementation) or parental F0 males were not examined. There was no gross evidence of polycystic kidneys in pups that died during lactation, but histopathology was not performed. Investigations of F1 weanlings on PD 21 revealed polycystic kidneys in group 3 (exposure during gestation and lactation), group 4 (pre-breeding, mating, gestation, and lactation), and group 5 (pre-breeding, mating, gestation, and lactation plus iron). Microscopic findings at scheduled sacrifice revealed polycystic kidneys with a high incidence reaching 96 % (24/25) in F1 females and 97 % (37/38) in F1 males in group 3; 91 % (29/32) in F1 females and 95 % (38/40) in F1 males in group 4; and 100 % (48/48) in F1 females and 91 % (31/34) in F1 males in group 5. Results of group 4 and 5 show, that iron supplementation did not affect the incidence of polycystic kidneys. There were no indications of polycystic kidneys in group 2 (exposure during pre-breeding and mating) or controls.

Literature data show that diphenylamine (DPA) induced polycystic kidneys (cystic dilation of the renal tubules, mainly collecting tubules and distal convoluted tubules) in different animal species after subchronic exposure (at least 6 months) (Evan and Gardner, 1976; Gardner *et al.*, 1976; Rohrbach *et al.*, 1993; Thomas *et al.*, 1967). Furthermore, in rats treated for two years cystic changes were accompanied with chronic nephritis (cystic tubule changes at 40.6 and 35.7 mg/kg/d in females and males, respectively, chronic interstitial nephritis at 203.0 and 178.5 mg/kg/d in females and males, respectively (Thomas *et al.*, 1967). Crocker and colleagues found out that diphenylamine derivatives chemically induced polycystic kidneys in newborn rats after treatment of dams from gestation day 14 until term (Crocker *et al.*, 1972). Subsequent investigations identified N,N,N'-triphenyl-p-phenylenediamine, a reaction product of diphenylamine, to induce polycystic kidneys in newborn rats (Clegg *et al.*, 1981). Diphenylamine (CAS: 122-39-4; EC: 204-539-4) and *other low molecular weight diphenylamine derivatives* are listed as impurities of BENPAT (for concentration ranges see Annex I). However, there is no further information available to the DS on the characterisation of *other low molecular weight diphenylamine derivatives*.

During the range-finding study for the prenatal developmental toxicity study, pregnant rats (8/dose group) were administered by gavage once daily, on GD 6 through 15, using doses of 600, 200, 70, and 20 mg/kg bw/d of the test substance. Four out of eight high dose dams died (two dams at GD 12 and two dams at GD 14). Among dams, there was evidence of vaginal bleeding (on GD 12, 13) in two dams. The other two pregnant

16 F0 parental animals, only kidneys with gross lesions were examined histologically in any group

17 kidneys of three F1 and F2 weanlings per sex per litter were examined histologically in any group

18 F1 parental animals, all kidneys were examined histologically in any group

females died or were sacrificed moribund on GD 12 with resorbing conceptuses. Dams exhibited pale organs and extremities, going along with internal bleeding. Furthermore, vaginal bleeding was evident in one dam at each 600 mg/kg bw/d, 70 mg/kg bw/d, and 20 mg/kg bw/d. Maternal body weights and weight changes were significantly reduced at 600 and 200 mg/kg bw/d, with an increased weight gain in the post-exposure period. Maternal food consumption was significantly reduced at 600 and 200 mg/kg bw/d (RTI, 1995). Observation of foetuses revealed significantly reduced body weight at 600 mg/kg/day and at 200 mg/kg/day.

For the main prenatal developmental toxicity study (OECD TG 414 (RTI, 1995)), rats were exposed to BENPAT by oral gavage at doses of 20.0, 70.0 and 200.0 mg/kg from GD 6-15. During the study, no female rat died, aborted, or delivered early. Pregnancy rates were high and equivalent across all groups (92.0 - 96.0 %). There were no statistically significantly differences in the number of pregnant females. All pregnant animals had one or more live foetuses at sacrifice (GD 20), without statistically significant differences in the number of pups/dam among dose groups. At 200 mg/kg bw/day, maternal body weight was significantly reduced on GD 12, and maternal weight gain was significantly reduced for GD 6-9, and 6-15 (dosing period). There were no specific treatment-related clinical signs, although dams at 200 mg/kg bw/d exhibited a significant decrease in weight gain from GD 6 to 15. Maternal feed consumption was significantly reduced at 200 mg/kg bw/d for GD 6-9, 9-12, and 6-15 (treatment period) and was significantly increased for GD 18-20 (in the post-treatment period). There were no treatment-related effects on any gestational parameters, including pre- or post-implantation loss, number of live foetuses per litter, foetal sex ratio (% males per litter) or foetal body weight per litter. Foetal body weight per litter exhibited a significant dose-related downward trend at 200 mg/kg bw/d (for sexes pooled and separately). There were no treatment-related statistically or biologically significant changes in the incidence of individual or pooled external, visceral (including craniofacial), skeletal or total foetal malformations or variations in this study.

There was no evidence of malformation in embryonic kidneys analysed in the prenatal developmental toxicity study with dams exposed from GD 6 to GD 15. However, in rats the metanephros, forming the permanent and functional adult kidneys, start to develop between embryonic day E12 and E13. Therefore, exposure time might be insufficient to investigate adverse effects of BENPAT on embryonic kidney development as well as adverse effect on fertility, identified in the two-generation developmental toxicity study and one-generation mechanistic study, namely dystocia.

Table 14: Effects of BENPAT on developmental toxicity (OECD TG 414; (RTI, 1995))

Dose (mg/kg bw/d)	**0**	**7.5**	**25**	**100**
No. of dams	23	23	23	24
No. of implantation sites per litter	15.9 ± 0.6	16.0 ± 0.5	16.4 ± 0.4	16.5 ± 0.4
% pre-implantation loss	14.1 ± 3.7	8.9 ± 2.7	11.2 ± 2.2	7.8 ± 1.7
% resorptions/ litter	2.7 ± 0.9	2.4 ± 0.9	2.2 ± 0.9	3.4 ± 1.0
No. of litters with resorptions (non-live implants)	7	7	6	10
% litters with resorptions	30.4	30.4	26.1	41.7
% late foetal deaths/ litter	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
% adversely affected implants/ litter	8.8 ± 1.7	9.9 ± 2.2	6.3 ± 1.4	7.3 ± 1.4
% litters with adversely affected implants	69.6	65.2	60.9	66.7
No. of live foetuses/ litter	15.4 ± 0.6	15.6 ± 0.5	16.0 ± 0.4	15.9 ± 0.5

10.10.3 Short summary and overall relevance of the provided information on adverse effects on development

A large body of evidence resulting from reliable animal studies indicates that BENPAT causes, with a high incidence, polycystic kidneys in F1 and F2 offspring. While polycystic kidneys were detected at low rates in F0 maternal animals, there was no evidence of polycystic kidneys in male or female adult rats exposed to BENPAT, from a 28-day oral toxicity study (similar to OECD TG 407; ca. 7.5, 30, and 120 mg/kg bw) or from a one-year dietary toxicity study (none-guideline; ca. 3.3, 20 and 120 mg/kg-day; studies are summarised in the Annex I).

The mechanistic study with BENPAT shows that exposure of F0 dams during gestation and lactation generated polycystic kidneys in the F1 weanlings. Exposure during the pre-breeding/mating periods did not increase the effects produced from gestation/lactation exposures only. Furthermore, polycystic kidneys are not related to iron deficiency. Because there were no groups only exposed during gestation or only during lactation, it is not possible to further define how the timing of exposure affects this endpoint (possible exposure of pups at the end of lactation caused by self-feeding might also be involved). Furthermore, from the two-generation developmental toxicity study (OECD TG 416) and the one-generation mechanistic study performed with BENPAT, comprising treatment of dams during the whole gestation, there is no information on microscopic investigation of newborn kidneys. In the developmental prenatal developmental toxicity study (OECD TG 414) dams were exposed from GD 6 to GD 15. However, the chosen time window limits to investigate adverse effects of BENPAT on embryonic kidney development. Altogether, the above-mentioned studies do not allow concluding that the high incidence of polycystic kidneys in F1 and F2 weanlings are caused through BENPAT-exposure in utero or are a consequence of exposure of pups during lactation and/or self-feeding.

However, there is evidence from literature data that DPA and a DPA derivative cause polycystic kidneys in newborn pups after exposure of dams during late gestation (GD 14 until term). DPA and *other low molecular weight diphenylamine derivatives* (without further specification) are listed as BENPAT impurities. Therefore, it cannot be excluded that BENPAT induces polycystic kidneys in embryos during prenatal development. Most likely, the developing embryonic kidney is more sensitive to BENPAT, compared to the adult kidney, supported by the low incidence of polycystic kidneys in F0 females and the high incidence in F1 and F2 offspring.

Polycystic kidneys are accompanied by structural abnormalities and impaired kidney function and the manifestation of developmental toxicity includes functional deficiency of the kidneys.

In summary, BENPAT causes adverse effects on development of the offspring, namely polycystic kidneys. There is no robust data on the MoA to conclude that the effects of BENPAT are not relevant to humans or to raise doubt about the human relevance.

10.10.4 Comparison with the CLP criteria

There are no human data available. Therefore, classification of BENPAT as a reproductive toxicant in Category 1A is not warranted.

The proposed classification of BENPAT is based on data from animal studies. A chemical is classified as presumed human reproductive toxicant (Category 1B), if *"data provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects"* (Guidance on the Application of CLP Criteria, 2017, 3.7.2.2).

Doubt about the relevance in humans strengthens classification in Category 2. "Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification" (Guidance on the Application of CLP Criteria, 2017, 3.7.2.2).

Toxic effects in the mother may be caused through non-specific mechanisms related to stress and the disruption of maternal homeostasis, or by specific maternally mediated mechanisms. Maternal toxicity shall be considered in the context of offspring development throughout gestation and during the early postnatal stages (Guidance on the Application of CLP Criteria, 2017, Annex I: 3.7.2.4).

A large body of evidence resulting from animal studies indicates that BENPAT causes adverse effects on sexual function. BENPAT prolongs parturition time, induces dystocia, and impairs oestrous cycling. Obstructed labours result in an increased number of pups born dead. Furthermore, an elevated number of maternal rats died/were euthanised during the process of prolonged delivery or during lactation. Dams showed retained dead foetuses *in uterus/in vagina*, vaginal bleeding, and alterations in the lungs, livers, and kidneys. Kidney and lung alterations (vascular congestion or haemorrhage, degeneration or necrosis and inflammatory lesions including vascular thrombosis) are most likely changes associated with dystocia and maternal infection subsequent to resorbing foetuses *in utero*. Altogether, it is warranted to classify BENPAT as a presumed human reproductive toxicant on fertility. There are no data available on the mode of action of BENPAT. However, BENPAT constituent DPPD was found to act as a prostaglandin inhibitor. Nevertheless, data on DPPD give just supportive information as no mechanistic data on BENPAT are available that demonstrate prostaglandin involvement.

Furthermore, BENPAT causes adverse effects on development, namely a high incidence of polycystic kidneys in F1 and F2 offspring. According to the CLP regulation *"any effect which interferes with normal development of the conceptus either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation"*, in the widest sense, should be considered as an adverse effect on development of the offspring. *"However, it is considered that classification under the heading of developmental toxicity is primarily intended to provide a hazard warning for pregnant women, and for men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity essentially means adverse effects induced during pregnancy or as a result of parental exposure"* (CLP regulation, Annex I, section 3.7.1.4). Available data does not show that polycystic kidneys in F1 and F2 weanlings were induced after treatment of dams only during gestation. However, data of DPA and its derivative (induction of polycystic kidneys in newborn rats after treatment of dams from gestation day 14 until term) indicate BENPAT-induced developmental toxicity. Therefore, it is warranted to classify BENPAT as a presumed human reproductive toxicant on development.

There is no information that the mode of action on sexual function and fertility, and development is not relevant in humans or is of doubtful relevance for humans. Therefore, the effects of BENPAT observed in the animal studies are potentially relevant also for humans.

In conclusion, it is warranted to classify BENPAT as a presumed human reproductive toxicant on fertility and development, Category 1B.

ED_{10} values were calculated (using BMDS 2.7.0.4 software) resulting in a value of 23.8 mg/kg bw/d for post-implantation loss of F0 dams with F1 litters and 4.3 mg/kg bw/d for polycystic kidneys in F1 females weanlings (PND 21). No SCL is proposed.

10.10.5 Adverse effects on or via lactation

A one-generation mechanistic study was performed with BENPAT, herein 20 CD (SD) rats per sex and groups received dietary doses of BENPAT (2500 ppm in corn oil, corresponding to approx. 250 mg/kg bw/d). Exposure groups were defined as follows: (1) no exposure (negative control), exposure during (2) pre-breeding (four weeks) and mating (up to two weeks), (3) gestation (three weeks) and lactation (three weeks), (4) pre-breeding, mating, gestation, and lactation, and (5) pre-breeding, mating, gestation, and lactation plus supplementation of 600 ppm of iron gluconate in the drinking water. The study design does not give information if exposure of F0 dams only during gestation or only during lactation is sufficient to induce polycystic kidneys in F1 weanlings. Therefore, it is unclear if polycystic kidneys in the F1 generation are due to the transfer of test chemical in the milk during lactation.

10.10.6 Conclusion on classification and labelling for reproductive toxicity

Based on the available data, classification of BENPAT as a "presumed human reproductive toxicant" in Category 1B, H360FD is warranted. It is recommended to place BENPAT into the medium potency group, with the (lower) ED_{10} value of 4.3 mg/kg bw/d.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Adverse effects on sexual function and fertility

The DS proposed classification for effects on sexual function and fertility in category 1B (H360F) based on a dose-dependent increased incidence of dystocia (obstructed labour) and increased gestational length in mid and high dose F0 female rats, and low, mid and high dose F1 female rats, observed in a GLP compliant two-generation reproduction toxicity study (OECD TG 416; RTI, 2001a). BENPAT induced dystocia and impaired oestrous cycling, and was associated with pallor, piloerection and vaginal bleeding in the high dose groups (mainly F0 dams). In addition, increased pup mortality as result of dystocia was noted. These findings were supported by similar findings in a one-generation mechanistic study (non-guideline study; RTI, 2000), where adverse effects on fertility and development upon exposure to BENPAT during pre-breeding, mating, gestation and lactation, with or without iron supplementation were observed. The DS concluded that these adverse effects on fertility were not secondary to maternal toxicity, as data on body and organ weights did not present severe maternal toxicity. Furthermore, the DS noted that observed effects in liver and kidney in dams are likely associated with dystocia and maternal infection as result of resorbing foetuses *in utero*.

Developmental effects

The DS proposed classification for effects on development in category 1B (H360D) based on a dose-dependent increase and high incidence of polycystic kidneys in rat weanlings (F1 and F2) in a GLP-compliant two-generation reproduction toxicity study (OECD TG 416). Polycystic kidneys were also observed upon treatment with BENPAT during gestation and lactation in F1 rat weanlings in a one-generation mechanistic study (non-guideline study). No polycystic kidneys were noted in rat pups in a prenatal developmental toxicity study (similar to OECD TG 414), possibly due to limitations of the chosen time window (gestation day (GD) 6-15). The DS noted that these latter studies do not allow conclusions on the effects of exposure of BENPAT on increased incidence of polycystic kidneys in weanlings upon exposure to BENPAT *in utero*, lactation and/or self-feeding. However, evidence of these renal abnormalities due to exposure to diphenylamine (DPA) and its derivates in late gestation (GD 14 onwards) in new-born rat, supported these findings (Crocker *et al.*, 1972), as DPA and its derivates are listed impurities of BENPAT. Incidence of polycystic kidneys was (mostly) not noted in dams, nor observed in two repeated dose toxicity studies (a one-year and a 28-day repeated dose toxicity study at comparable dose levels). According to the DS this demonstrated the sensitivity of embryonic kidneys to BENPAT as compared to adult kidneys.

Effects on or via lactation

Data from the one-generation mechanistic study do not allow to draw conclusion regarding increased incidence of polycystic kidneys in weanlings during lactation.

Overall conclusion of the DS

No human studies are available addressing adverse effects of BENPAT on sexual function, fertility and development. However, there is no robust data on the mode of action to conclude the observed adverse effects are not relevant to humans.

The DS proposed classification of BENPAT as presumed human reproductive toxicant in category 1B (H360FD).

The DS derived ED_{10} values of 4.3 mg/kg bw/d for polycystic kidneys in F1 female weanlings (on post-natal day (PND) 21) and 23.8 mg/kg bw/d for post-implantation loss of F0 dams with F1 litters. These values are all within the medium potency group. As a consequence, the DS did not propose a SCL.

Comments received during consultation

Adverse effects on sexual function and fertility

One Member State Competent Authority (MSCA) supported the proposal for classification on sexual function and fertility, and agreed this is warranted because of dystocia observed in the one- and two-generation studies. The MSCA noted that maternal toxicity observed in these studies, as possible cause of dystocia, should be further elaborated to assess the classification in either category 1B or 2. It was suggested that available data on hormonal levels in female rats from repeated dose toxicity studies could help to give insight in the mode of action of toxicity.

One industry representative questioned the relevance and benefits of a harmonised classification for reproductive toxicity since the exposure to the general population is extremely low and exposure control measures are applied for workers. Furthermore, it was acknowledged that dystocia observed in Sprague-Dawley rats is an established adverse effect on fertility leading to maternal and pup mortality. However, there were doubts raised about whether dystocia was observed in absence of (maternal) toxicity and the relevance of these adverse effects to humans. It was pointed out that the NOAEL (16 mg/kg bw/d) reported in a 52-week repeated dose toxicity study in F344 rats (AHF, 1996), based on changes in organ weights (liver, kidney, spleen), was at considerably lower concentrations than adverse effects noted in the two-generation reproduction toxicity study. Furthermore, this could suggest that these adverse effects could thus be strain dependent and it cannot be presumed that observed effects (toxicity or developmental) would exist in other species or strains. Another point raised by the industry representatives was the possibly less significant effect of prostaglandins on the parturition process in humans in comparison to rats. Therefore the relevance of DPPD-induced inhibition of prostaglandin in rats to humans is not been demonstrated. The proposal did not consider known analogous effects with salicylic acid (SA) and acetyl salicylic acid (ASA). A comparison could be considered as it was demonstrated for these chemicals that prostaglandin inhibition could not be presumed to cause equal effects in rats vs. other species.

The DS acknowledged that the process of parturition differ between humans and rats but noted that the prostaglandin PGF2alpha has a relevant role in delivery in humans too. DPPD data provide supportive information, however, BENPAT is a multiconstituent and other constituents might contribute to the effects seen.

Regarding the comparison with SA and ASA, the DS noted that these chemicals present a different target profile in comparison to BENPAT (e.g. malformations (amongst which cranioschisis, and dose-related growth retardation). A different target profile is also observed in repeated dose studies.

With regard to the remarks on dystocia and maternal toxicity, the DS responded with detailed information on the high dose F0 dams with split information on earlier euthanized animals and terminal sacrificed animals. The maternal toxicity was observed not in all dams with dystocia: liver necrosis (centrilobular hepatocyte; minimal to moderate) in 7 high dose dams and kidney necrosis (cortex; minimal to marked) in 5 high dose out of 9 dams, therefore maternal toxicity may be discussed as primary cause resulting in dystocia in some dams. However, due to the fact that some dams with dystocia did not show necrotic lesions in any of these organs and the observation that the majority of necrotic lesions was only minimal to mild, it appears as not likely that these necrotic lesions are the cause of dystocia. Moreover, the necrosis could also be secondary to dystocia and haemorrhagic lesions at multiple sites. Additional information on the F1 dams resulted in the conclusion by the DS that dystocia and post-implantation loss are not secondary to the mild to moderate effects observed in liver and kidney.

Developmental effects

One MSCA noted that offspring generations (F1 and F2) are more likely to develop polycystic kidneys as compared to their parental generation. Furthermore it was commented that these effects could be considered as systemic toxicity rather than developmental toxicity in case structural disturbances of the development of the kidney have not occurred.

One industry representative acknowledged that presence of polycystic kidney was demonstrated in Sprague-Dawley rats upon exposure to BENPAT in the available reproductive toxicity studies. However, doubts were raised whether 1) these kidney effects were a developmental effect, but rather a result of direct exposure and 2) sufficient evidence was provided to expect similar effects would occur in humans. Renal effects were acknowledged but could be due to direct toxic effects and due to the low water solubility of the substance, according the industry representative. It was noted by industry representatives that there was evidence of reversibility (especially renal tubular regeneration) of renal effects, suggesting a toxicity-related effect and not a developmental effect. The tubular regeneration observed indicates that renal effects are likely reversible. As the kidneys develop, the ability to clear toxic effects induced by BENPAT increases. Hence, the incidence of polycystic kidneys decreased in F1 adults and the renal effects are thus not permanent, according to industries.

It was also noted that these kidney effects could be strain-dependent as they were not observed in repeated dose toxicity studies in F344 rats. This statement could not be verified because BENPAT has not been studied in other reproductive studies using other strain or species. The representative therefore concluded it cannot be presumed that observed effects (toxicity or developmental) would exist in other species and humans. Another point raised was regarding literature data for DPA and its derivatives causing polycystic kidneys in new-born

pups as supporting evidence by the DS. The industry representative noted that no harmonised classification for reproductive toxicity is currently in place for DPA and therefore raised doubts regarding the legal basis for using literature data for DPA as supportive evidence in this proposal for BENPAT.

The DS responded that the terminology used for polycystic kidney in the two-generation study was to distinguish from spontaneous renal cysts (cortical or medullary). In addition, formation of cysts is not associated with a primary hydronephrotic mechanism according to study author. The DS concluded that the developing kidneys are more sensitive than the adult kidneys and therefore assessed the effect as foetal toxicity.

Assessment and comparison with the classification criteria

Adverse effects on sexual function and fertility

There are two reliable and relevant studies available for assessing the effects on sexual function and fertility upon exposure to BENPAT; a two-generation reproduction toxicity study and a one-generation mechanistic (supportive) study. The findings are described below per study.

In a two-generation reproduction toxicity study (OECD TG 416; Klimisch score 1; RTI, 2001a) exposure to BENPAT (0, 120, 400, 1500 ppm or 0, 7.5, 25, 100 mg/kg bw/d) via feed (in corn oil) was studied in Sprague-Dawley rats (n = 30/sex/dose group) during pre-breeding (F0-1; 10 weeks), mating (F0-2; 2 weeks), gestation (F0-2; 3 weeks) and lactation (F0-2; 3 weeks). Mortality in pregnant F0 dams was observed in the mid dose (3/24 (12.5 %)) and the high dose group (8[19]/25 (32.0 %)), occurring mostly during lactation (Table 11 CLH dossier). Increased mortality was associated with extended parturition, dystocia and highly associated with the delivery of litters with all-dead pups. No mortality was observed in F0 males. Maternal mortality in F1 animals was relatively low (25/100 mg/kg bw/d: 1/22 (4.5 %) and 1/24 (4.2 %), respectively) and only observed in F1 dams during the postnatal period. Clinical signs such as pallor, piloerection and vaginal bleeding were noted, and likely associated with dystocia. Body weight (Table 11, CLH dossier) and body weight gain were affected in F0 and F1 dams during pre-breeding, mating, gestation and lactation at the highest dose tested, but not at the end of lactation and was (mostly) not excessive. See also a summary of mortality and histopathological findings upon exposure to BENPAT in a two-generation study by RAC in the Supplemental information.

Table: *Effects of BENPAT, general toxicity and reproductive parameters from an OECD TG 416 study*

	F0 animals for F1 litters				**F1 animals for F2 litters**			
Dose (mg/kg bw/d)	**0**	**7.5**	**25**	**100**	**0**	**7.5**	**25**	**100**
Mortality no. dams (%)	1/24[a] (4.2)	0	3/24 (12.5)	8/25 (32.0)	0	0	1/22 (4.5)	1/24 (4.2)
Body weight dams (%) – pre-breeding[b]	-	-	-	-7	-	-	-	-7
Body weight dams (%) – mating[b]	-	-	-	-	-	-	-	-7

[19] Please note an inconsistency: Table 10 in CLH report notes 8 mortalities, DS reaction in RCOM reports 9. According to DS, correct mortalities is 8.

Body weight dams (%) – gestation[b]	-	-	-	**-10***	-	-	-	**-**
Body weight dams (%) – lactation (PND 0, 4, 7) [b]	-	-	-	**-11***	-	-	-	**-9***
General toxicity (scheduled necropsy)								
Organ weights								
Relative paired kidney weight (%) ↑				10.7 ♂ 12.0♀				10.1♂
Absolute liver weight (%) ↑				14.0♀				
Relative liver weight (%) ↑				19.8♀				
Absolute uterine weight (%) ↓			-20.4	-23.0				
Relative uterine weight (%) ↓			-20.7	-19.6				
Relative paired ovary weight (%) ↓							-17.61	
Effects on sexual function and fertility								
Abnormal cycles (%)	17.2	6.7	6.7	13.3	6.7	10	28.6**	43.3**
Mean cycle length (d)	4.7	4.5	4.7	4.7	5.1	4.8	5.5	7.2
No. of mating pairs	29	30	30	30	30	30	30	30
No. of ♂ sperm positive	26	30	26	27	26	26	24	26
No. of ♀ pregnant	24	27	24	25	22	23	22	24
No. of ♀ with live litters, PND 0	24	26	23	15**	22	22	20	21
No. of dams with live & dead pups, PND 0	2	4	3	6	3/22	5/23	4/22	11/24
No. of dams with no live litters, PND0	0	1/27[c]	1/24[c]	10/25[d]	0	1/23	2/22[c]	3/24
Gestational length (d)	22.2	22.4	22.8**	23.5**	22.2	22.8**	23.1**	23.2**
Dystocia[e]			3/24	9/25		1/23	3/22	3/24
No. of implantation sites/litter	16.9	15.9	15.6	14.5	16.5	16.4	15.1	15.0
Post-implantation loss /litter (%)	10.7	14.9	26.1**	52.3**	6.8	18.5	20.2	32.6**
No. of total pups/litter, PND 0	15.7	14.9	12.3**	12.1**	15.7	14.5	15.2[cf]	13.3
No. of live pups/litter, PND 0	15.6	14.1	11.9*	7.6**	15.6	13.7	13.4	10.8**
No. of dead pups/litter on PND 0	0.1	0.3	0.4	4.1**	0.1	0.7	0.4	2.5**
Live birth index (%)	99.2	98.0	97.0	57.5**	99.2	91.9	97.2	77.8**
Stillbirth index (%)	0.8	2.0	3.0	42.5**	0.8	7.6	2.8	22.2**
Sex ratio (% males)	50.4	54.1	55.2	44.1	44.4	47.1	46.2	48.6
Pup body weights (g) on PND 0	6.4	6.8**	6.9**	6.6	6.3	6.9**	7.0**	6.6*

* = p < 0.05 versus control group value; ** = p < 0.01 versus control group value

[a] One female control was euthanized on 17th day of prebreed dosing due to apparent broken hind limb

[b] Based on % compared to body weight in corresponding control group and information in CLH dossier or Annex. Information not available for all dose groups.

[c] Implantation sites only

[d] One female with implantation sites only

[e] From Table 2 and 3 in the RCOM. Dystocia resulted in 4 of the 8 or 9 mortalities reported.

[f] Figure from the CLH report. Seems to be incorrect and does not reflect sum of live and dead pups

Gestational length was statistically significantly increased at ≥ 25 mg/kg bw/d in F0 dams (25/100 mg/kg bw/d: 22.8/23.5 days, vs. 22.2 days in control) and at ≥ 7.5 mg/kg bw/d in F1 dams (7.5/25/100 mg/kg bw/d: 22.8/23.1/23.2 days, vs. 22.2 days in control). Dose-

dependent and statistically significant changes in other reproductive parameters were noted in the high dose group in F0 dams, such as reduced live birth index (57.5 % vs. 99.2 % in control), and increased stillbirth index (42.5 % vs. 0.8 % in control) and number of dead pups per litter (4.1 vs. 0.1 in control). In F1 dams, similar statistically significant changes were reported at 100 mg/kg bw/d for live birth (77.8 % vs. 99.2 % in control) and stillbirth index (22.2 % vs. 0.8 % in control). No statistically significant changes in hormonal cycles were noted in any dose groups in F0 dams. However, there was an increased incidence of abnormal hormonal cycles (females in metestrus and upward trend cycle length) observed in F1 females at ≥ 25 mg/kg bw/d (25/100 mg/kg bw/d: 28.6 % (8/28; 2/30 not cycling) / 43.3 % (13/30), vs. 6.7 % (2/30)) in control. The abnormal hormonal cycling could be a result of exposure to BENPAT *in utero* and during lactation. No other treatment-related effects on mating, pregnancy rates, fertility indices or histopathological findings on reproductive organs were noted in F0 and F1 females in the two-generation reproduction toxicity study. No reproductive toxicity was observed in F0 and F1 males.

Signs of dystocia were noted in 3/24 (12.5 %) in the mid dose group and 9/25 (36 %) in the high dose group in F0 females. In F1 females signs of dystocia were noted in the low, mid and high dose groups (1/23 (4.3 %), 3/22 (13.6 %), 3/24 (12.5 %), respectively). Dystocia was accompanied with pallor, piloerection, vaginal bleeding (mainly F0 dams), haemorrhage and/or inflammation in uterus (mild) during late gestation (GD 23-24). Minimal, mild or moderate liver (centrilobular hepatocyte) necrosis (25/100 mg/kg bw/d: 1/3 and 7/9, respectively) and minimal, mild or marked kidney cortex necrosis (1/3 or 5/9) in F0 dams presenting dystocia were observed. No histopathological changes in liver and kidney were noted in F0 males. Liver lesions were less prominent in F1 dams (1/3 for both 25 and 100 mg/kg bw/d) presenting dystocia, while necrotic lesions in the kidney was not noted.

Some of the F0 dams presenting dystocia had no histopathological changes in the liver (minimal to moderate) or kidneys (minimal to marked; Table 2 in the RCOM): 1/3 (female #84) and 1/9 (female #108) at 25 and 100 mg/kg bw/d, respectively. In F0 dams, most histopathological changes in the liver and kidneys noted were in dams with signs of dystocia. Although histopathological changes in the kidneys (minimal to moderate) were observed in all F1 dams with signs of dystocia (Table 3 in the RCOM), the majority of incidence (polycystic kidney, inflammation acute/chronic, renal tubule regeneration) were noted in dams not presenting dystocia. In addition, histopathological changes in the liver (mild or moderate) were noted only in some F0 dams with signs of dystocia (1/3 at both 25 and 100 mg/kg bw/d). Indicating that necrotic lesions in the liver and/or kidneys could be secondary to dystocia and haemorrhagic lesions. The body weight in F0 dams in the high dose group that died or that were euthanised (mostly due to dystocia) were not different from those sacrificed in this dose group. Not all F0 dams with dystocia showed necrotic lesions in liver and kidney, and these effects were observed to be minimal to mild. On the other hand, incidence of clinical signs (piloerection, vaginal bleeding) was higher in F0 dams that died or that were euthanised in the high dose group. This together suggests that the dystocia is not secondary to the effects in liver or kidney. It cannot be excluded, however, that these lesions in the liver and kidney are the result of dystocia instead.

In a one-generation mechanistic study (non-guideline study; Klimisch score 2; RTI, 2000) Sprague-Dawley female rats (n = 20/group) were exposed to 0 (group 1) or 2500 ppm (157-436 mg/kg bw/d) BENPAT via feeding (in corn oil) during pre-breeding and mating (group 2), gestation and lactation (group 3), pre-breeding up to and including lactation (group 4) or pre-breeding up to and including lactation with iron (600 ppm iron gluconate in drinking water)

supplementation (group 5). Mortality in euthanised dams was noted in group 3 (gestation: 1 (GD 19), lactation: 5 (PND 0, 3 or 4)) and in group 4 (1 (GD 24), 4 (PND 0, 3)). Clinical signs of toxicity (alopecia, pallor, piloerection, chromodacryorrhea, pale eyes and tail) during gestation and lactation were noted in all treated groups. In addition, lower body weight (statistically significant) was noted in all exposed groups, during pre-breeding (group 2, 4 and 5; male/females, -4.5 to -9.8 %), gestation (group 3 and 5; -8.0 to -12.3 %) or lactation (group 3, 4 or 5; females, -8.8 to -12.8 %). Evidence of macrocytic anaemia (on PND 21) and increased (statistically significant) absolute/relative liver weight (not specified) and relative kidney weight (not specified) were observed in group 4 and 5.

Increased gestational length (statistically significant) associated with dystocia was observed in groups 3-5 compared to control and group 2. Other statistically significant effects on reproductive parameters included: decreased number of live pups and increased number of dead pups per litters, were observed in groups 3-5 compared to control. In group 3 and 4, gestational index and live birth index were decreased and post-implantation loss was increased (both statistically significant). In addition, number of implantation sites per litter was decreased and foetal body weight per litter was increased (14.1 % at PND 0) in group 5 (both statistically significant). This study demonstrated BENPAT-induced effects on reproductive parameters during gestation and lactation, but this was not associated to an iron-deficiency.

Table: *Effects of BENPAT on reproductive parameters from the one-generation mechanistic study (copied from the CLH dossier)*

Dose groups and effects	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**
Pre-breeding and mating, BENPAT (mg/kg bw/d)	0	250	0	250	250[a]
Gestation and lactation, BENPAT (mg/kg bw/d)	0	0	250	250	250[a]
No. of animals started	20	20	20	20	20
Mating index (%)	95.0	95.0	100	95.0	85.0
Fertility index (%)	78.9	73.7	90.0	78.9	70.6
Gestational index (%) = no. females with live litters/no. females pregnant	100	92.9	**64.7[b]**	**71.4[c]**	100
Gestational length (d), (No. animals)	22.2 ± 0.1 (13)	22.3 ± 0.1 (13)	**23.6 ± 0.2 (14)[d]**	**23.8 ± 0.2 (13)[d]**	**23.5 ± 0.2 (11)[d]**
No. of live litters, PND 0	15	13	11	10	11
No. of implantation sites/ litter	14.7 ± 0.8	13.9 ± 0.6	11.6 ± 1.2	13.5 ± 0.8	**10.6 ± 1.4[e]**
Post-implantation loss/litter (%)	12.2 ± 2.8	16.9 ± 6.8	**57.5 ± 10.4[d]**	**55.8 ± 10.6[d]**	18.0 ± 5.9
No. of live pups/litter, PND 0	12.6 ± 0.6	12.8 ± 0.6	**5.8 ± 1.5 [f]**	**6.8 ± 1.6[f]**	**8.2 ± 1.0[f]**
No. of dead pups/litter, PND 0	0.4 ± 0.2	0.2 ± 0.2	**4.1 ± 1.3[g]**	**5.3 ± 1.4[f]**	**1.3 ± 0.7**
Live birth index (%)	96.9 ± 1.3	98.9 ± 1.1	**54.6 ± 12.0[g]**	**54.0 ± 12.0 [f]**	90.0 ± 5.2
Sex ratio (% males) (PND 0)	57.0 ± 4.3	47.6 ± 3.5	64.9 ± 6.3	44.9 ± 9.4	39.4 ± 4.6

[a] Iron supplementation
[b] $p < 0.01$, Fisher's Exact Test
[c] $p < 0.05$, Fisher's Exact Test
[d] $p < 0.01$, Dunnett's Test

[e] $p < 0.05$, Dunnett's Test
[f] $p < 0.01$, Mann-Whitney U Test
[g] $p < 0.05$, Mann-Whitney U Test

Exposure to BENPAT was associated with dystocia and increased gestational length in rat one- and two-generation studies. No marked maternal toxicity was observed in both studies. Clinical signs and histopathological changes (liver and kidney) observed were likely the result of dystocia and thus secondary to dystocia. This is supported by absence of marked toxicity in two repeated dose toxicity studies available for BENPAT; a one-year dietary repeated dose toxicity study (none-guideline; ca. 3.3, 20 and 120 mg/kg bw/d) and a 28-day oral repeated dose toxicity study (similar to OECD TG 407; ca. 7.5, 30, and 120 mg/kg bw/d) in F344 rat (AHF, 1994; AHF, 1996). Besides increased relative weights of liver and kidney in the mid and high dose groups, no substance related deaths or clinical findings and no histopathological changes in liver and kidney were reported in these repeated dose toxicity studies.

There are no human data available regarding adverse effects on sexual function or fertility upon exposure to BENPAT and there is no evidence available that adverse effects on (female) fertility observed in animal studies are not relevant to humans.

Potential mode of action

Other animal studies demonstrated similar effects on dystocia and prolonged parturition upon exposure to DPPD (a BENPAT constituent), which acts as prostaglandin inhibitor (Fujimoto *et al.*, 1984; Marois, 1998). In humans, prostaglandins have an important role in various physiological mechanisms, such as pregnancy (Bakker *et al.*, 2017; Mitchell *et al.*, 1978; Reece *et al.*, 1996; Romero *et al.*, 1994). DPPD induced dystocia and prolonged parturition in animals is supportive evidence of a possible mode of action. However, it is noted that BENPAT consists of other constituents and impurities, with unknown toxicity/modes of action. As a consequence the effects observed for BENPAT cannot be solely attributed to DPPD. There is no mechanistic information indicating that the observed effects are not relevant for humans, therefore the adverse effects on sexual function and fertility reported in rats are considered relevant for classification and these effects are considered relevant to humans.

Conclusion

BENPAT exposure resulted in a dose-dependent increase in gestational length in the available two-generation study (F0 and F1) and in the one-generation study, without marked maternal toxicity. Furthermore, in the F1 generation, an increase in abnormal cycles was noted. The studies show that BENPAT results in dystocia (prolonged parturition or obstructed labour), which in most cases resulted in dead dams and pups. The histopathological effects in liver and kidneys in the highest dose group were minimal to mild and not observed in all dams presenting dystocia. Increased gestational length was also observed in the mid dose group and low dose group in F0 and F1 dams, respectively.

RAC agrees with the DS that classification for Repr. 1B is warranted for BENPAT, based on adverse effects observed on female fertility (abnormal cycles, gestational length, dystocia, and pup mortality) which are already observed in the absence of marked toxicity and are considered relevant to humans.

Developmental effects

There are four relevant studies available for assessing the effects on development upon exposure to BENPAT; two key studies and two supportive studies. The findings are described below per study.

In a two-generation reproduction toxicity study (RTI, 2001a), a statistically significant and dose-dependent post-implantation loss in F0 dams was observed (mid and high dose: 26.1/52.3 %, vs. 10.7 % in control). A dose-dependent post-implantation loss was also noted in F1 animals reaching statistical significance at the highest dose (6.8, 18.5, 20.2 and 32.6 % for control, low, mid and high dose, respectively).

Foetal body weight was increased in F1 (7.5/25 mg/kg bw/d: 6/7 %) and in F2 pups (7.5/25/100 mg/kg bw/d: 9/11/5 %), but showing no consistent pattern. Increased pup mortality (F1 and F2) occurred mostly on PND 0 and some on PND 1-4. Pup mortality was strongly associated with prolonged parturition/dystocia.

Patent ductus arteriosus accompanied with no air in lungs and no milk in stomach were observed in many dead F1 and F2 pups in all treated groups. However, many pups died with closed ductus and air in lungs. It should be noted that many pups had autolysed abdominal organs and data should be taken with care due to low animal numbers, so no robust conclusion is possible on basis of the available information.

A dose-dependent increased incidence of polycystic kidneys was noted in F1 and F2 weanlings from the lowest dose (7.5 mg/kg bw/day) onwards. In F1 adults, polycystic kidneys were also increased in all treated groups, although incidences were lower in F1 adults in comparison to F1 weanlings. No polycystic kidneys were noted in the controls group. In the high dose F0 females, only animals with gross lesions were examined histologically, resulting in 3/9 animals showing polycystic kidneys. Polycystic kidneys were characterised by the presence of renal tubule cysts primarily in the outer medulla and occasionally in the inner medulla (papilla) and cortex. No information is available on the severity of the polycystic kidneys in F1 and F2 weanlings or on kidney function (e.g. urine analysis).

Table: *Incidence of polycystic kidneys in animals from OECD TG 416 (copied from the CLH dossier).*

	Polycystic kidneys in male animals				**Polycystic kidneys female animals**			
[mg/kg bw/d]	0	7.5	25	100	0	7.5	25	100
F0 adults [a]	0 %	0 %	0 %	0 % 0/1	0 %	0 %	0 % 0/2	33 % 3/9
F1 weanlings [b]	0 % 0/23	4 % 1/25	40 % 8/20	91 % 10/11	0 % 0/22	19 % 5/26	39 % 7/18	100 % 11/11
F1 adults [c]	0 % 0/30	17 % 5/30	33 % 10/30	70 % 21/30	0 % 0/30	7 % 2/30	3 % 1/30	60 % 18/30
F2 weanlings [b]	0 % 0/60	5 % 3/64	32 % 6/19	94 % 15/16	0 % 0/60	8 % 5/64	42 % 8/19	100 % 15/15

[a] F0 parental animals, only kidneys with gross lesions were examined histologically in any group
[b] Kidneys of three F1 and F2 weanlings per sex per litter were examined histologically in any group
[c] F1 parental animals, all kidneys were examined histologically in any group

In a prenatal developmental toxicity study (OECD TG 414 but with deviating exposure window from the current OECD TG: GD 6-15; GLP; Klimisch score 2; RTI, 1995) Sprague-Dawley rats (n = 25/dose) were exposed to 0, 20, 70, 200 mg/kg bw/d BENPAT (in corn oil) via gavage

on GD 6-15. No statistically significant changes on corrected maternal body weight gain (subtracted gravid uterine weight) were noted at any dose level. No substance-related clinical signs or effects on sexual function and fertility (pregnancy rates 92-96 % in all groups) were observed in dams. A (statistically significant) dose related decrease in foetal body weight per litter was noted at 200 mg/kg bw/d. However, no notable (statistically significant) changes on developmental variations or malformations were observed in any dose groups compared to control. In addition, no evidence of polycystic kidneys in adults or pups was found in this study. However, the short exposure window is a deviation from the current test guidelines but in alignment with the test guideline applicable in 1995. According to the current OECD TG 414, a pregnant animal is exposed to a test substance from implantation to one day prior to the day of sacrifice. As indicated by the DS, the exposure window used is not sufficient to study embryonic development of the kidney.

In a range-finding study (RTI, 1995), conducted prior to the above-described prenatal developmental toxicity study, pregnant Sprague-Dawley rats (n = 8/group) were exposed to 0, 20, 70, 200 and 600 mg/kg bw/d BENPAT (in corn oil) via gavage (once daily) at GD 6-15. In the highest dose group (600 mg/kg bw/day) 4/8 animals died (2 were found dead and 2 animals were sacrificed due to moribund condition). No unscheduled deaths were noted in other dose groups. Vaginal bleeding (1/8 (12.5 %) at 20 and 70 mg/kg bw/d, and 3/8 (33 %) at 600 mg/kg bw/d) were noted, but not at 200 mg/kg bw/d. Pale organs and internal bleedings at 600 mg/kg bw/d were observed. Body weight (-6.5 % to -16.8 %, not corrected for gravid uterine weight) and body weight gain (corrected for gravid uterine weight) were statistically significantly changed at ≥ 200 mg/kg bw/d. Foetal body weight per litter was statistically significantly reduced at ≥ 200 mg/kg bw/d (-6.3 to -12.6), but this decrease was not statistically significant in male pups at 200 mg/kg bw/d. No statistically significant changes on reproductive parameters or external malformations were noted.

In a one-generation mechanistic study (RTI, 2000), polycystic kidneys were observed in dams of group 5 (15 % (3/20) vs. 0 % in control), but examination in other treated groups did not provide gross evidence. In weanlings, haematological changes (increased haemoglobin and mean corpuscular haemoglobin concentration) and increased (relative) organ weights (liver and heart) were noted in groups 3-5. In addition, increased incidence of polycystic kidneys was observed in group 3 (female/male: 24/25 (96 %)/ 37/38 (97 %)), 4 (29/32 (91 %)/ 38/40 (95 %)) and 5 (48/48 (100 %)/ 31/34 (91 %)). In control and group 2 no incidences of polycystic kidneys were found. There was no gross evidence of polycystic kidneys in pups that died during lactation, but histopathology was not performed. Post-implantation loss was statistically significantly increased in group 3 and 4, but not in the iron-supplemented group 5. It is noted that implantation sites/litter was (statistically significantly) decreased only in the BENPAT exposed group with iron supplementation. The study indicates that the effects observed were not (fully) associated with iron deficiency. This study does not elucidate whether the BENPAT induced increase of polycystic kidneys is due to *in utero* exposure only or is a resultant of *in utero* exposure and exposure during lactation and/or diet.

Assessment of developmental effects

In a two generation study a dose-dependent increase of post-implantation loss was observed in F0 and F1 dams. Post-implantation loss was also observed in a one-generation study upon exposure during pre-breeding up to and including lactation (group 3 and 4). The post-implantation loss in the two-generation study was observed from 25 mg/kg bw/day onwards in the F0 and was dose related increased in the F1 reaching statistical significance at the highest dose (6.8 %, 18.5 %, 20.2 %, 32.6 %; for controls, low, mid and high dose,

respectively). No marked non-specific toxicity was seen in the mid and high dose groups. The mortality seen in the mid and high dose F0 females was associated with dystocia. Body weight of the high dose females during gestation was about 10 % lower compared to controls (no exact figures given in the report) and no marked differences in body weight in the mid dose group as compared to the controls were reported. Effects on liver and kidney weight were noted in the high dose group and accompanied by minimal to moderate histopathological changes. All in all no marked non-specific maternal toxicity was observed and effects on post-implantation loss were dose related and statistically significant from the mid dose onwards. Post-implantation loss was also noted in the one generation reproduction mechanistic study in the groups exposed during gestation. For classification for developmental effects, post-implantation loss is considered a relevant adverse effect.

Patent *ductus arteriosus* noted in a two-generation study could indicate an adverse developmental effect on blood vessels. However, the incidence is not clear due to many cannibalised pups or pups with autolysed abdominal organs (unable to evaluate). Closure of the *ductus arteriosus* is mediated through various factors, such as oxygen and prostaglandin concentration (Hundscheid *et al.*, 2019). Patent *ductus arteriosus* together with no milk in stomach and no air in lungs were noted in dead pups, indicating this was likely due to dystocia-mediated pup mortality rather than a development effect.

Polycystic kidneys upon exposure to BENPAT were noted in F1 and F2 weanlings, and to a lesser extent in F0 (high dose group) and F1 adults (low, mid and high dose groups) in the two-generation study. These effects were noted from the lowest dose onwards in weanlings, in absence of marked non-specific maternal toxicity. This is supported by similar findings in weanlings in group 3-5 in the one-generation study. No polycystic kidneys were reported in two repeated dose toxicity studies with BENPAT using similar dose levels (0-120 mg/kg bw/d) in F344 rat (other rat strain) or in the prenatal rat developmental toxicity. However, the exposure window in the prenatal developmental toxicity study (GD 6-15) might not cover the metanephros where the permanent and functional kidney are developed (Moritz and Wintour, 1999), and deviates from the current test guidelines (OECD, 2018).

Polycystic kidneys were thus more pronounced in weanlings as compared to the effects seen in adult rats. Polycystic kidneys in weanlings can be the result of exposure to BENPAT *in utero*, to a higher sensitivity for the formation of polycystic kidneys in weaning or, of exposure to BENPAT via lactation and self-feeding. It is not possible to rule out any of these options. No information is provided on the severity/grade of the observed polycystic kidneys in weanlings. Cysts on the kidney reflect permanent change, but is mainly considered a 'gray zone' variation (Solecki *et al.*, 2003)[20], whether this relates to kidney cysts in general or polycystic kidneys as observed in weanlings after BENPAT exposure is not clear. The fact that polycystic kidneys are also observed in F1 adults does indicate the effect is not (fully) reversible. Overall, it is not clear whether polycystic kidneys are the effect of exposure *in utero,* via lactation or via the diet (or a combination thereof) and the effects seem to be non-reversible.

For DPA and its derivatives (reported impurities of BENPAT), renal cystic disease in new-born rats were reported upon exposure to DPA (1.5-2.5 % in feeding or 20 mg via tube feeding, exposure during last 7 days of gestation) by Crocker *et al*. (1972). RAC agrees with DS that, together with evidence from BENPAT, it appears that developing embryonic kidneys are more sensitive to BENPAT in comparison to adult kidneys to develop polycystic kidneys in rat. It is

[20] "Cyst on kidney" was assessed with an IA (Index of Agreement) (%) of -35.00, classification as Gray zone/V (Variation) and Minimal information needed S (severity grading).

unclear from the available data whether this is a developmental effect due to *in utero* exposure or a post-natal developmental effect due to post-natal exposure.

Mode of action

The DS notes studies with DPPD (one of the BENPAT constituents) that also result in dystocia and longer gestational length. Mechanistic studies with co-exposure to DPPD and prostaglandin F2α show a total cancelling of the effects of DPPD (see Annex I, CLH report). It is suggested that DPPD inhibits the prostaglandin formation. Whether this mode of action is the only cause of the effects exerted by BENPAT is not clear because mechanistic studies on this mode of action with BENPAT are lacking. There are no human data available regarding adverse effects on development upon exposure to BENPAT, its constituents or impurities.

Conclusion

- Pup mortality is strongly associated with dystocia and is therefore not considered solely a developmental effect.
- The post-implantation loss was noted in the reproduction toxicity studies. The effects observed in the two-generation study were dose related and consistent in the F0 and F1 generation and were noted at dose levels without marked non-specific maternal toxicity. Post-implantation loss is relevant for classification for adverse effects on development.
- Effects on blood vessel (patent *ductus arteriosus*) were observed in F1 and F2 pups. The incidence is not clear due to many cannibalised pups or pups with autolysed abdominal organs (unable to evaluate). This effect in dead pups was observed together with absence of air in lungs and no milk in stomach, suggesting pups did not breathe after parturition and thus seems likely to be associated to dystocia.
- A dose-dependent increase in polycystic kidneys was noted in rat F1 and F2 weanlings, and to a lesser extent in F1 adults in a two-generation study. These effects were also noted in a one-generation study. In F0 females polycystic kidneys were observed in a few animals of the high dose only. No polycystic kidneys were reported in the available repeated dose studies (AHF, 1994; AHF, 1996). Although weanlings appear to be more sensitive to BENPAT induced polycystic kidneys, from the information available it is unclear whether these effects are solely due to *in utero* exposure or due to exposure duration lactation and the diet. According to CLP Annex I paragraph 3.7.1.4, classification for developmental toxicity is primarily intended for effects induced during pregnancy and due to parental exposure. Therefore, it is unclear whether the increase in polycystic kidneys in weanlings can be considered a developmental effect warranting classification on its own.
- There is no mechanistic information indicating that adverse effects observed in rats upon exposure to BENPAT are not relevant for humans.

The DS considered the adverse effects on development, as seen by the high incidence of polycystic kidneys in F1 and F2 offspring, as key effects for classification. The DS noted that according to the CLP regulation: *"any effect which interferes with normal development of the conceptus either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation", in the widest sense, should be considered as an adverse effect on development of the offspring. "However, it is considered that classification under the heading of developmental toxicity is primarily intended to provide a hazard warning*

for pregnant women, and for men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity essentially means adverse effects induced during pregnancy or as a result of parental exposure" (CLP regulation, Annex I, section 3.7.1.4).

The available data does not allow to conclude that polycystic kidneys in the F1 and F2 weanlings were induced after treatment of dams exclusively during gestation. However, according to the DS, data on DPA and its derivative (induction of polycystic kidneys in newborn rats after treatment of dams from gestation day 14 until term) are supportive of BENPAT-induced developmental toxicity, and on this basis the DS proposes to classify BENPAT as a presumed human reproductive toxicant, development Repr. 1B.

Although RAC concurs with the DS that BENPAT warrants classification as Repr. 1B for development, the line of reasoning is somewhat different. The consistent and dose-related post-implantation loss is key for classification for development. Polycystic kidneys in weanlings are considered as supportive evidence for this conclusion, because the effects are considered as permanent and serious but it is not clear whether this is due to *in utero* exposure only.

Effects on or via lactation

The available one and two generation studies do not allow a conclusion regarding the effects of BENPAT due to do exposure via lactation.

There are no human data available regarding adverse effects on lactation upon exposure to BENPAT, its constituents or impurities.

RAC concurs with the DS that no classification is warranted for lactation.

Overall conclusion

There is no evidence for reproductive toxicity of BENPAT in humans. Therefore category 1A is not warranted for BENPAT.

Effects on sexual function and fertility (abnormal cycles, gestational length, dystocia, and pup mortality) were observed in animals. In addition, clear evidence for adverse effects on foetal development (post-implantation loss, supported by polycystic kidneys in weanlings) in animals is available. These effects are not considered secondary to marked general toxicity and are considered relevant for humans. Therefore RAC concludes that category 1B is warranted for sexual function and fertility and on development, in agreement with the DS.

For BENPAT ED_{10} values of 4.3 mg/kg bw/d for polycystic kidneys in F1 female weanlings (on PND 21) and 23.8 mg/kg bw/d for post-implantation loss of F0 dams with F1 litters are derived by the DS. RAC agrees that these values are both within the limits of the medium potency group (4 to 400 mg/kg bw/d) for the GCL, and thus a SCL is not justified.

RAC agrees with no classification for effects on or via lactation due to inconclusive data. Together this results in a recommendation for **classification as Repr. 1B; H360FD**.

Supplemental information - In depth analyses by RAC

Summary of mortality and histopathological findings upon exposure to BENPAT in a two-generation study (OECD TG 416).

Table: *Mortality and histopathological findings of BENPAT from an OECD TG 416 study*[a]

	F0 animals for F1 litters								F1 animals for F2 litters			
Dose (mg/kg bw/d)	**0**	**7.5**	**25**		**100**				**0**	**7.5**	**25**	**100**
		sched.	**unsch.**	**sched.**	**unsch.**	**sched.**	**unsch.**	**sched.**				
					Info from RCOM table		**Info from Table 10**					
Number of mortalities (day found dead)			3= 1 GD17), 1 (PND0), 1 (PND2)				8 = 4 (during del, PND0, PND1) 4				1 (PND 3)	1 (PND 0)
Vagina												
Bleeding					5/8	2/16	5/30					
pup stuck in vagina					1/8	1/17						
Inflammation, chronic					-	1/17						
Haemorrhage (mild, marked)					1/8	-						
Uterus												
inflammation, acute (mild/moderate marked); chronic-active (moderate)				1	4/8	-	3					
Haemorrhage (mild/ moderate)				2	2/8	-	2					
Ovary												
Cyst, follicle (minimal, mild); mineralisation; oviduct (moderate); cyst, germinal epithelium (minimal)					2/8	2/17	1					
Liver												
Vacuolisation Cytoplasmic, Hepatocyte, Centrilobular (mild/moderate)					2/8	-						
Hematopoietic Cell Proliferation (minimal/mild/moderate)					4/8	-		4/30				
Inflammation, acute (mild)					1/8	-	1					

Haemorrhage, Centrilobular (mild)					1/8	-	1♀					
Necrosis, Hepatocyte, Centrilobular (5x minimal or mild/2x moderate)				1/11 ♀	7/8	-	7♀	7/30 ♀				
Kidney												
Congestion, medulla (moderate)					1/8	-						
Inflammation, acute (mild); chronic (mild)					2/8	-	1♀			2/30 ♀	4/30 ♀	9/30 ♀
Polycystic kidney (1x minimal/ 1x minimal or 1x moderate)					1/8	2/17		3/30 ♀		5/30 ♂ 2/30 ♀	10/30 ♂ 1/30 ♀	21/30 ♂ 18/30 ♀
Necrosis, Cortex (1x minimal, 2x mild, 2x marked)			1	1/11 ♀	5/8	-	5♀	5/30 ♀				
Tubule dilatation in renal papilla											1/30 ♂ 1/30 ♀	3/30 ♂ 4/30 ♀
Renal tubule regeneration										1/30 ♂	1/30 ♂ 1/30 ♀	16/30 ♂ 14/30 ♀
Lungs												
Congestion (mild)					1/8	-	1♀					
Inflammation, acute, artery (marked)					2/8	-	2♀	2/30 ♀				
Thrombosis, artery (marked)					2/8	-	2♀	2/30 ♀				
Congestion							1♀					
Metaplasia, osseous, minimal					-	-						
Adrenal cortex												
Degeneration (moderate/marked)					3/8	-	2♀	2/30 ♀				
Haemorrhage (mild)					1/8	-	2♀					

[a] Number of unscheduled necropsies is 8, of scheduled necropsies is 17 in F0 at 100 mg/kg bw/d and confirmed by DS. This is different from Table 1 at comment 3 in the RCOM.

10.11 Specific target organ toxicity-single exposure

Hazard class not assessed in this dossier.

10.12 Specific target organ toxicity-repeated exposure

Hazard class not assessed in this dossier.

10.13 Aspiration hazard

Hazard class not assessed in this dossier.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Hazard class not assessed in this dossier.

12 EVALUATION OF ADDITIONAL HAZARDS

Hazard class not assessed in this dossier.

13 ADDITIONAL LABELLING

Hazard class not assessed in this dossier.

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15 ANNEXES

Annex I

Conf. Annex

CLH report

Annex I

International Chemical Identification:

1,4-Benzenediamine, N,N'-mixed Ph and tolyl derivs. ;

Reaction mass of N-phenyl,N'-o-tolyl-phenylene diamine, N,N'-diphenyl-p-phenylene diamine and N,N'-di-o-tolyl-phenylene diamine

EC Number: **273-227-8**

CAS Number: **68953-84-4**

Index Number: **-**

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CONTENTS

1 HUMAN HEALTH HAZARD ASSESSMENT

1.1 Reproductive toxicity studies with BENPAT

1.1.1 Two-Generation Reproductive Toxicity Study (OECD TG 416)

Reproductive Toxicity of BENAPT was investigated in a GLP-compliant two-generation reproductive toxicity study, performed according to OECD TG 416 (RTI, 2001a). Rats (Sprague-Dawley) received dietary doses of BENPAT at 0, 120, 400, and 1500 ppm (corresponding to approx. 0, 7.5, 25, and 100 mg/kg bw/d of BENPAT, vehicle: corn oil). The DS considers this study as a key study for adverse effects on sexual function and fertility, and on development. Test animals were exposed as follows: F0 male & females - continuously for 10 weeks prior to mating, and then during the mating period. F0 females & males continued on diet until deliveries occurred. Female exposures continued through a 21-day lactation period post-delivery. For F1 generation rats, animals were exposed via diet upon their ingestion of maternal diet. Their exposures to both sexes continued through a 10-week period after which housing was arranged for mating of F1 animals. Again, exposures continued for females through the gestation and lactation periods post-deliveries whereas F1 male exposures ended upon F2 deliveries. The F2 generation was not exposed to treated diets except late in the 21-day lactation period (diets, when offered, are available 24 hr/day, 7 days/week).

1.1.1.1 Characterisation of polycystic kidneys

"Polycystic kidneys were characterized by the presence of renal tubule cysts primarily in the outer medulla but occasionally in the inner medulla (papilla) and cortex. In general, cysts were lined by cuboidal to flattened epithelial cells and were surrounded by a variable amount of fibrous tissue and chronic-active inflammation. The majority of the cysts appeared empty; however, some contained either red blood cells, mixed inflammatory cells, desquamated tubular epithelial cells or a mixture of the aforementioned cells…", cited from the Two-Generation Reproductive Toxicity Study with BENPAT, OECD TG 416 (RTI, 2001b).

1.1.1.2 Body and organ weights of parental animals and weanlings, cited from Two-Generation Reproductive Toxicity Study with BENPAT (OECD TG 416)

F0 male body weight "exhibited a statistically significant reduction at 1500 ppm [100 mg/kg bw/d] for all weeks evaluated, through the end of the pre-breed dosing period (day 70) and through the two-week mating period (to day 84) […]. F0 male weekly body weight changes exhibited statistically significant reductions at 1500 ppm [highest dose] […] for the entire pre-breed period (sd 0-70), at 400 ppm [mid dose] and 120 ppm [low dose] for week 3 (sd 14-21), and at 120 ppm [low dose] for week 8 (sd 49-56)." During pre-breeding and mating, "F0 male feed consumption, expressed asg/day, was significantly lower at 1500 ppm [100 mg/kg bw/d] only for week 1 (days 0-7) during the ten-week pre-breed dosing period. When the data were expressed as g/kg/day, the values at 1500 ppm [100 mg/kg bw/d] were significantly increased for week 5 (sd 28-35), 7 (sd 42-49), 9 (sd 56-63) and 10 (sd 63-70)… "

F0 female body weight of high dose animals were significantly lower, "beginning at the end of week 1 (pre-breed sd day 7) and continuing through nine of the ten-week pre-breed exposure period. […] F0 female weekly body weight changes [during pre-breeding] exhibited only a statistically significant reduction, at 1500 ppm [100 mg/kg bw/d] for week 1 (sd 0-7), and only a statistically significant increase, at 400 ppm [25 mg/kg bw/d] for week 10 (sd 63-70)." F0 female feed consumption (g/day) was significantly increased at mid dose for week 10 (sd 63-70) during the ten-week pre-breed dosing period. "When the data were expressed on g/kg/day, maternal feed consumption values were significantly increased at 400 ppm [25 mg/kg bw/d] for week 1 (sd 0-7), 5 (sd 28-35), 6 (sd 35-42), and for the entire pre-breed period (sd 0-70), and at 400 [mid] and 1500 ppm [high dose] for weeks 7 (sd 42-49), 8 (SD 49-56) and 10 (SD 63-70)." F0 female body weight and body weight gain during gestation were statistically significantly reduced at 100 mg/kg bw/d. Maternal gestational feed

consumption (g/day) was significantly reduced at high dose for GD 7-14; "when expressed as g/kg/day, feed consumption was significantly increased at 400 [mid] and 1500 ppm [high dose] for gd 14-20. […]"

Body weight of dams during lactation was statistically significantly reduced at 100 mg/kg bw/d for PND 0, 4, and 7, but lactation weight change (PND 0-21) was significantly increased at the highest dose.

F0 male body weight at scheduled sacrifice was significantly reduced at 100 mg/kg bw/d. In F0 male animals," relative liver weights were significantly increased (109.2 % of control value) at 1500 ppm [100 mg/kg bw/d]. Relative paired kidney weights were significantly increased at 400 ppm [25 mg/kg bw/d] (106.0 % of control value) and at 1500 ppm [100 mg/kg bw/d] (110.7 % of control value)"; there were no apparent treatment-related gross necropsy findings in males. There were no apparent treatment- or dose-related findings in males who died on study or who were sacrificed on schedule. Two kidneys at 120 ppm [low dose] exhibited "pits" and one kidney at 1500 ppm [100 mg/kg bw/d] exhibited a clear cyst on the surface. Similarly, there were no treatment- or dose-related histopathologic findings in F0 male reproductive organs or in organs with gross lesions (including retained kidneys)."[…]

F0 female terminal "body weights were statistically equivalent across groups (with a significant downward trend, $p<0.05$)." […] In F0 females, "relative liver weights were significantly increased at 1500 ppm [100 mg/kg] bw/d (119.8 %)[at scheduled sacrifice]. Relative paired kidney weights were significantly increased at 400 ppm [25 mg/kg bw/d] (108.7 % of control) and 1500 ppm [100 mg/kg bw/d] (112.0 % of control). [Furthermore,] relative paired ovary weight was statistically significantly reduced at 120 ppm [7.5 mg/kg bw/d] (88.0 % of control) and increased, but not statistically significant, at 400 ppm [25 mg/kg bw/d] (130.9 % of control) and at 1500 ppm [100 mg/kg bw/d] (168.0 % of control). Relative uterine weights were significantly reduced at 400 ppm [25 mg/kg bw/d] (79.3 %) and at 1500 ppm [100 mg/kg bw/d] (80.4 %). […] At scheduled sacrifice of surviving females, gross effects (probably treatment-related) were observed on the kidneys at 120 ppm [7.5 mg/kg bw/d] (one animal) and at 1500 ppm [100 mg/kg bw/d] (three females)." […] Histopathologic findings in F0 females were observed at 100 mg/kg bw/d in kidneys ("specifically polycystic and cortical necrosis") and livers ("specifically hematopoietic cell proliferation, hepatocellular centrilobular necrosis").

"**F1 weanling [PND 21] male body weight** was significantly increased (109.2 % of control) at 400 ppm [25 mg/kg bw/d] (and statistically but not significantly reduced at 1500 ppm [100 mg/kg bw/d], 96.4 % of the control value). […] Relative male thymus weight (as percentage of sacrifice weight) was significantly reduced at 1500 ppm [100 mg/kg bw/d] (91.7 % of controls); relative male spleen weight was significantly increased at 120 ppm [7.5 mg/kg bw/d] (113.7 % of control) and 400 ppm [25 mg/kg bw/d] (123.8 % of control) and slightly, but not statistically significantly, increased (113.2 % of control) at 1500 ppm [100 mg/kg bw/d]."

F1 male body weights of high dose animals were significantly reduced during the last three weeks of the pre-breeding period and during mating. F1 male body weight changes were significantly reduced at 100 mg/kg bw/d and 25 mg/kg bw/d "for the entire 70-day pre-breed period. […] Feed consumption [(g/day)] was significantly reduced at 400 ppm [mid dose] and 1500 ppm [high dose] for weeks 7 (sd 42-49) and 10 (sd 63-70). When feed consumption data were expressed as g/kg/day, feed consumption was significantly decreased at 120 ppm [low dose] for week 1 (sd 0-7) and at 400 ppm [mid dose] for week 8 (sd 49-56) during the 12-week period. […]

F1 weanling (PND 21) female body weight "was significantly increased at 400 ppm [25 mg/kg bw/d] (109.1 % of control value)." […] Relative F1 female spleen weight on PND 21 "was significantly increased at 120 ppm [7.5 mg/kg bw/d] (112.7 % of controls), [25 mg/kg bw/d] 400 ppm (113.8 % of controls) and at 1500 ppm [100 mg/kg bw/d] (116.6 %). Relative female brain weight was significantly reduced at 400 ppm [25 mg/kg bw/d] (88.8 % of control) and at 1500 ppm [100 mg/kg bw/d] (89.8 % of control)."

F1 female body weights and weight gains during the ten-week pre-breed dosing period […] were essentially unaffected." […] During gestation, "maternal gestational body weights were statistically equivalent for all time points." However, "maternal gestational weight change (gd 0-20) was significantly reduced at 400 [25 mg/kg bw/d] and 1500 ppm" [100 mg/kg bw/d]. Food consumption of dams, expressed as g/kg/day, was significantly increased at high dose for gd 14-20. During lactation maternal F1 body weight "exhibited significant reductions at 1500 ppm on PND 0, 4, and 7."

At scheduled sacrifice, F1 male body weight was significantly reduced at 100 mg/kg bw/d. […] F1 male relative liver weights and relative paired kidney weights were significantly increased at 100 mg/kg bw/d (108.8 % of control liver weight and 110.1 % of control kidney weight). Gross findings in F1 males "were limited to the kidneys" (hydronephrosis, polycystic kidneys, irregular renal cortex, the latter two parameters being dose-dependent)

At scheduled sacrifice, F1 female body weight "was equivalent across all groups". Furthermore, relative liver weight was increased at 100 mg/kg bw/d (112.2 %), but not statistically significantly different from control. Relative uterine weights of the mid and high dose groups were decreased, but not statistically significantly (80.7 % and 81.2 % of the control value, respectively). Additionally, "relative paired ovary weight was significantly reduced at 400 ppm [25 mg/kg bw/d] (82.4 % of controls); the value at 1500 ppm [100 mg/kg bw/d] was 91.2 % of controls, not statistically significantly different." […] Gross findings in F1 females were observed in the kidneys at 100 mg/kg bw/d. Microscopic treatment-related findings were limited to the kidneys in all dose groups (dose-dependent; tubule dilatation in the renal papilla, chronic inflammation, nephropathy, polycystic kidneys, and renal tubule regeneration).

Body weights of male F2 weanlings (PND 21) "were significantly increased at 120 ppm [7.5 mg/kg bw/d] and 400 ppm" [25 mg/kg bw/d], compared to controls.[…] "Male relative brain weight was significantly reduced at 1500 ppm [7.5 mg/kg bw/d] (93.8 % of controls) and 400 ppm [25 mg/kg bw/d] (87 % of controls)". […] F2 weanling female body weight was significantly increased at 400 ppm [25 mg/kg bw/d.] […] Relative female brain weight was significantly reduced at 400 ppm [25 mg/kg bw/d] (91.4 % of controls). […] All of the gross findings involved the kidneys (except for one male at 1500 ppm [100 mg/kg bw/d] with an enlarged eye and polycystic kidneys)." Examinations revealed "renal lesions presented as one or more clear cysts on the surface or within the kidney, one or more white foci on the surface, and hydronephrosis (dilation of renal pelvis), predominantly at 1500 ppm [100 mg/kg bw/d]. […] Microscopic findings were also limited to the kidneys.; treatment-related lesions included polycystic kidneys" in males and females at 7.5, 25, and 100 mg/kg bw/d (RTI, 2001a).

1.1.2 One-generation mechanistic study - study design

The objective of this study was to provide additional information on the previous findings from a multi-generation reproductive toxicity study and a chronic study of dietary Wingstay 100 (BENPAT) in rats. The study design involved 5 groups: group 1 is a control group and groups 2-5 are dosed with 2500 ppm of Wingstay 100 via the diet. The dose groups were differentiated by the exposure duration and timing. Group 2 was dosed only during pre-breed and mating. Group 3 was dosed only during gestation and lactation. Group 4 was dosed during all phases: pre-breed, mating, gestation and lactation. Group 5 was also dosed during all phases, but additionally received 600 ppm iron supplementation via the drinking water. Duration of the phases were as follows: 4 weeks of pre-breeding; up to 2 weeks of mating phase (depending on when the females became sperm positive); 3 weeks of gestation phase; 3 weeks of lactation phase. Males and females were paired within groups (1:1) for the 2-week mating period. Once a given female was found sperm positive (= date designated GD 0) "her" male was euthanized and discarded. Other than mating periods, animals were housed individually. On [PND] 4, litters were culled to 10, counted, sexed and weighed. On [PND] 7, 14 and 21, pups were counted, sexed and weighed. All F1 pups were euthanized and one/sex/litter necropsied on [PND] 21. [F0] Females were euthanized on [PND] 21 and maternal blood samples were assessed: WBC, RBC, Platelet count, Hgb, Hct, MCV, MCH, MPV, MCHC, RDW, WBC differential and methemoglobin. Male animals were not examined histopathologically. The following maternal organs were weighed and retained in fixative: spleen, liver, kidneys (2). Kidneys of the control group and of Group 5 were examined histopathologically. The following organs from the one pup/sex/litter, for a maximum of ten/sex/group (and 80 total), were weighed and retained in fixative: spleen, liver, kidneys (2) and heart. The retained offspring kidneys from all groups were examined histologically. Dead pups [on PND] 0 and 1 were examined macroscopically for polycystic kidneys. All dead pups during lactation were necropsied, if possible, for cause of death.

1.2 Reproductive/Developmental toxicity studies with BENPAT constituent DPPD

Table 1: Summary table of other studies relevant for toxicity on sexual function and fertility – studies with BENPAT constituent DPPD

<table>
<tr><th>Type of study/data</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr>
<td>Reproductive/ Developmental Toxicity Screening Test, according to OECD TG 421

Reliability: 2, reliable with restriction

(no study report available to the DS)

None GLP-compliant

<u>Deviations:</u>

Dams with offspring killed on day five post-partum, instead of on day 13

(Matsumoto et al., 2013)</td>
<td>Dosed by gastric intubation of N,N'-diphenyl-p-phenylene-diamine (DPPD) at 0 (control: sodium carboxymethyl cellulose), 8, 50, or 300 mg/kg bw/d

CAS: 74-31-7

Purity: 99.9 % (Lot No. KWR0015, purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan))</td>
<td>Rats, [Crl:CD(SD)] SPF, N=13/sex/group

Exposure males: 42 days in total, starting with a 14 days prebreed period;

Exposure females: 42-46 days in total, starting with a 14 days prebreed period, dosing continued throughout mating and gestation period, until four days of lactation</td>
<td><u>F0 generation - effects on sexual function and fertility</u>
<u>300 mg/kg bw/d</u>
↑ Gestation length
<u>50 mg/kg bw/d</u>
↑ Gestation length

Dose-dependent ↓ no. of pups born, delivery index, no. of live pups, birth index, and live birth index on PND 0 (not statistically significant)

<u>F0 generation - general toxicity</u>
No effects on BW, BWC, and feed consumption
<u>300 mg/kg bw/d</u>
Two ♀ died/ sacrificed GD 23 due to dystocia; hemorrhage in uterus lumen, incomplete retention, red colour in lungs, and dark red medulla and hardness on kidneys; for another female nesting and nursing were not observed, and this female was sacrificed on day 1 of lactation due to total litter loss.

One ♀ (50 mg/kg bw/day) sacrificed on day 9 of administration for incorrect operation at time of dosage

<u>F1 generation - general toxicity</u>
<u>300 mg/kg bw/d</u>
↓ live pups and viability index on PND 4 (not statistically significant)

<table>
<tr><th>DPPD [mg/kg bw/d]</th><th>0</th><th>8</th><th>50</th><th>300</th></tr>
<tr><td>Fertility index [%]</td><td>100</td><td>92.3</td><td>100</td><td>100</td></tr>
<tr><td>No. with live litters, PND 0</td><td>13</td><td>12</td><td>12</td><td>11</td></tr>
<tr><td>No. of dams died/sacrificed during gestation and lactation</td><td>0</td><td>0</td><td>0</td><td>3</td></tr>
<tr><td>Gestational length [d]</td><td>22.4 ± 0.5</td><td>22.8 ± 0.5</td><td>23.0 ±0.0[1]</td><td>23.0 ± 0.4</td></tr>
<tr><td>No. implantations</td><td>15.9 ± 1.5</td><td>16.3 ± 2.7</td><td>16.2 ± 1.0</td><td>15.8 ± 1.9</td></tr>
</table>
</td>
</tr>
</table>

[1] Significantly different from the control group, $p < 0.01$, Kruskal–Wallis followed by the Dunnett type test (Matsumoto et al., 2013).

<table>
<tr><th>Type of study/data</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr><td></td><td></td><td></td><td>
<table>
<tr><td>No. of pups born, PND 0</td><td>14.8 ± 2.1</td><td>14.8 ± 3.1</td><td>14.3 ± 1.5</td><td>13.7 ± 3.1</td></tr>
<tr><td>No. live pups/litter, PND 0</td><td>14.7 ± 2.1</td><td>14.4 ± 2.7</td><td>13.8 ± 1.5</td><td>12.8 ± 4.1</td></tr>
<tr><td>Live birth index [%]</td><td>99.5 ± 1.7</td><td>97.7 ± 5.4</td><td>97.2 ± 5.3</td><td>92.0 ± 20.7</td></tr>
<tr><td>Sex ratio [% males]</td><td>44.3 ± 18.3</td><td>39.4 ± 12.1</td><td>47.6 ± 14.1</td><td>48.1 ± 13.2</td></tr>
</table>
</td></tr>
<tr><td>Non-guideline study,

Reproductive studies on DPPD and its impurities as a food additive to the diets of white rats

Reliability: 4, not assignable

(Study report not available to DS, data available from US EPA provisional peer-reviewed toxicity values for DPPD)

GLP-none compliant

(EPA-US, 2009)</td><td><u>DPPD</u>, diets of 300 and 1000 ppm, corresponding to approx. 31 and 103 mg/kg/bw day (EPA-US, 2009)

CAS: 74-31-7

Purity: 99.5 %</td><td>Rats, Wistar; N=approx. 20/ group

F0 ♀ exposed during pregnancy and lactation

F0 ♂ exposed to control diet, except for pairing with females (same diet females were exposed to)

Analysis of minimum, maximum and mid-point of estimated gestation, number of live and dead offspring, number of pups raised to 21 days, and weight of pups on PND 21; No data on maternal food consumption or body weight</td><td>- ↑ no. of dams that hemorrhaged abnormally during delivery at all doses of DPPD
- ↑ no. of anemic dams (among survivors) at all doses of DPPD

Data for F1 effects on sexual function and fertility of F0 generation and for offspring toxicity are summarised below:

<table>
<tr><th>DPPD [mg/kg bw]</th><th>Fertility rate [%]</th><th>Maternal mortality [%]</th><th>Mean live births[2]</th><th>Mean dead births[2]</th><th>Mean gestation time [d][3]</th><th>Litters weaned [%][4]</th><th>Mean weanling weight, PND 21 [g]</th></tr>
<tr><td>0 (Control)</td><td>85</td><td>0</td><td>9.0</td><td>0.4</td><td>20.9</td><td>80</td><td>46.1</td></tr>
<tr><td>31</td><td>75</td><td>5 (1/20)</td><td>4.6</td><td>4.1</td><td>22.6</td><td>38</td><td>47.0</td></tr>
<tr><td>103</td><td>81</td><td>10 (2/20)</td><td>1.9</td><td>5.4</td><td>22.6</td><td>7.7</td><td>36.0</td></tr>
</table>
Table adopted from (EPA-US, 2009)</td></tr>
<tr><td>Non-guideline study,

Antioxidants, prostaglandins and preterm labor</td><td>NN'-Diphenyl-p-phenylene-1-4-diamine (DPPD), in olive</td><td>Adult pregnant Wistar rats were subcutaneously dosed with DPPD at:

200 mg/kg bw/d from GD14</td><td>- 200 mg/kg bw/d DPPD from GD 17: upsets parturition

- 400 mg/kg bw/d DPPD from GD14: prolongs pregnancy duration, often prevents birth of living fetuses; causes female mortality during delivery</td></tr>
</table>

2 "Ashe (1956) reports that these values may not be absolutely accurate due to unquantifiable cannibalism, but they are still useful for comparing across groups" (EPA-US, 2009).

3 Data are uncertain, timing of conception was not accurately determined.

4 "Number litters weaned divided by the number dams pregnant × 100; determined on Postnatal Day 21" (EPA-US, 2009).

<table>
<tr><th>Type of study/data</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr>
<td>Reliability: 2, reliable with restriction
GLP: No information

(Marois, 1998)</td>
<td>oil
Purity: No data</td>
<td>(N=24) and GD17(N=8) until parturition,
300 mg/kg bw/d (N=16) and 400 mg/kg bw/d (N=12) from GD14until parturition,
N=10/control group
♀ treated with 400 mg/kg bw DPPD were subcutaneously injected with 500 µg/kg bw Prostaglandin F2α (PGF2α), four times, every hour from GD20 or 21
Examination: gestation length; non-delivering ♀ were sacrificed at 25d or 26d of pregnancy; analysis of no. & viability of pups</td>
<td>- Increased number of non-delivering females (DPPD-treated) with living fetus in utero at day 25 of pregnancy:
<table>
<tr><th>DPPD [mg/kg bw/d]</th><th>Number of ♀, sacrificed day 25 of pregnancy</th><th>Number of living fetus in utero</th></tr>
<tr><td>200</td><td>4/24</td><td>11 (per ♀)</td></tr>
<tr><td>300</td><td>6/16</td><td>25 (in total)</td></tr>
<tr><td>400</td><td>8/12</td><td>10 (in total)</td></tr>
</table>
- 2 mg/kg bw (in total) of PGF2α injected on GD21 almost totally cancel effects of DPPD
<table>
<tr><td>No. ♀</td><td>10</td><td>6</td><td>4</td><td>12</td><td>6</td><td>6</td></tr>
<tr><td>PGF2α injection, [d] of gestation</td><td>-</td><td>20</td><td>21</td><td>-</td><td>21</td><td>22</td></tr>
<tr><td>DPPD injection, [d] of gestation</td><td>-</td><td>-</td><td>-</td><td>14</td><td>14</td><td>14</td></tr>
<tr><td>Date of parturition, [d] of gestation</td><td>22-23</td><td>21 - 22</td><td>22</td><td>23-25*</td><td>21 -22</td><td>22 - 23</td></tr>
<tr><td>Average number of living fetus/ ♀</td><td>10-15</td><td>3.7</td><td>9</td><td>1.3</td><td>6</td><td>8.3</td></tr>
</table>
PGF2a: 2 mg/kg bw in total
DPPD: 400 mg/kg bw/d
* Eight ♀ sacrificed on day 25</td>
</tr>
<tr>
<td>Non-guideline study,
Inhibition of prostaglandin synthesis in rabbit kidney medulla slices by antioxidants
Reliability: 2, reliable with restriction
GLP: No information

(Fujimoto et al., 1984)</td>
<td>NN'-Diphenyl-p-phenylene-1-4-diamine (DPPD)</td>
<td>Kidney medullary slices were prepared from ♀ rabbits and incubate with antioxidants (DPPD, 1 µM); medium was assayed for prostaglandin E (PGE), using HPLC (PGE extracted with ethyl acetate (approx. pH3) was measured after based-catalysed conversion to prostaglandin B);
Slices were incubated in presence of arachidonic acid, to stimulate prostaglandin synthesis</td>
<td>- Treatment with DPPD (1 µM) resulted in decrease of PGE release from rabbit kidney medulla slices
- Dose-dependent stimulation of PGE production after treatment of slices with arachidonic acid
- Arachidonic acid (50, 100 µM) induced stimulation of PGE formation was not blocked by DPPD
- The authors suggest that DPPD inhibits prostaglandin formation by affecting a phospholipase pathway (not affecting the cyclooxygenase enzyme)</td>
</tr>
</table>

<table>
<tr><th>Type of study/data</th><th>Test substance</th><th>Relevant information about the study (as applicable)</th><th>Observations</th></tr>
<tr><td>Non-guideline study,
Effect of DPPD on the Reproductive Process in the Rat
Reliability: 4, not assignable
Only abstract available (data available from US EPA provisional peer-reviewed toxicity values for DPPD)
GLP-none compliant
(Ames et al., 1956)</td><td>Dietary doses of <u>DPPD</u>, at 0.0125, 0.0625, 0.313, and 1.55 %, corresponding to approx. 11, 55, 275, and 1360 mg/kg bw/ day (EPA-US, 2009)
CAS: 74-31-7
Purity: No data “feed grade”</td><td>Rats (strain: No information), N=10-17 ♀
Exposure: starting 10 days prior to mating, during parturition and lactation
Examinations: Fertility index (no. pregnant ♀/ no. mated ♀), litter efficiency (% pregnant ♀ with ≥1 viable foetus), mortality index (no. dams dying at parturition/no. pregnant ♀), duration of pregnancy, litter size, viability index (no. pups alive PND 3/ no. pups born), and lactation index (no. young weaned/ no. alive PND 3)</td><td>- Signs of difficult parturition occasionally observed (vaginal bleeding, prolapse of the uterus) at all doses of DPPD
- ↑ pup mortality at all doses of DPPD (deaths occurred mainly during parturition)
- Maternal mortality at all doses of DPPD (deaths occurred mainly during parturition)
<table><tr><th>DPPD [mg/kg bw/day]</th><th>Mean duration of gestation [d]</th><th>Mean litter size</th><th>Maternal mortality</th><th>Pup mortality (PND 3)</th></tr><tr><td>0</td><td>23</td><td>10.6</td><td>1/17</td><td>33/104 (32 %)</td></tr><tr><td>11</td><td>24</td><td>7.9</td><td>0/12</td><td>75/79 (95 %)[5]</td></tr><tr><td>55</td><td>25</td><td>4.9</td><td>5/17</td><td>49/49 (100 %)[5]</td></tr><tr><td>275</td><td>25</td><td>5.3</td><td>5/10[5]</td><td>16/16 (100 %)[5]</td></tr><tr><td>1360</td><td>25</td><td>4.7</td><td>7/13[5]</td><td>14/14 (100 %)[5]</td></tr></table>Table adopted from (EPA-US, 2009)</td></tr>
<tr><td>Non-guideline study,
Investigations of DPPD on gestation and parturition in rats
Reliability: 2, reliable with restriction
GLP-none compliant
(Oser and Oser, 1956)</td><td>Dietary doses of N,N'diphenyl-p-phenylenediamine (<u>DPPD)</u> at 0.025, 0.10, 0.40, and 1.60 %, corresponding to approx. 22, 88, 350, and 1400 mg/kg bw/ day (EPA-US, 2009)
Purity: ≥95 %</td><td>Rats (strain: No information), N=10 ♀/dose (each having previously produced and weaned a normal litter)
Exposure F0 generation: Prebreed (two weeks), mating, parturition, and lactation
Examinations for body weight, duration of pregnancy, number and weight of pups cast or found</td><td>- ↓ post-partum survival of dams at all doses of DPPD
- no evidence of organic injury of the pituitary, after histopathological examinations of rats that died in parturition (GD 24 and 25, N=5), compared to normal rats (sacrified GD 22),
- ↑ body weight (>10 %) of pups born dead or found in uterus of ♀ who died at parturition (pups born alive were of normal weight), at all doses of DPPD
- normal proportion of pregnancies at all doses
<table><tr><th>DPPD [mg/kg bw/day]</th><th>Duration of gestation mean ±SEM [d]</th><th>Litters born complete (partial)</th><th>Maternal mortality, parturition</th><th>Pups mortality (PND 0)</th><th>Mean pup weight, born dead or in utero (born alive) [g]</th></tr><tr><td>0</td><td>22.1± 0.23</td><td>10</td><td>0/10</td><td>18/107 (17 %)</td><td>5.5 (5.5)</td></tr><tr><td>22</td><td>22.9± 0.23[6]</td><td>9</td><td>1/10</td><td>42/79 (53 %)[7]</td><td>6.6 (6.2)</td></tr></table></td></tr>
</table>

[5] Statistically significantly different from control ($p < 0.05$) by Fisher exact test conducted for the “Provisional peer-reviewed toxicity values for DPPD” (EPA-US, 2009).

[6] “Statistically significant difference from controls, Student’s t-test, p-value not specified” (EPA-US, 2009)

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations					
	(Good-rite DPPD feed grade, a product of the B. F. Goodrich Co.)	*in utero*, mortality up to the end of a normal gestation and lactation period	88	24.1± 0.30[6]	7	3/10	21/35 (60 %)[7]	6.3 (5.4)
			350	25.2± 0.68[6]	6 (1)	3/10	18/20 (90 %)[7]	6.6 (5.0)
			1400	24.7± 0.54[6]	4 (3)	5/10[7]	20/24 (83 %)[7]	5.9 (5.0)

[7] Statistically significant difference from controls, ($p < 0.05$) by Fisher exact test conducted for the "Provisional peer-reviewed toxicity values for DPPD" (EPA-US, 2009)

Numerous animal studies are available, that analysed the effect of BENPAT constituent N,N'-Diphenyl-p-phenylenediamine (DPPD) on developmental toxicity, especially on gestation and parturition. Because studies show similar effects comparable to those of BENPAT, the DS decided to include studies performed with DPPD as supporting evidence. Furthermore, studies with DPPD hypothesise a possible mode of action of BENPAT.

In a reproductive/ developmental toxicity screening test, according to OECD TG 421, Matsumoto and colleagues (Matsumoto et al., 2013) gastric intubated rats with DPPD at doses of 0, 8, 50, or 300 mg/kg bw (Matsumoto et al., 2013).

Furthermore, numerous none-guideline studies from the 1950s were found during literature, addressing an effect of DPPD on gestation and parturition in rats. Ashe (1965) fed Wistar rats with diets of 300 and 1000 ppm (corresponding to 31 and 103 mg/kg bw/d of pure DPPD (EPA-US, 2009)). Study report was not available to the DS. However, data were adopted from US EPA's "Provisional Peer-Reviewed Toxicology Values for DPPD" (EPA-US, 2009). US EPA reported that some data of this study are uncertain, due to e.g. inaccurate determination of timing of conception. Furthermore, a statistical analysis is missing.

US EPA reviewed another non-guideline study, not available by the DS. In this study female rats of unspecified strain were exposed with dietary doses of DPPD, at concentration of 0.0125, 0.0625, 0.313, and 1.55 % (corresponding to an average intake of approx. 11, 55, 275, and 1360 mg/kg bw/day (Ames et al., 1956; EPA-US, 2009)). Ames and colleagues investigated females during pre-breed, parturition and lactation. Examinations were performed for the fertility index, litter efficiency, and mortality index. Furthermore, duration of pregnancy, litter size, a viability index, and a lactation index were analysed.

Finally, (Oser and Oser, 1956) investigated the effect of DPPD in female rats (unspecified strain) from a breeding colony; each one of them has previously produced and weaned a normal litter. Females were fed diets with DPPD concentrations of 0.025, 0.10, 0.40, and 1.60 % (corresponding to approx. 22, 88, 350, and 1400 mg/kg bw/ day (EPA-US, 2009) during a two week premating period, gestation, parturition, and lactation, while male rats remained unexposed. The authors examined the proportion of pregnancies, duration of parturition, live and dead pups, and complete and partial litters, pup body weights, and maternal (during parturition and postpartum) and pup (PND 4 and 21) survival.

1.2.1 Adverse effects of BENPAT constituent DPPD on sexual function and fertility

In a Reproductive/ Developmental Toxicity Screening Test, conducted according to OECD TG 421, gastric intubation of DPPD at 50 and 300 mg/kg bw/d resulted in a statistical significant prolonged gestation compared to control animals. The number of pups born, delivery index, number of live pups, birth index, and live birth index on day 0 of lactation were dose-dependently decreased, but data were not statistical significantly different from controls. On day four of lactation, the number of live pups and viability were decreased in DPPD-treated animals (300 mg/kg bw/d), compared to controls. There were no changes observed in litter weights and body weights of pups on days 0 and 4 of the lactation. Furthermore, gross examinations revealed no external or internal abnormalities in pups.

Each of the aforementioned none-guideline studies with DPPD from the 1950s identified a prolonged pregnancy in DPPD treated rats compared to controls. Ashe (1956) indicated a possible increase in gestation time in females treated with DPPD at 31 and 103 mg/kg bw/d. However, the author reported approximate data because timing of conception was not accurately determined for all animals (EPA-US, 2009). In the same study, dose-related offspring mortality was shown and pups died primarily at birth. Mean number of live pups at birth and the ratio of the numbers of litters weaned divided by the number of pregnant dams were decreased at doses of DPPD at 31 and 103 mg/kg bw/d. It was reported that the "affected offspring were deeply cyanotic" and the authors concluded that "foetal deaths were due to anoxia resulting from partial or complete placental separation at term with inadequate uterine contraction" (EPA-US, 2009). Ames and colleagues identified prolonged gestations in rats treated with DPPD at doses equal to and higher than 55 mg/kg bw/d. Furthermore, significantly increased pup mortality, compared to control animals, was observed on and before PND 3, and most of the deaths occurred during parturition (DPPD≥11 mg/kg bw/d). Mean litter size from all DPPD treated females was decreased. Investigations of (Oser and Oser, 1956) revealed a significantly prolonged gestation in all DPPD treated females (DPPD ≥22 mg/kg bw/d) accompanied with a delayed parturition, "in some cases

as much as 6 days" (Oser and Oser, 1956). The stillbirth index was significantly increased from dams treated with DPPD at concentrations ≥ 22 mg/kg bw/d. Number of litters born complete were dose-dependently reduced in DPPD treated rats, compared to controls. The mean body weight of pups born dead or in utero from DPPD exposed females was 10 to 20 % increased (no dose-response), whereas the mean body weight of pups born alive was increased (DDPD: 22 mg/kg bw/d), or reduced (350 and 1400 mg/kg bw/d).

During the reproductive/ developmental toxicity screening test, gastric intubation of DPPD (8, 50, or 300 mg/kg bw/d) did not result in effects on the number of mated pairs, number of copulated pairs, copulation index, number of fertile males, or fertility index. There were no effects on the length of estrus cycle, pairing days until copulation, number of corpora lutea, number of implantations, implantation index, and number of pregnant females.

Ashe (1956) reported a possible slight decrease in fertility of females treated with DPPD at approx. 31 and 103 mg/kg/bw day, compared to controls (EPA-US, 2009). However, (Oser and Oser, 1956) did not identify effects on fertility of DPPD treated females (approx. 22, 88, 350, and 1400 mg/kg bw/day), shown by a normal proportion of pregnancies. From the study of Ames and colleagues (Ames et al., 1956) no data were available concerning adverse effects on sexual function and fertility.

1.2.2 General toxicity from studies performed with BENPAT constituent DPPD

Gastric intubation of DPPD at 8, 50, and 300 mg/kg bw/day did not reveal effects on male or female body weights, body weight gains, and food consumption**.** In males, neither death nor clinical toxicity was observed. Among females, two rats were observed with piloerection, hypothermia, and pale skin on day 23 of pregnancy. One of these two females died and the other was sacrificed due to dystocia on day 23 of pregnancy. Another female with piloerection and pale skin delivered only three live pups. This female was sacrificed on day 1 of lactation due to total litter loss. Gross pathological findings of two females that died or were sacrificed on day 23 of pregnancy revealed "hemorrhage in the lumen of the uterus, incomplete retention and red colour in the lung, and dark red medulla and hardness on the kidney in both animals; hydrothorax in the thoracic cavity, attachment of red content in mucosa of the glandular stomach and recessed area, or red spots in the duodenum in either animal. In the histopathological examination, slight hemorrhage in the endometrium, and very slight edema, very slight foam cell accumulation in alveolus, and very slight capillary fibrinous thromboses in the lung were observed in the two females. The histopathological examination revealed no toxicological effects in other males and females" (Matsumoto et al., 2013).

Possible treatment-related effects on maternal mortality in females fed with 31 and 103 mg/kg bw/day pure DPPD were observed by Ashe (1956) and all deaths occurred during parturition. Furthermore, dams of all DPPD-treated groups, but not controls, hemorrhaged abnormally during delivery. The authors reported that surviving dams were severely anemic for many weeks. However, no data were presented to expound these effects. Ashe conducted gross and microscopic examinations of rats that died during labor and of those that failed to become pregnant. However, the authors did not specify the test material (pure DPPD, commercial DPP, or contaminants) (EPA-US, 2009) and therefore data show a high uncertainty and were not considered for assessment. During another study maternal mortality was significantly increased in dams treated with DPPD ≥275 mg/kg bw (Ames et al., 1956). Also in this study, females died mainly during labor and signs of difficult parturition were occasionally observed, e.g. vaginal bleeding or prolapse of the uterus. (Oser and Oser, 1956) also observed maternal mortality, with a significant increase in highest dose (1400 mg/kg bw/day), compared to control animals. The post-partum survival of females was diminished at levels of DPPD ≥350 mg/kg bw/d (not statistically different from controls). The authors histopathological examined the pituitary of DPPD-fed rats that died in parturition (GD 24 and 25, N=5). Investigations did not give any evidence of organic injury of the pituitary between treated animals and controls.

In summary, adverse effects of DPPD on fertility (dystocia) are considered to be due to prostaglandin inhibition.

1.3 Repeated dose toxicity studies with BENPAT – for information only

Table 2: Summary table of animal studies on repeated dose toxicity

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels	Results
Oral 4-week dietary study; similar to OECD TG 407 **Key study** GLP-compliant Rat, Fischer 344 8 animals/sex/dose group sacrificed after 28 days of exposure; for 6 male and female control and high dose animals, a treatment-free period of 2 weeks followed (AHF, 1994)	BENPAT, CAS 68953-84-4; dietary doses of 0, 7.5, 30 and 120 mg/kg bw/d Purity: no data	No compound related deaths occurred **120 mg/kg bw/d** ↓ body weight gain in ♀, started after first week administration; reduction recovered within first treatment-free week ↓ food consumption in ♂ by day 12, which continued until day of sacrifice & ↓ food consumption in ♀ with a plateau effect at the end of 2 week exposure-free period ↑ mean corpuscular volume (MCV) in ♂ & ♀ ↓ mean corpuscular hemoglobin concentration (MCHC) in ♂ & ♀; recovery trend by being of lesser degree of significance at 42 days During the recovery phase, ↑ reticulocyte count in ♂ & ♀ ↑ total bilirubin in ♂ at day 28, levels returned to control values during recovery ↑ cholesterol in ♂ & ♀ at day 28, finding recovered completely in ♂, degree of significance was reduced during recovery in ♀ ↑ total protein and albumin in ♂ during recovery ↑ absolute & relative liver weights in ♂ & ♀ at day 28, finding completely recovered by day 42 ↑ relative kidney weights in ♂ & ♀ at day 28, by day 42 this finding remained significant in males, but not in females. ↑ relative heart and splenic weights in ♀ at 28 days, finding showed a recovering trend by day 42 ↓ mean renal cell nuclei labelling (PCNA) index in ♀, during recovery at 42 days, the labelling index had increased significantly over control animals ↑ mean urothelial cell nuclear (PCNA) labelling index in ♂ & ♀ at 28 days **30 mg/kg bw/d** ↓ food consumption in ♂ by day 26 & in ♀ ↓ mean corpuscular hemoglobin concentration (MCHC) in ♀ ↑ total bilirubin in ♂ at day 28, levels returned to control values during recovery ↑ mean hepatocellular nuclear labelling (PCNA) index ♂ at day 28 ↑ mean urothelial cell nuclear labelling (PCNA) index in ♂ & ♀ at 28 days **7.5 mg/kg bw/d** reduction of food consumption in ♂ by day 26 ↑ mean hepatocellular nuclear labelling (PCNA) index ♂ & ♀ at day 28

		↑ mean urothelial cell nuclear labelling (PCNA) index in ♀ at 28 days There was no pertinent or compound-related gross finding in either gender: Most changes observed during microscopic evaluation were not compound related LOAEL of 7.5 mg/kg bw/d should be derived, based on urothelial cell proliferation (occurring) in different studies
Oral 52 week dietary study, none-guideline study **Supporting study** GLP-compliant Rat, Fischer 344 N=6 animal/sex/dose sacrificed after 38 weeks; 20 animals/ sex/dose (control: 12 animals per sex) sacrificed after 52 weeks; 6 animals/ sex/dose exposure-free for 12 weeks after 52-week administration period (AHF, 1996)	BENPAT, CAS 68953-84-4; dietary doses of 53, 310 and 1900 ppm (corresponding to nominal doses of 0, 3.3, 20 and 120 mg/kg bw/d of the test substance)	No compound-related deaths or clinical findings occurred in this study. **120 mg/kg bw/d** ↓ body weight gain in ♀ (-18 %), including the recovery period (-10 %, not significant) ↓ body weight in ♀ & ♂ during exposure period, body weight of ♀ lower compared to controls during exposure-free period ↓ food consumption in ♂ (weeks 16-30 and at 52 weeks) & ♀ (started with week 12, remained reduced during recovery) ↑ MCV in ♀ & ♂ at week 52, recovery during exposure-free period ↓ MCHC in ♀ & ♂ at week 52, recovery during exposure-free period ↓ red blood cell count (RBC) in ♂ at week 52, recovery during exposure-free period ↓ hemoglobin (HGB) in ♂ & ♀, recovery during exposure-free period ↑ total bilirubin in ♂ at 52-week, returned to control values during exposure-free period; (consistently) ↓ total bilirubin in ♀ at 38 & 52 weeks ↑ urinary bilirubin in ♂ at 38 week ↑ relative liver weight , ↑ relative kidney weight in ♂ & ♀; ↑ relative heart weight in ♀; recovered during exposure-free period ↑ relative spleen weight in ♂ & ♀, still ↑ in ♂ during exposure-free period ↑ urothelial cell nuclei labelling index in ♂ & ♀, still increased during exposure-free period **20 mg/kg bw/d** ↑ urothelial cell nuclei labelling index in ♂ & ♀; still increased during exposure-free period ↑ relative liver weight & ↑ relative heart weight in ♀; recovered during exposure-free period **3.3 mg/kg bw/d** ↑ urothelial cell nuclei labelling index in ♂; still increased during exposure-free period

Three oral repeated dose toxicity studies performed with BENPAT are available. An oral 3-week gavage study was conducted as a range-finding study. Furthermore, a 4-week dietary study and a 52-week chronic dietary study are available. The two latter studies were identified as key studies by the registrants and are summarised below. Repeated dose toxicity studies with other exposure routes (dermal, inhalation) are not available.

1.3.1 Oral 4-week dietary study

The study did not adhere to any guideline. However, many elements were similar to OECD TG 407 and the study was performed in accordance with GLP principles. The DS considers this study as the key study on specific target organ toxicity after repeated exposure of BENPAT. Male and female Fischer 344 rats were exposed via diet to BENPAT doses of 0, 7.5, 30 and 120 mg/kg bw/d. After 28 days of exposure, eight animals/sex/dose group were killed. For six male and female control and high dose animals, a treatment-free period of two weeks followed. At sacrifice, hematology, clinical chemistry, urinalysis, and complete necropsies were performed. The absolute and relative weights of selected tissues (liver, kidneys, pituitary, uterus, heart, brain, spleen, thyroids, adrenals, testes, and ovaries) were recorded; sampled tissues were processed and subjected to macroscopic evaluation. Slides of liver, kidneys, and urinary bladder were subjected to immunohistochemical staining for proliferating cell nuclear antigen (PCNA), but not for standard H&E staining.

Results:

The following effects were observed: increased liver and kidney weights in mid-dose females and in high-dose males and females; increased heart and spleen weight and decreased body weight in high dose females (with reduced food consumption), but no pertinent or compound-related gross findings. Increased cell division for liver and decreased cell division for kidney cells as evidenced by PCNA staining: at 28 days, there was a significant increase of the mean hepatocellular PCNA labelling index in males and females of the low dose ($p<0.01$). High dose females exhibited a decrease in the mean renal cell PCNA labelling index ($p<0.05$).

Reversible hematological signs (including significantly ($p<0.01$) increased mean corpuscular volume, and significantly ($p<0.01$) decreased mean corpuscular hemoglobin concentration in high dose animals of both sexes) and clinical chemistry changes (including significantly increased blood bilirubin at high- ($p<0.01$) mid- ($p<0.05$) dose animals and significantly increased cholesterol levels in high dose animals ($p<0.01$).

At 28 days, mean urothelial PCNA indices of the low and mid dose groups were increased (statistically significant ($p<0.05$) only for low-dose females), but heavily decreased in high dose animals.

1.3.2 Oral 52-week dietary study

The study did not adhere to any guideline; however, it was performed in accordance with GLP principles. Groups of male and female Fischer 344 rats received dietary levels of 0 (control), 53, 310 and 1900 ppm of the test substance (corresponding to nominal doses of 0, 3.3, 20 and 120 mg/kg bw/d of the test substance). Six animal/sex/dose were sacrificed after 38 weeks; 20 animals/sex/dose (control: 12 animals per sex) were sacrificed after 52 weeks. Another six animals/sex/dose were kept exposure-free for 12 weeks after the 52-week administration period. Clinical observations, body weights, and food consumption were monitored. At sacrifices (after 38, 52 and 64 weeks) hematology, clinical chemistry, urinalysis, and complete necropsies were performed and the absolute and relative weights of selected tissues (liver, kidneys, pituitary, heart, spleen, thyroids, adrenals, brain, uterus, ovaries and testes) were recorded. All sampled tissues were processed and subjected to microscopic evaluation. Slides of liver, urinary bladder, and kidneys were also subjected to immunohistochemical staining for proliferating cell nuclear antigen (PCNA).

Results:

During the 52-week dietary study, no compound related deaths or clinical findings occurred. Compared to controls, body weight gains were non-significantly and reversibly decreased in high dose males and significantly and non-reversibly decreased in high dose females. Relative kidney and heart weights were increased at all sacrifices in the high dose animals.

The mean corpuscular volume (MCV) was statistically significantly increased in male and female high-dose animals ($p<0.01$ for both sexes), whereas the mean corpuscular hemoglobin concentration (MCHC) was decreased at 38 and 52 weeks of the high dose groups (statistically significant at 52 weeks in both sexes, $p<0.01$). At week 52, red blood cell counts (RBC) were statistically significantly decreased (-13.2 %) in high-

dose males ($p<0.01$) and hemoglobin values were statistically significantly decreased (-10.7 %) in high dose males ($p<0.01$) and females ($p<0.05$). Hematological parameters returned to control values in the treatment-free period. Liver and splenic weights were also increased at 38 and 52 weeks. Extramedullary erythropoiesis was evident in these organs at the highest dose tested in both male and female animals indicating the presence of a chronic macrocytic anemia. In the high dose males only, total (blood) bilirubin was increased at both intervals, accompanied with increases in urinary bilirubin (at 38 weeks only). There was also an increase in serum cholesterol in high dose groups of both sexes and at both time intervals. Urothelial cell PCNAs were significantly increased in a dose- and time-related manner at all sacrifices (weeks 38, 52 and 64). PCNAs in liver and kidneys were not significantly different from controls. Urinary pH remained constant through all sacrifices and dosages. There were no compound-related findings in any of the organs or tissues examined microscopically by H&E staining, except for the evidence of extramedullary erythropoiesis in the spleen and liver of high doses animals of both sexes at the 52-week sacrifice. No indication on treatment-related carcinogenic activity was found in the study. Main chronic toxic effects were a mild chronic macrocytic anemia and increases in urinary bladder cell proliferation. At the end of the study, no compound-related pre-neoplastic changes or neoplasms were present.

In summary, significant anemic effects (reductions in RBC and hemoglobin) were observed at the high dose level only. Increases in blood bilirubin in high dose males only indicated hemolysis as a possible cause of the loss of erythrocytes. Extramedullary hematopoiesis in the liver and the spleen indicates compensatory erythropoiesis and was consistently observed in high dose males and females.

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