Section A 6.4.1 **Subchronic toxicity (rodent)**

Annex Point II 6.4 **Oral toxicity in rats**

			Official use only
		1 REFERENCE	
1.1	**Reference**	A 6.4.1/01 [redacted] (1991) Report on the study of the oral toxicity of Bis-(N-cyclohexyl-diazeniumdioxy)-copper in rats Administration via the diet for 3 months Report: 30C0679/89041, [redacted]	
1.2	**Data protection**	Yes	
1.2.1	Data owner	BASF	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes OECD Guideline 408 "Subchronic Oral Toxicity - Rodent: 90-day Study"	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material 1**	Cu-HDO	
3.1.1	Lot/Batch number	Reu E 7360	
3.1.2	Specification		
3.1.2.1	Description	Solid, metallic violet	
3.1.2.2	Purity	Purity 99%	
3.1.2.3	Stability	The stability of the test substance in the food for a period of 10 and 32 days was checked and confirmed before the start using a comparable batch (Reu E 7339; ZST No. 88/124)	X
3.2	**Test material 2**	Copper sulphate ($CuSO_4$)	
3.2.1	Lot/Batch number	846A308 291	
3.2.2	Specification		
3.2.2.1	Description	Solid, grey powder	
3.2.2.2	Purity	Pro analysis	
3.2.2.3	Stability	The stability of $CuSO_4$ in the food over a period of 10 and 32 days was proven.	
3.3	**Test Animals**	Non-entry field	
3.3.1	Species	Rats	
3.3.2	Strain	Wistar rat	
3.3.3	Source		
3.3.4	Sex	Males and females	

Section A 6.4.1	**Subchronic toxicity (rodent)**
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3.3.5	Age/weight at study initiation	Age: 42 days Weight at study initiation: male 196g / female 153g	
3.3.6	Number of animals per group	10 male and 10 female	
3.3.7	Control animals		
3.4	**Administration/ Exposure**	Oral	
3.4.1	Duration of treatment	3 months	
3.4.2	Frequency of exposure	Daily	
3.4.3	Postexposure period	Fasting period of about 16-20 hours before necropsy	X
3.4.4	**Oral**		
3.4.4.1	Type	In food	
3.4.4.2	Concentration	Food, drinking water 0, 500, 2000, 4000ppm Food consumption per day: The mean amount of daily ingested test substance (in mg per kg body weight) was calculated taking into account food consumption, dosage and body weight data:	

	Males		Females	
Test group	Test substance (mg/kg bw)	Cu^{2+} (mg/kg bw)	Test substance (mg/kg bw)	Cu^{2+} (mg/kg bw)
Test group 1 500 ppm Cu-HDO	35.2	6.4	41.4	7.5
Test group 2 2000 ppm Cu-HDO	139.8	25.4	166.9	30.4
Test group 3 4000 ppm Cu-HDO	275.4	50.1	322	58.6
Test group 4 1800 ppm $Cu-SO_4$	125.5	50.3	144.4	57.9

X

3.4.4.3	Vehicle	Kliba rats/mice/hamsters maintenance diet	
3.4.4.4	Concentration in vehicle	0, 500, 2000, 4000ppm	
3.4.4.5	Total volume applied		

Section A 6.4.1	**Subchronic toxicity (rodent)**
Annex Point II 6.4	**Oral toxicity in rats**

3.4.4.6	Controls	Untreated animals (10 males and 10 females) To differentiate the toxicity of Cu^{2+} from the toxicity of the test substance Cu-HDO, a group of 10 male and 10 female animals was dosed with 1800ppm copper sulphate ($CuSO_4$), representing the equimolar amount of Cu^{2+} in the diet of this test group as in the diet of the 4000ppm Cu-HDO group.
3.5	**Examinations**	
3.5.1	Observations	
3.5.1.1	Clinical signs	Yes General health state was checked at least daily, thorough clinical examinations were performed once a week
3.5.1.2	Mortality	Yes check twice a day and once a day (Saturday, Sunday)
3.5.2	Body weight	Yes time periods for determinations: weekly
3.5.3	Food consumption	Yes time periods for determinations: weekly
3.5.4	Water consumption	Yes
3.5.5	Ophthalmoscopic examination	Yes at the beginning and at the end of the study
3.5.6	Haematology	Yes Time points: Four weeks after the beginning and at the end of the test Number of animals: All animals Parameters: - Leukocytes - Erythrocyte - Hemoglobin - Hematocrit - Mean corpuscular volume - Mean corpuscular hemoglobin - Mean corpuscular hemoglobin concentration - Platelets - Differential blood count - Reticulocytes - Hemoglobin derivatives: total hemoglobin, oxyhemoglobin, carboxyhemoglobin, methemoglobin, content of oxygen bound to hemoglobin, oxygen saturation, reduced (deoxygenated) hemoglobin, oxygen capacity - Clotting analysis
3.5.7	Clinical Chemistry	Yes Time points:

Section A 6.4.1	Subchronic toxicity (rodent)
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		Four weeks after the beginning and at the end of the test Number of animals: All animals Parameters: **Enzymes:** - alanine aminotransferase - aspartate aminotransferase - alkaline phosphatase - serum-γ-glutamyltransferase **blood chemistry:** - sodium - potassium - chloride - inorganic phosphate - calcium - urea - creatinine - glucose - total bilirubin - total protein - albumin - globulines - triglycerides - cholesterol - magnesium
3.5.8	Urinalysis	Yes Time points: Four weeks after the beginning and at the end of the test Number of animals: All animals Parameters: - appearance - volume - nitrite - pH - protein - glucose - ketones - urobilinogen - bilirubin - blood - sediment
3.6	**Sacrifice and pathology**	
3.6.1	Organ Weights	Yes All animals organs: liver, kidneys, adrenal glands, testes,
3.6.2	Gross and histopathology	Yes all dose groups The following organs were fixed in 4% formaldehyde solution: all gross

lesions, brain, pituitary gland, thyroid glands, parathyroid glands, thymus, trachea, lungs, heart, aorta, salivary glands, liver, spleen, kidneys, adrenal glands, pancreas, testes/ovaries, uterus/vagina, accessory genital organs (epididymides, prostate, seminal vesicle, coagulation glands), skin, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, mesenteric lymph nodes, female mammary gland, musculature, sciatic nerve, sternum, bone marrow (femur), eyes, femur with knee joint, spinal cord (cervical, mid thoracic, lumbar cord)

3.6.3 Other examinations

After the organs were fixed, processing, the examination by light microscopy and the evaluation of findings was performed according to the following table:

	test groups (dose)				
organs	0 (control)	1 (500 ppm Cu-HDO)	2 (2000 ppm Cu-HDO)	3 (4000 ppm Cu-HDO)	4 (1800 ppm Cu SO4)
all gross lesions	A2	A2	A2	A2	A2
brain	A1	A1	A1	A1	A1
pituitary gland	A1			A1	A1
thyroid glands	A1			A1	A1
parathyroid glands	A1			A1	A1
thymus	A1			A1	A1
trachea	A1			A1	A1
lungs	A1	A1	A1	A1	A1
heart	A1			A1	A1
aorta	A1			A1	A1
liver	A1 B1	A1	A1	A1 B1	A1 B1
spleen	A1			A1	A1
kidneys	A1	A1	A1	A1	A1
adrenal glands	A1			A1	A1
pancreas	A1			A1	A1
testes/ovaries	A1			A1	A1
uterus	A1			A1	A1
esophagus	A1			A1	A1
stomach	A1	A1	A1	A1	A1
duodenum, jejunum, ileum	A1 B1	A1 B1	A1 B1	A1 B1	A1 B1
cecum, colon, rectum	A1	A1	A1	A1	A1
urinary bladder	A1			A1	A1
mesenteric lymph node	A1			A1	A1
sciatic nerve	A1			A1	A1
sternum (with marrow)	A1			A1	A1
bone marrow (femur)	A1			A1	A1

Scope of examinations:
1 = all animals per group
2 = all animals affected per group

Methods:
A = hematoxylin-eosin
B = Perls reaction for iron

3.6.4 Statistics

For the statistical evaluation of the study, means and standard deviations were calculated for the variables food consumption, body weight, body weight change and substance intake for the animals in each test group, and printed out in the form of tables.

Statistical significance of the clinical data (food consumption, body weight, body weight change) was determined by analysis of variance (ANOVA) followed by a DUNETT'S test.

The results of the $CuSO_4$ group were compared with the results of the control group by means of an analysis of variance (ANOVA) and STUDENT'S t-test.

3.7 Further remarks

4 RESULTS AND DISCUSSION

4.1 Observations

4.1.1 Clinical signs

Clinical signs: animals of test group 1 (500ppm) showed black-brown coloured faeces, and the animals from test group 2 (2000ppm), 3

Section A 6.4.1	**Subchronic toxicity (rodent)**
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		(4000ppm) and 4 (1800ppm $CuSO_4$) the faeces were coloured black. This finding was considered to represent a chemical reaction of the test substance in the digestive tract rather than being the consequence of a toxic effect on the animals. No other abnormal clinical symptoms were observed.
4.1.2	Mortality	No mortalities at any dose
4.2	**Body weight gain**	No substance related effects
4.3	**Food consumption and compound intake**	No effects
4.4	**Ophtalmoscopic examination**	No pathological changes
4.5	**Blood analysis**	
4.5.1	Clinical Chemistry, Haematology, Urinalysis	**Test group 3 (4000ppm Cu-HDO)** - decrease in alkaline phosphatase and globulins in the serum of the females - increase in alanine aminotransferase, aspartate aminotransferase and cholesterol in the serum of the males - decrease in triglycerides in the serum of the male - increase in granulated casts in the urine sediment of the males **Test group 4 (1800ppm $CuSO_4$)** - increase in alanine aminotransferase in the serum of the males - decrease in alkaline phosphatase and globulins in the serum of the females
4.6	**Sacrifice and pathology**	
4.6.1	Organ weights	
4.6.2	Gross and histopathology	**Test group 3 (4000ppm Cu-HDO)** - Minimal to slight hepatic single cell necrosis (males) - Swelling and pigmentation of Kupffer's cells (in females weaker than in males) - Slight reduction in hepatocellular lipid content (males) - Minimal and slight bile duct hyperplasia (2 males only) - Hyaline droplets in the proximal tubular epithelial cells and protein precipitates in the renal tubular lumina (both sexes) - Minimal to slight diffuse hyperkeratosis in the forestomach (both sexes) - Iron-positive pigment in the tunica propria of the small intestine (both sexes) **Test group 2 (2000 ppm Cu-HDO):** - Minimal hepatic single cell necrosis and swelling and pigmentation of Kupffer's cells (both sexes) - Hyaline droplets in the proximal tubular epithelial cells and protein precipitates in the renal tubular lumina (males only) - Minimal diffuse hyperkeratosis in the forestomach (both sexes)

- Iron-positive pigment in the tunica propria of the small intestine (both sexes)

Test group 1 (500ppm Cu-HDO)

- No substance-induced changes

Test group 4 (1800ppm $CuSO_4$)

- Swelling of the mucosa at the transition between forestomach and glandular stomach (both sexes)
- Minimal to slight single cell necrosis in the liver (males only)
- Swollen and pigmented Kupffer's cells (minimal to severe in males and minimal in females)
- Minimal bile duct hyperplasia in one male rat
- Hyaline droplets in the proximal tubular epithelial cells of the kidneys (males only)
- Protein precipitates in the renal tubular lumina (both sexes)
- Minimal hyperkeratosis in the forestomach (both sexes)

4.7 Other

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

OECD Guideline 408 "Subchronic Oral Toxicity - Rodent: 90-day Study"

To differentiate the toxicity of Cu^{2+} from the toxicity of the test substance, a group of 10 male and 10 female animals was dosed with 1800ppm copper sulphate ($CuSO_4$) (test group 4), representing the equimolar amount of Cu^{2+} in the diet of this test group as in the diet of the 4000ppm Cu-HDO group.

5.2 Results, discussion and conclusion

The administration of Bis-(N-Cyclohexyldiazeniumdioxy)-copper (Cu-HDO) at doses of 4000, 2000, and 500ppm or of copper sulphate ($CuSO_4$) at a dose of 1800 ppm to male and female Wistar rats resulted in a discoloration of the faeces in all treated groups. However, this was assessed as being a chemical reaction of the test substance in the digestive tract without having a toxicological relevance.

By contrast the findings below were assessed as being related to the test substance administration:

Test group 3 (4000ppm Cu-HDO)

<u>Clinical chemistry, haematology and urinalyses:</u>

- Increase in alanine aminotransferase, aspartate aminotransferase and cholesterol in the serum of the males
- Decrease in triglycerides in the serum of the males
- Increase in granulated casts in the urine sediment of the males
- Decrease in alkaline phosphatase and globulins in the serum of the females

<u>Pathology:</u>

- Minimal to slight hepatic single cell necrosis (males)
- Swelling and pigmentation of Kupffer's cells (in females weaker than in males)
- Slight reduction in hepatocellular lipid content (males)
- Minimal and slight bile duct hyperplasia (2 males only)

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- Hyaline droplets in the proximal tubular epithelial cells and protein precipitates in the renal tubular lumina (both sexes)
- Minimal to slight diffuse hyperkeratosis in the forestomach (both sexes)
- Iron-positive pigment in the tunica propria of the small intestine (both sexes)

Test group 2 (2000ppm cu-HDO):

Pathology:

- Minimal hepatic single cell necrosis and swelling and pigmentation of Kupffer's cells (both sexes)
- Hyaline droplets in the proximal tubular epithelial cells and protein precipitates in the renal tubular lumina (males only)
- Minimal diffuse hyperkeratosis in the forestomach (both sexes)
- Iron-positive pigment in the tunica propria of the small intestine (both sexes)

Test group 1 (500ppm Cu-HDO):

- No substance-induced changes

Test group 4 (1800ppm $CuSO_4$):

Clinical chemistry, hematology and urinalysis:

- Increase in alanine aminotransferase in the serum of the males
- Decrease in alkaline phosphatase and globulins in the serum of the females

Pathology:

- Swelling of the mucosa at the transition between forestomach and glandular stomach (both sexes)
- Minimal to slight single cell necrosis in the liver (males only)
- Swollen and pigmented Kupffer's cells (minimal to severe in males and minimal in females)
- Minimal bile duct hyperplasia in one male rat
- Hyaline droplets in the proximal tubular epithelial cells of the kidneys (males only)
- Protein precipitates in the renal tubular lumina (both sexes)
- Minimal hyperkeratosis in the forestomach (both sexes)

Both Cu-HDO and $CuSO_4$ (at concentrations of 2000 and 4000ppm Cu-HDO and 1800ppm $CuSO_4$) appear to be stored in the Kupffer's cells in the liver and bring about swelling of cells there. They are probably also present in the kidneys of the male animals.

However, Cu-HDO caused iron pigment deposits in the tunica propria of the duodenum and jejunum at 4000 and 2000ppm which were not observed in either the male or female animals in group 4 ($CuSO_4$)

Although there is no evident dose-response relationship, the pigment deposits are probably due to the substance administration. The iron pigment in duodenum and jejunum probably has a hematogenic origin and represents residues of degraded erythrocytes as a result of slight irritation or a possible permeability disturbance with erythrodiapedesis or bleeding. However, such iron pigments may also be the consequence of injuries caused by food particles, parasites spontaneous bleeding, and

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		the like. The hyperkeratosis in the forestomach may also be attributable to a slight irritant effect of the two substances at concentrations of 4000ppm Cu-HDO and 1800ppm $CuSO_4$. Taking into account all findings above, the 3 months administration of Bis-(N-Cyclohexyldiazeniumdioxy)-copper (Cu-HDO) at doses of 4000 ppm (= approximately 140mg/kg body weight for males and 167mg/kg body weight for female animals) let to substance-induced changes in liver, kidney, forestomach and small intestine. At 500ppm (= approximately 35mg/kg body weight for male and 42mg/kg body weight for female animals) no substance-induced changes were observed. The administration of 1800ppm copper sulphate ($CuSO_4$) (= approximately 126mg/kg body weight for male and 144mg/kg body weight for female animals) caused substance induced changes in forestomach, liver, and kidneys being similar to that observed in the high dose Cu-HDO group. This indicates, that the effects observed in these organs might be caused by the Cu^{2+} ion. The "No observed adverse effect level" for Bis-(N-Cyclohexyldiazeniumdioxy)-copper is therefore 500ppm (= approximately 35mg/kg body weight for male and 41mg/kg body weight for female animals).	
5.3	**Conclusion**		
5.3.1	LO(A)EL	1800ppm	
5.3.2	NO(A)EL	500ppm, approx. 38mg/kg/day	X
5.3.3	Other		
5.3.4	Reliability	1	
5.3.5	Deficiencies	No	

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	December 2005
Materials and Methods	**3.4.4.2 Concentration** 1800 ppm CuSO4 contains same molar amount of Cu as 4000 ppm Cu-HDO **3.1.2.3 Stability** Also confirmative results from the 1-year study are available, see A.6.5. **3.4.3 Post-exposure period** is recommended by the OECD method 408, but not mandatory **3.6.1 Organ weights** OECD method 408 recommends furthermore epididymis, uterus, ovaries, thymus, spleen, brain, heart. However this deviation is not considered crucial.
Results and discussion	Agree with applicant's version
Conclusion	**NOAEL** = 38 mg/kg bw/day based on minimal hepatic single cell necrosis and swelling and pigmentation of Kupffer´s cell (m+f)), Hyaline droplets in the proximal tubular epithelial cells and protein precipitates in the renal tubular lumina (m), Minimal diffuse hyperkeratosis in the forestomach (m+f), Iron-positive pigment in the tunica propria of the small intestine (m+f) at ca. 153 mg/kg bw/day.
Reliability	1
Acceptability	Acceptable
Remarks	-

Table A6_4.1.1.-1 Results of clinical chemistry haematology and urinalysis

	Controls Cu-HDO				low dose 500ppm Cu-HDO				medium dose 2000ppm Cu-HDO				high dose 4000ppm Cu-HDO				1800 ppm $CuSO_4$			
Parameter changed	♂	days after start of treatment	♀	days after start of treatment	♂	days after start of treatment	♀	days after start of treatment	♂	days after start of treatment	♀	days after start of treatment	♂	days after start of treatment	♀	days after start of treatment	♂	days after start of treatment	♀	days after start of treatment
Serum alanine aminotransferase													↑	**29 & 87**			↑	**29 & 87**		
Serum aspartate aminotransferase													↑	**29 & 87**						
Serum cholesterol													↑	**87**						
Serum triglycerides													↓	**87**						

Serum alakaline phosphatase														↓	**87**			↓	**87**
Serum globulin														↓	**29**			↓	**29**
Granulated casts in the urine sediment													↑	**86**					

Table A6_4.1.1-2 Results *(specify)* of repeated dose toxicity study,

Parameter	**Control**		**low dose 500ppm Cu-HDO**		**medium dose 2000ppm Cu-HDO**		**high dose 4000ppm Cu-HDO**		**1800ppm $CuSO_4$**		**dose-response +/-**
	m[a]	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m**	**f**	
Number of animals examined	10	10	10	10	10	10	10	10	10	10	
Mortality	0	0	0	0	0	0	0	0	0	0	
Clinical signs*											
Body weight											
Food consumption											
Clinical chemistry*											
Ophthalmoscopy											
Haematology*											
Urinalysis*											
Organ x											
Organ weight*											

Parameter	**Control**		**low dose 500ppm Cu-HDO**		**medium dose 2000ppm Cu-HDO**		**high dose 4000ppm Cu-HDO**		**1800ppm $CuSO_4$**		**dose-response +/-**
	m[a]	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m**	**f**	
Gross pathology*				Discoloration of the gastrointestinal content (2 animals)	Discoloration of the gastrointestinal content	Discoloration of the gastrointestinal content	Discoloration of the gastrointestinal content (10 animals)	Discoloration of the gastrointestinal content (10 animals)	Discoloration of the gastrointestinal content (6 animals)	Discoloration of the gastrointestinal content (8 animals)	
									Swelling of the mucosa at the transition between forestomach & glandular stomach	Swelling of the mucosa at the transition between forestomach & glandular stomach	
Microscopic pathology*:											
Gastrointestinal tract:					Minimal diffuse hyperkeratosis in the forestomach	Minimal diffuse hyperkeratosis in the forestomach	Minimal diffuse hyperkeratosis in the forestomach (9 animals)	Minimal to slight diffuse hyper-keratosis in the forestomach (7 animals)	Minimal hyperkera-tosis in the forestomach (4 animals)	Minimal hyperkera-tosis in the forestomach (8 animals)	
					Iron-positive pigment in the tunica propria of the small intestine	Iron-positive pigment in the tunica propria of the small intestine	Iron-positive pigment in the tunica propria of the small intestine (7 animals)	Iron-positive pigment in the tunica propria of the small intestine (5 animals)			

Parameter	**Control**		**low dose 500ppm Cu-HDO**		**medium dose 2000ppm Cu-HDO**		**high dose 4000ppm Cu-HDO**		**1800ppm $CuSO_4$**		**dose-response +/-**
	m[a]	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m**	**f**	
Liver:					Swelling & pigmentation of Kupffer's cells (6 animals)	Swelling & pigmentation of Kupffer's cells	Swelling & pigmentation of Kupffer's cells	Swelling & pigmentation of Kupffer's cells	Swelling & pigmentation of Kupffer's cells (minimal to severe, 10 animals)	Swelling & pigmentation of Kupffer's cells (minimal, 2 animals)	+
					Minimal hepatic single cell necrosis (3 animals)		Minimal to slight hepatic single cell necrosis (10 animals)		Minimal to slight hepatic single cell necrosis (6 animals)		+
							Minimal & slight bile duct hyperplasia (2 males only)		Minimal bile duct hyper-plasia (1 male only)		
							Slight reduction in hepatocellular lipid content				
Kidneys:					Hyaline droplets in the proximal tubular epithelial cells		Hyaline droplets in the proximal tubular epithelial cells (10 animals)	Hyaline droplets in the proximal tubular epithelial cells (7 animals)	Hyaline droplets in the proximal tubular epithelial cells (10 animals)		

Parameter	**Control**		**low dose 500ppm Cu-HDO**		**medium dose 2000ppm Cu-HDO**		**high dose 4000ppm Cu-HDO**		**1800ppm $CuSO_4$**		**dose-response +/-**
	m[a]	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m**	**f**	
					Protein precipitates in the renal tubular lumina		Protein precipitates in the renal tubular lumina	Protein precipitates in the renal tubular lumina (8 animals)	Protein precipitates in the renal tubular lumina (10 animals)	Protein precipitates in the renal tubular lumina (9 animals)	

[a] *give number of animals affected/total number of animals, percentage, or just ↑ or ↓ for increased or decreased*

Section A6.4.1 **Subchronic toxicity (non rodent)**

Annex Point IIA6.4 ***Subchronic oral toxicity in beagle dogs***

			Official use only
		1 REFERENCE	
1.1	**Reference**	A 6.4.1/02 [redacted] (1995) Subchronic oral toxicity study with Bis-(N-Cyclohexyldiazeniumdioxy)-copper in beagle dogs, Report: 31D0141/92060, [redacted]	
1.2	**Data protection**	Yes	
1.2.1	Data owner	BASF	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes Directive 87/302/EEC, part B, p. 12 "Sub-chronic oral toxicity test: 90-day repeated oral dose using non-rodent species"	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Cu-HDO	
3.1.1	Lot/Batch number	Reu E 7360 B	
3.1.2	Specification		
3.1.2.1	Description	Solid, metallic violet	
3.1.2.2	Purity	99%	
3.1.2.3	Stability	Guaranteed throughout the study	
3.2	**Test Animals**	Non-entry field	
3.2.1	Species	Dogs	
3.2.2	Strain	Beagle	
3.2.3	Source	[redacted]	
3.2.4	Sex	Male and female	
3.2.5	Age/weight at study initiation	Age: 5-9 months Weight at study initiation: 12kg (male) / 10.6kg (female)	
3.2.6	Number of animals per group	5 male and 5 female per test group	
3.2.7	Control animals	Yes	
3.3	**Administration/ Exposure**	Oral	
3.3.1	Duration of treatment	Duration of test/exposure: 3 months	
3.3.2	Frequency of exposure		

Section A6.4.1 **Subchronic toxicity (non rodent)**
Annex Point IIA6.4 ***Subchronic oral toxicity in beagle dogs***

3.3.3	Postexposure period	fasting period of 16 hours		X
3.3.4	**Oral**			
3.3.4.1	Type	food		
3.3.4.2	Concentration	food, ~~drinking water~~	ppm + mg/kg bw	X
		food consumption per day	~~ad libitum~~/certain amount per day	X
3.3.4.3	Vehicle	feed		
3.3.4.4	Concentration in vehicle	0, 300ppm, 900ppm, 2700ppm		
3.3.4.5	Total volume applied			
3.3.4.6	Controls			
3.4	**Examinations**			
3.4.1	Observations			
3.4.1.1	Clinical signs	Yes State of health was checked each day		
3.4.1.2	Mortality	Yes		
3.4.2	Body weight	Yes Determined once a week		
3.4.3	Food consumption	Yes Daily determination		
3.4.4	Water consumption	Yes		
3.4.5	Ophthalmoscopic examination	Yes Before the beginning and towards the end of the administration period		
3.4.6	Haematology	Yes Number of animals: all animals Time points: once before and 2 times during the study Parameters: - Leukocytes - Erythrocyte - Haemoglobin - Haematocrit - Mean corpuscular volume - Mean corpuscular haemoglobin - Mean corpuscular haemoglobin concentration - Platelets - Differential blood count - Clotting analysis		
3.4.7	Clinical Chemistry	Yes time points: once before and 2 times during the study number of animals: all animals		

Section A6.4.1	**Subchronic toxicity (non rodent)**
Annex Point IIA6.4	***Subchronic oral toxicity in beagle dogs***

		Parameters: **Enzymes:** - alanine aminotransferase - aspartate aminotransferase - alkaline phosphatase - serum-γ-glutamyltransferase **blood chemistry:** - sodium - potassium - chloride - inorganic phosphate - calcium - urea - creatinine - glucose - total bilirubin - total protein - albumin - globulines - triglycerides - cholesterol - magnesium	
3.4.8	Urinalysis	Yes number of animals: all animals time points: once before and 2 times during the study Parameters: - appearance - volume - nitrite - pH - protein - glucose - ketones - urobilinogen - bilirubin - blood - sediment	
3.5	**Sacrifice and pathology**		
3.5.1	Organ Weights	Yes organs: liver, kidneys, adrenal glands, thyroid glands, brain, ovaries, testes, epididymides	X
3.5.2	Gross and histopathology	Yes all animals organs: brain, pituitary gland, thyroid and parathyroid glands, thymus, trachea, lungs, aorta, heart, tongue, salivary glands, liver, gallbladder, spleen, kidneys, adrenal glands, esophagus, stomach, duodenum, jejunum and ileum, cecum, colon and rectum, uterus and vagina, oviducts, urinary bladder, lymph nodes, pancreas, testes / ovaries,	

Section A6.4.1 **Subchronic toxicity (non rodent)**
Annex Point IIA6.4 ***Subchronic oral toxicity in beagle dogs***

		epididymides, prostate, female mammary gland, skin, skeletal muscle, sciatic nerve, spinal cord, sternum with bone marrow, femur with knee joint and marrow, eyes	
3.5.3	Other examinations		
3.5.4	Statistics	performed with Kruskal-Wallis-H-test and Wilcoxon test	
3.6	**Further remarks**		
		4 RESULTS AND DISCUSSION	
4.1	**Observations**		
4.1.1	Clinical signs	**Test group 3 (2700ppm; approx. 69mg/kg bw)** Clinical findings: – Vomiting in both sexes mainly in the first week of administration – Marked impairment of food efficiency, especially in the males – Body weight losses in both sexes (males about 12%, females about 5%) – Reduced food consumption in both sexes (males about 22%, females about 26%) **Test group 2 (900ppm; approx. 26mg/kg bw)** – No substance induced changes **Test group 1 (300ppm; approx. 9mg/kg bw)** – No substance-induced changes	
4.1.2	Mortality	No mortalities at any dose	
4.2	**Body weight gain**	See 4.1.1	
4.3	**Food consumption and compound intake**	See 4.1.1	
4.4	**Ophtalmoscopic examination**		X
4.5	**Blood analysis**		
4.5.1	Haematology		
4.5.2	Clinical chemistry	**Test group 3 (2700ppm; approx. 69mg/kg bw)** – Increase in alanine aminotransferase, aspartate aminotransferase and potassium in both sexes – Prolonged prothrombin time in the males – Decrease in calcium, total protein, albumin, globulins and cholesterol in both sexes – Decrease in glucose in the females **Test group 2 (900ppm; approx. 26mg/kg bw)** – No substance induced changes **Test group 1 (300ppm; approx. 9mg/kg bw)** – No substance-induced changes	
4.5.3	Urinalysis	At the end of the study, statistically significantly increased bilirubin levels were detected in the urine specimens of the high dose males. No	

Section A6.4.1	Subchronic toxicity (non rodent)
Annex Point IIA6.4	*Subchronic oral toxicity in beagle dogs*

		further treatment-related changes were seen in the other urinary parameters.
4.6	**Sacrifice and pathology**	
4.6.1	Organ weights	**Test group 3 (2700ppm; approx. 69mg/kg bw)** – Significantly decreased mean absolute and relative liver weights in male dogs – Significantly decreased mean relative liver weight in female dogs **Test group 2 (900ppm; approx. 26mg/kg bw)** – No substance induced changes **Test group 1 (300ppm; approx. 9mg/kg bw)** No substance-induced changes
4.6.2	Gross and histopathology	**Test group 3 (2700ppm; approx. 69mg/kg bw)** <u>Macroscopic and histopathological findings</u> – Gross lesions in the liver of four male and three female dogs indicative for liver cell damage represented by foci, necrosis and/or capsular retractions – Chronic hepatitis in all male and female dogs – Liver cirrhosis in five male and in three female dogs – Copper pigment storage in hepatocytes and Kupffer cells of all animals – Edema in the gall bladder wall of two male and four female animals – Edema in the pancreas and in the mesentery of two male dogs – Minimal hyperplasia in the mucosa of the esophagus of three males and one female – Lymphoid depletion in the thymus of three males **Test group 2 (900ppm; approx. 26mg/kg bw)** – No substance induced changes **Test group 1 (300ppm; approx. 9mg/kg bw)** No substance-induced changes
4.7	**Other**	

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1	**Materials and methods**	Directive 87/302/EEC, part B, p. 12 "Sub-chronic oral toxicity test: 90-day repeated oral dose using non-rodent species"
5.2	**Results, discussion and conclusion**	Vomitus occurred frequently in all animals of test group 3 (2700ppm) mainly in the first week of the test substance administration. This finding together with reduced food consumption in both sexes and the distinct reduction of food efficiency and slight to moderate body weight losses in male and female dogs of the 2700-ppm group are assessed as substance-related effects. The marked decreases in total protein, albumin, globulins and cholesterol in the high dose dogs are also associated with treatment. Furthermore, the changes seen in calcium, potassium, glucose, and prothrombin time are difficult to relate to a specific toxic effect of the test compound administered. Due to the reduced food consumption and

body weight gain of the high-dose animals and the changes seen in the liver, it is likely that all the aforementioned findings in clinical pathology testing are probably the combined result of indirect toxic effects associated with the decreases in food consumption and body weight of the animals of test group 3 and the direct effects observed in the liver.

The increases in alanine aminotransferase and aspartate aminotransferase activities in the high dose animals are considered to be treatment-related effects which may have been a result of hepatocellular damage. The degree of changes in aminotransferase activities was slight and nearly constant throughout the treatment period indicating a mild, persistent hepatotoxic effect of the test compound.

Bilirubinuria noted in the urine specimens of the high dose males at the end of the study is possible also a treatment-related effect which is probably associated with the changes seen in the liver. However small quantities of bilirubin are commonly in concentrated urine samples obtained from normal dogs. Since some of the high dose males excreted low amounts of urine, the detection of Bilirubinuria in these animals seems to be normal. Therefore, it is questionable if increased elimination of urinary bilirubin by the high-dose dogs is really a treatment-related effect.

These findings have to be seen in context with the pathological finding of continuous liver cell damage resulting in chronic hepatitis in all animals and in liver cirrhosis in all male and three female dogs of test group 3. As liver cirrhosis can lead to congestive symptoms by interference with blood circulation, the occurrence of edema in different organs (gallbladder wall, pancreas, mesentery) in some animals of the 2700-ppm group has to be regarded as a consequence of the cirrhosis. Moreover, the decreased liver weights are most likely also a result of liver cirrhosis.

The administration of Bis-(N-Cyclohexyldiazeniumdioxy)-copper led to copper storage in liver cells and Kupffer cells of the high-dose animals. Although there was no acute cell damage in the copper laden cells it seems most likely that the source of liver cell damage is related to copper toxicity.

Basal cell hyperplasia of the mucosa of the esophagus in test group 3 animals is possibly the result of an irritating effect of the test substance.

Lymphoid depletion, which was exclusively found in the thymus of 3 males of test group 3, was judged as a consequence of generally altered metabolism and body function and not as a sign of specific organ toxicity.

Taking into account the above mentioned findings it can be stated that the 3-month oral administration of Bis-(N-Cyclohexyldiazeniumdioxy)-copper to male and female dogs caused the following substance-induced changes:

Test group 3 (2700ppm):

- Clinical findings:
  - Vomiting in both sexes mainly in the first week of administration
  - Reduced food consumption in both sexes (males about 22%,

females about 26%)
  - Marked impairment of food efficiency, especially in the males.
  - Body weight losses in both sexes (males about 12%, females about 5%)
- Clinicochemical findings:
  - Increase in alanine aminotransferase, aspartate aminotransferase and potassium in both sexes
  - Prolonged prothrombin time in the males
  - Decrease in calcium, total protein, albumin, globulins and cholesterol in both sexes
  - Decrease in glucose in the females
- Organ weights
  - Significantly decreased mean absolute and relative liver weights in male dogs
  - Significantly decreased mean relative liver weights in female dogs
- Macroscopic and histopathological findings
  - Gross lesions in the liver of four male and three female dogs indicative for liver cell damage represented by foci, necrosis and/or capsular retractions.
  - Chronic hepatitis in all male and female dogs
  - Liver cirrhosis in five male and in three female dogs
  - Copper pigment storage in hepatocytes and Kupffer cells of all animals.
  - Edema in the gall bladder wall of two male and four female animals
  - Edema in the pancreas and in the mesentery of two male dogs
  - Minimal hyperplasia in the mucosa of the esophagus of three males and one female
  - Lymphoid depletion in the thymus of three males

Test group 2 (900ppm):

No substance-induced changes

Test group 1 (300ppm):

No substance-induced changes

In conclusion it can be stated that Cu-HDO administered with the diet to Beagle dogs in a dose of 2700ppm (approx. 69mg/kg bw/day) led to distinct clinical signs (lack of appetite, bodyweight losses, impairment of food efficiency, vomitus,) as well as to hepatotoxic effects characterized by hepatocellular damage associated with deviations in clinicochemical parameters (eg increase in ALT and AST).

The administration of the test article resulted in chronic hepatitis in all animals and in liver cirrhosis in all male and three female dogs of test group 3 associated with edema in the gallbladder wall (2 male and 4 female dogs) and in the pancreas and mesentery (2 male animals). Although no acute damage of liver cells was noted in the present study,

Section A6.4.1	Subchronic toxicity (non rodent)
Annex Point IIA6.4	*Subchronic oral toxicity in beagle dogs*

		the latter findings are interpreted as sequelae thereof. This is justified by the finding of hepatocellular single cell necrosis in the previous 4-week feeding study employing a similar high dose (2500ppm) Moreover, copper storage was found in liver cells, and Kupffer cells of the high dose animals. The source of liver cell damage in the present study is most likely related to copper toxicity. The administration of 900 ppm (approx. 26mg/kg bodyweight/day) and of 300ppm (approx. 9mg/kg bodyweight/day) was tolerated by the male and female dogs without any changes that could be related to the test substance administered. Therefore, the "no observed adverse effect level" (NOAEL) for male and female Beagle dogs under the chosen test conditions is 900 ppm.	
5.3	**Conclusion**		
5.3.1	LO(A)EL		
5.3.2	NO(A)EL	900ppm	X
5.3.3	Other		
5.3.4	Reliability	1	
5.3.5	Deficiencies	No	

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	December 2005
Materials and Methods	**3.3.3 Post-exposure period** is recommended by the OECD method 409, but not mandatory **3.3.4.2 Concentration** 300 ppm, 900 ppm, 2700 ppm corresponding to 8,3; 25,2; 64,6 in males and 9,3; 27,4; 71,9 mg/kg bw/day in females. unconsumed food was subtracted from the original amount offered **3.5.1 Organ weights** OECD method 409 recommends to also weight: uterus, spleen, heart. However this deviation is not considered to be crucial
Results and discussion	**4.4 Ophtalmoscopic examination** no pathological changes which can be attributed to the test substance administration
Conclusion	**5.3.2 NOAEL** 900 ppm corresponding to 25.5 mg/kg bw/day (male) based on vomiting, reduced food consumption, body weight loss and clinicochemical effects, organ weight, gross and histopatholocial effects on the liver and edema in the gall bladder wall and the pancreas and mesentery and minimal hyperplasia in the esophagus mucosa and lymphoid depletion in the thymus at 2700 ppm corresponding to ca. 65 mg/kg bw/day (male).
Reliability	1
Acceptability	Acceptable
Remarks	-

Table A6_4.1.2-1 Results of clinical chemistry haematology and urinalysis

(Use this or similar table, if relevant effects occur and if time sequence is important.Give either symbols for increases or decreases (↑↓) or abbreviations inc., dec. Only if more information is needed, give figures or percentages.)

parameter changed	Unit	Controls	low dose	medium dose	high dose
weeks after start of treatment					
Males					– Alanine aminotransferase ↑ – Aspartate aminotransferase ↑ – Potassium ↑ – Prothrombin time ↑ – calcium, total protein, albumin, globulins, cholesterol ↓ – bilirubin level ↑
Females					– Alanine aminotransferase ↑ – Aspartate aminotransferase ↑ – Potassium ↑ – calcium, total protein, albumin, globulins, cholesterol ↓ – glucose ↓

* $p < 0,05$

Give only those parameters which are changed in at least one dose group compared to control. Usually only statistically significant effects

Depending on number of parameters changed one table each for Haematology, Clinical Chemistry, Urinalysis

Table A6_4.1.2-2 Results *(specify)* of repeated dose toxicity study

Parameter	**Control**		**low dose 300ppm**		**medium dose 900ppm**		**high dose 2700ppm**		**dose-response +/-**	
	m[a]	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m[a]**	**f[a]**	**m**	**f**
Number of animals examined	5	5	5	5	5	5	5	5		
Mortality	0	0	0	0	0	0	0	0		
Clinical signs*							Vomiting	Vomiting	+	
Food efficiency							↓	↓	+	
Body weight							↓	↓	+	
Food consumption							↓	↓	+	
Clinical chemistry*										
Alanine aminotransferase							↑	↑	+	
Aspartate aminotransferase							↑	↑	+	
Potassium							↑	↑	+	
Calcium, total protein, albumin, globulins, cholesterol							↓	↓	+	
Prothrombin time							↑		+	
Glucose								↓	+	
Haematology*										
Urinalysis*										
Bilirubin level							↑		+	
Liver:										
Organ weight (mean absolute)							↓		+	
Organ weight (relative)							↓	↓	+	
Gross pathology*							– Gross lesions in the liver – Foci, necrosis and/or capsular retractions – Chronic hepatitis – Liver cirrhosis – Copper pigment storage in hepatocytes and Kupffer cells	– Gross lesions in the liver – Foci, necrosis and/or capsular retractions – Chronic hepatitis – Liver cirrhosis – Copper pigment storage in hepatocytes and Kupffer cells	+	
Microscopic pathology*										

Parameter	**Control**		**low dose 300ppm**		**medium dose 900ppm**		**high dose 2700ppm**		**dose-response +/-**	
Digestive organs										
Gross pathology							Edema in gallbladder wall Edema in pancreas and mesentery Minimal hyperplasia in the mucosa of the esophagus		+	
Lymphatic system										
Gross pathology							Lymphoid depletion in the thymus		+	

** specify effects; for different organs give special findings in the order organ weight, gross pathology and microscopic pathology if there are effects*

[a] *give number of animals affected/total number of animals, percentage, or just ↑ or ↓ for increased or decreased*

Section A 6.5 Chronic toxicity (rodent)

Annex Point IIA6.5

			Official use only
		1 REFERENCE	
1.1	**Reference**	A 6.5 [redacted] (1993) Report on the study of the chronic toxicity of Bis-(N-Cyclohexyl-diazeniumdioxy)-copper in rats, Report: 50C0679/89080, [redacted]	
1.2	**Data protection**	Yes	
1.2.1	Data owner	BASF AG	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes - EC Commission directive 87/3027EEC of 18. Nov. 1987, Part B: Methods for the determination of Toxicity; Chronic Toxicity Test; Official Journal of the European Communities No. L133, p. 27-31 (1988) - OECD Guidelines for Testing of Chemicals; No. 452: Chronic Toxicity Studies; May 12, 1981	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	1. Cu-HDO 2. Copper-(II)-sulfate water free	
3.1.1	Lot/Batch number	1. Reu E 7360 2. 846A308 291	
3.1.2	Specification		
3.1.2.1	Description	1. Solid, metallic violet 2. Solid, grey powder	
3.1.2.2	Purity	1. 99% 2. pro analysi	
3.1.2.3	Stability	1. The stability of Cu-HDO over the study period has been guaranteed by the supplier, but in addition it has been proven by reanalysis after termination of the study 2. The stability of copper sulfate over the duration of the study can be assumed. A reanalysis will, however, be performed after the experimental part of the onogenicity study (Project No.: 70C0679/89113)	X
3.2	**Test Animals**	~~Non-entry-field~~	
3.2.1	Species	Rats	
3.2.2	Strain	Wistar rats	
3.2.3	Source	[redacted]	

Section A 6.5 Chronic toxicity (rodent)

Annex Point IIA6.5

3.2.4	Sex	Males and females	
3.2.5	Age/weight at study initiation	42 days 178g (male) / 148g (female)	
3.2.6	Number of animals per group	40 (20 males and 20 females per group)	
3.2.7	Control animals	Yes	
3.3	**Administration/ Exposure**	Oral	
3.3.1	Duration of treatment	12 months	
3.3.2	Frequency of exposure	The animals were daily fed with the test substance preparations until the start of the fasting period (withdrawal of food) of about 16 – 20 hours before necropsy.	
3.3.3	Postexposure period	16 – 20 hours before necropsy	
3.3.4	**Oral**		
3.3.4.1	Type	in food	
3.3.4.2	Concentration	0, 100, 300, 1000, 3000ppm Cu-HDO 1350ppm $CuSO_4$ food consumption per day: ad libitum	X
3.3.4.3	Vehicle	Food	
3.3.4.4	Concentration in vehicle	0, 100, 300, 1000, 3000ppm Cu-HDO 1350ppm $CuSO_4$	
3.3.4.5	Total volume applied		
3.3.4.6	Controls	Plain diet	
3.4	**Examinations**		
3.4.1	Observations		
3.4.1.1	Clinical signs	Yes Time periods for observation: checked at least daily, additional examinations and palpations were performed once a week	
3.4.1.2	Mortality	Yes Time periods for observation: checked twice a day and once a day (Sunday)	
3.4.2	Body weight	Yes Time periods for determinations: determined before the start of the administration period, during the first 13 weeks each week and thereafter every 4 weeks until the end of the study	
3.4.3	Food consumption	Yes Time periods for determinations: determined once a week during the first 13 weeks and then every 4 weeks until the end of the study	
3.4.4	Water consumption		

Section A 6.5 Chronic toxicity (rodent)

Annex Point IIA6.5

3.4.5	Ophthalmoscopic examination	Yes Time periods for examinations: at the beginning and the end of the study the eyes of the male and female animals of the control group (0 ppm), test group 4 (3.000ppm) and test group 5 (1350ppm CuSO4) were examined for pathological changes
3.4.6	Haematology	Yes Number of animals: All animals Time points: The blood sampling procedure and the subsequent analysis was done in randomised sequence: 92/93, 184/185, 359/360 days after the beginning of the administration period Parameters: Leukocytes; erythrocytes, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets, thromboplastin time
3.4.7	Clinical Chemistry	Yes Number of animals: All animals Time points: End of study or other Parameters: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, serum-glutamyltransferase, sodium, potassium, chloride, inorganic phosphate, calcium, urea, creatinine, glucose, total bilirubin, albumin, globulins, triglycerides, cholesterol, magnesium
3.4.8	Urinalysis	Yes Number of animals: All animals Time points: The urine samples were evaluated in general in randomised sequence: 86/87, 177/178, 352/353 days after the beginning of the administration period Parameters: volume, colour, turbidity, nitrite, pH, protein, glucose, ketones, urobilinogen, bilirubin, blood, specific gravity, sediment
3.5	**Sacrifice and pathology**	
3.5.1	Organ Weights	Yes Organs: kidney, brain, liver, testis, adrenal glands, epididymis
3.5.2	Gross and histopathology	Yes all dose groups Organs: - brain - pituitary gland - thyroid and parathyroid glands - thymus

Section A 6.5 Chronic toxicity (rodent)

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- trachea
- lungs
- aorta
- heart
- salivary glands
- liver
- spleen
- kidneys
- adrenal glands
- esophagus
- stomach
- duodenum, jejunum and ileum
- cecum, colon and rectum
- uterus and vagina
- urinary bladder
- lymph nodes
- pancreas
- testes / ovaries
- accessory genital organs
- prostate
- female mammary gland
- skin
- skeletal muscle
- sciatic nerve
- spinal cord
- sternum with bone marrow
- marrow (femur)
- femur with knee joint
- eyes

3.5.3 Other examinations

3.5.4 Statistics — Dunnett test, Student's T-test, used for body weights, absolute and relative organ weights

3.6 Further remarks

4 RESULTS AND DISCUSSION

4.1 Observations

4.1.1 Clinical signs — Throughout the administration period in male and female animals of test group 3, 4, and 5 the faeces were coloured black from day 2 of the administration period onwards until the end of the study. This finding was considered to represent a chemical reaction of the test substance in the digestive tract rather than being the consequence of a toxic effect of the test substance. All other clinical findings, like palpable mass in the abdomen, reduced general state, cataract etc. were assessed as being incidental without dose-response relationship.

4.1.2 Mortality — The mortality was not affected by the test substance administration. Only 2

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Annex Point IIA6.5

male animals of test group 0 (0ppm) and 2 female animals of test group 3 (1000ppm) died intercurrently.

4.2 Body weight gain

Test groups 1 – 4 (100, 300, 1000, and 3000ppm Cu-HDO) compared with group 0 (0ppm)

Regarding the absolute body weights, there were no remarkable differences between test groups 1 – 4 in comparison to test group 0. The bodyweight change values in the male animals of test groups 1 to 4 and in female animals of test groups 4 were statistically significant decreased at the start of the study. However, this was assessed as being incidental and not substance-related. X

Test group 4 (3000ppm Cu-HDO) compared with test group 5 (1350ppm $CuSO_4$)

Compared with test group 5 the body weight / body weight change values in male and female animals of test group 4 were statistically significantly decreased at the start of the study.

However all statistically significant deviations were assessed as being incidental and not related to the test substance administration.

4.3 Food consumption and compound intake

In all test groups, no remarkable substance-induced changes of food consumption were observed. The deviations from the values of the control group were assessed as being incidental and not related to the test substance administration.

The approximate mean test substance intake as well as the uptake of Cu^{2+} was calculated based upon the weekly-determined food consumptions and the respective body weights. The results are shown in the following table:

	Male animals		Female animals		All animals	
	Test substance (mg/kg bw)	Cu^{2+} * (mg/kg bw)	Test substance (mg/kg bw)	Cu^{2+} * (mg/kg bw)	Test substance (mg/kg bw)	Cu^{2+} * (mg/kg bw)
Test group 1	5	1	7	1	6	1
Test group 2	16	3	20	4	18	3
Test group 3	54	10	67	12	61	11
Test group 4	161	29	205	37	183	33
Test group 5	74	30	91	37	82	33

* calculated for $C_{12}H_{22}N_4$=4Cu (= Cu-HDO): = 18.2% Cu^{2+} and for $CuSO_4$: = 40.1% Cu^{2+}

4.4 Ophtalmoscopic examination

The ophthalmological examinations carried out with an ophthalmoscope at the beginning and at the end of the study showed no pathological changes being attributable to the test substance administration.

4.5 Blood analysis

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4.5.1	Haematology	After 3 and 6 months of test substance administration, statistically significantly increased white blood cells were detected in the peripheral blood of the male animals of test group 4 (3000ppm). At the end of the study, increased leukocytes were also found in the blood of the high-dose males. However, there was only a trend toward increased values. In the differential blood count of the high dose males, the increase in white blood cells correlates with an increase in lymphocytes. An increase in white blood cells was also noted in the males of test group 4 (3000ppm) when compared with the results of test group 5 (1350ppm $CuSO_4$). Therefore, the increase in leukocytes and lymphocytes in the males of test group 4 is assessed as being treatment-related, although the cause of these findings cannot be explained. In the peripheral blood of the female animals, no changes were seen in the haematological parameters.
4.5.2	Clotting analysis	No substance-induced changes were observed in the clotting analysis of both sexes.
4.5.3	Enzymes	Throughout the study, statistically significantly increased alanine aminotransferase and aspartate aminotransferase activities were found in the serum of the high dose males. The activities of the alanine aminotransferase and aspartate aminotransferase are also increased in the males of test group 4 (3000ppm) when the results of the high dose group are compared with those of test group 5. Since alanine aminotransferase and aspartate aminotransferase activity is increased in test group 4 (3000ppm) when compared with test group 0 (0ppm) and test group 5 (1350ppm $CuSO_4$), these findings are considered to be substance-related. The increase in enzyme activities may be caused by liver damage, which is probably due to a slight hepatotoxic potential of the test substance. In the female animals, no changes in enzyme activities were detected.
4.5.4	Blood chemistry	After 3 and 6 months of test substance administration, statistically significantly increased total bilirubin and cholesterol concentrations were detected in the serum of the high dose males. In the females, only a statistically significant increase in total bilirubin was found in the highest dose group (3000ppm) at the end of the administration period. These changes are also substance-induced and may be related to the slight hepatotoxic potential of the test compound. Since there are no statistically significant differences between the high dose group (3000ppm) and test group 5 (1350ppm $CuSO_4$), it is assumed that the increase in total bilirubin and cholesterol is based on a reaction of the copper moiety of the test substance. In the other clinicochemical examinations, no changes were observed which are related to the test substance administered.
4.5.5	Urinalysis	After 6 and 12 months of the test substance administration, statistically significantly increased squamous epithelial cells were seen in the urine sediment of the high-dose females. This finding is probably also associated with the administration of the test compound and might indicate an injury of the urinary tract in the females of test group 4. The other urine examination revealed no substance related changes.
4.5.6	Other deviations	There are some further statistically inter-group differences in the results of the clinicochemical and urine examinations. These deviations are marginal and inconsistent, when compared with the other sex, or lack dosage relationship, or are caused by an incidental high control value. Accordingly, these changes are considered to be of no toxicological significance. Furthermore, several statistically significant differences were seen in results of

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		test group 4 (3000ppm) when compared with those of test group 5 (1350ppm $CuSO_4$). These deviations are generally not assessed as being substance-related because most of the statistically significant differences seen in this comparison are due to changes in the results of test group 5 and not to an effect of the test compound in the highest dose group, or they are based on the different statistical test used in this evaluation.	X
4.6	**Sacrifice and pathology**		
4.6.1	Organ weights	**Dose group 4 (3000ppm Cu-HDO)** - Significantly increased absolute and relative kidney weights in males - Significantly increased relative liver weight in females - Significantly increased absolute and relative liver weights in females of group 4 (3000ppm Cu-HDO) in comparison to female group 5 (1350ppm $CuSO_4$) - The liver (in males) and kidney weights (in males and females) were similar in both groups 4 (3000ppm Cu-HDO) and 5 (1350 ppm $CuSO_4$) **Dose group 3 (1000ppm Cu-HDO), Dose group 2 (300ppm Cu-HDO) and dose group 1 (100ppm Cu-HDO)** No significantly mean organ weight parameters	
4.6.2	Gross lesions	**Dose group 4 (3000ppm Cu-HDO)** Thickening of forestomach wall in 20/20 males and in 16/20 females This finding was similar in both groups 4 (3000ppm Cu-HDO) and 5 (1350ppm $CuSO_4$) **Dose group 3 (1000ppm Cu-HDO)** Thickening of forestomach wall in 2 males and 4 females **Dose group 2 (300ppm Cu-HDO) and dose group 1 (100ppm Cu-HDO):** No treatment related gross lesions	
4.6.3	Histopathology	**Dose group 4 (3000ppm Cu-HDO)** - Hyperkeratosis, hyperplasia of forestomach mucosa and edema in submucosa (males and females) - Hyperplasia of the glandular stomach mucosa (males and females) - Hyperplasia of duodenal mucosa (males and females) - swollen and pigmented Kupffer's cells in the liver (males and females) and single cell necrosis (males) - hyaline (fluorescent) droplets in the renal proximal tubules and proteinaceous casts in the tubular lumina (males) The incidence of hyperkeratosis in forestomach and of hyperplasia in forestomach, glandular stomach and duodenum was comparable in males and females of both groups 4 (3000ppm Cu-HDO) and 5 (1350ppm $CuSO_4$) The incidence of swollen and pigmented Kupffer's cells in the liver was comparable in males and females of both groups 4 (3000ppm Cu-HDO) and 5 (1350ppm $CuSO_4$) The incidence of edema in submucosa of the forestomach was higher in males and females of group 4 (3000ppm Cu-HDO) compared to group 5 (1350ppm $CuSO_4$)	

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Annex Point IIA6.5

The incidence of single cell necrosis in the liver was higher in males of group 4 (3000ppm Cu-HDO) compared to group 5 (1350ppm $CuSO_4$)

The incidence of hyaline (fluorescent) droplets in renal proximal tubules and proteinaceous casts in tubular lumina was higher in males of group 4 (3000ppm Cu-HDO) compared to group 5 (1350ppm $CuSO_4$)

The biological relevance of one duodenal adenoma (group 4, 3000ppm Cu-HDO) will be decided together with histopathological investigations of the 24-month study No. 70C0679/89113.

Dose group 3 (1000ppm Cu-HDO)

- Hyperkeratosis of forestomach mucosa (females)
- Hyperplasia of glandular stomach mucosa (females)
- Swollen and pigmented Kupffer cells in the liver (males and females)

Dose group 2 (300ppm Cu) and dose group 1 (100ppm Cu-HDO)

No treatment-related microscopic findings

4.7 Other

In test groups 3, 4, and 5 (1000; 3000ppm Cu-HDO and 1350ppm $CuSO_4$) a discoloration of the faeces was observed. However, this was assessed as being a chemical reaction of the test substance in the digestive tract without any toxicological relevance.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

- EC Commission directive 87/3027EEC of 18. Nov. 1987, Part B: Methods for the determination of Toxicity; Chronic Toxicity Test; Official Journal of the European Communities No. L133, p. 27-31 (1988)
- OECD Guidelines for Testing of Chemicals; No. 452: Chronic Toxicity Studies; May 12, 1981

5.2 Results and discussion

The administration of Bis-(N-Cyclohexyldiazeniumdioxy)-copper (Cu-HDO) at doses of 3000, 1000, 300, and 100ppm Cu-HDO or of Copper sulphate ($CuSO_4$) at a dose of 1350 ppm to male and female Wistar rats for 12 months resulted in a discoloration of the faeces in the test group 3 (1000ppm Cu-HDO) and 5 (1350ppm) $CuSO_4$). However, this was assessed as being a chemical reaction of the test substance in the digestive tract without having a toxicological relevance.

By contrast the findings below were assessed as being toxic effects induced by the test substance administration:

Test group 4 (3000ppm Cu-HDO $\triangleq$ approximately 183mg/kg body weight:

- Increase in total bilirubin in both sexes
- Increase in white blood cells, lymphocytes, alanine aminotransferase, aspartate aminotransferase and cholesterol in the males
- Increase in squamous epithelial cells in the urine sediment of the females
- Increased relative and absolute kidney weights in males
- Increased relative liver weight in females
- Thickening of the forestomach wall (males and females)

Section A 6.5 Chronic toxicity (rodent)

Annex Point IIA6.5

- Hyperkeratosis and hyperplasia of the forestomach mucosa and edema in the submucosa (males and females)
- Hyperplasia of the glandular stomach mucosa (males and females)
- Hyperplasia of the duodenal mucosa (males and females)
- Swollen and pigmented Kupffer's cells in the liver (males and females) and single cell necrosis (males)
- Hyaline (fluorescent) droplets in the renal proximal tubules (males) and proteinaceous casts in the tubular lumina (males)

Test group 3 (1000ppm Cu-HDO ≙ approximately 61mg/kg body weight):

- Thickening of the forestomach wall (males and females)
- Hyperkeratosis of the forestomach mucosa (females)
- Hyperplasia of glandular stomach mucosa (females)
- Swollen and pigmented Kupffer's cells in the liver (males and females)

Test group 2 (300ppm Cu-HDO ≙ approximately 18mg/kg body weight) and test group 1 (100ppm Cu-HDO ≙ approximately 6mg/kg body weight)

- No substance related findings

Comparison of test group 4 (3000ppm Cu-HDO ≙ approximately 183mg/kg body weight or 33mg Cu^+/kg body weight) with test group 5 (1350ppm $CuSO_4$ ≙ approximately 82mg/kg body weight or 33mg Cu^+/kg body weight)

- Increase in white blood cell count, lymphocytes, alanine aminotransferase, and aspartate aminotransferase in males of group 4
- Increase in squamous epithelial cells in the urine sediment of females of group 4
- Increase in total bilirubin (males and females) was observed in both groups
- Increase in cholesterol (males) was observed in both groups
- Increased absolute and relative liver weights in females of group 4
- The liver (males) and kidney weights (males and females) were similar in both groups
- The thickening of the forestomach wall (males and females) was similar in both groups
- The incidence of hyperkeratosis in the forestomach and of hyperplasia in the forestomach, glandular stomach and duodenum was comparable in males and females of both groups
- The incidence of swollen and pigmented Kupffer's cells in the liver was comparable in males and females of both groups
- The incidence of edema in the submucosa of the forestomach was higher in males and females of group 4.
- The incidence of single cell necrosis in the liver was higher in males of group 4.
- The incidence of hyaline (fluorescent) droplets in renal proximal tubules and proteinaceous casts in tubular lumina was higher in males of group 4

In summary it can be stated, that the administration of Cu-HDO for a period

Section A 6.5 Chronic toxicity (rodent)

Annex Point IIA6.5

		of 12 months in the diet caused adverse effects at 3000ppm (about 183mg/kg bw) and 1000ppm (about 61mg/kg bw). Target organs were digestive tract, liver, and kidneys. Comparing the administration of 3000ppm Cu-HDO with the administration of 1350ppm $CuSO_4$ (both resulting in an approximate intake of about 33mg Cu^{2+} /kg bw per day), some effects on the digestive tract, the liver and the kidneys were observed in both test groups and can therefore be attributed to the Cu^{2+} ion in both substances. However, the increase in white blood cell count, in lymphocytes, in alanine aminotransferase, in aspartate aminotransferase, in squamous epithelial cells in the urine sediment, increased liver weights in females, higher incidence of edema in the submucosa of the forestomach, higher incidence of single cell necrosis in the liver, and the higher incidence of hyaline droplets and proteinaceous casts in the kidneys were specific for Cu-HDO.	
5.3	**Conclusion**	The increase in white blood cell count, in lymphocytes, in alanine aminotransferase, in aspartate aminotransferase, in squamous epithelial cells in the urine sediment, increased liver weights in females, higher incidence of edema in the submucosa of the forestomach, higher incidence of single cell necrosis in the liver, and the higher incidence of hyaline droplets and proteinaceous casts in the kidneys were specific for Cu-HDO.	
5.3.1	LO(A)EL		
5.3.2	NO(A)EL	300ppm, approx. 18mg/kg/d	X
5.3.3	Other		
5.3.4	Reliability	1	
5.3.5	Deficiencies	No	

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	December 2005
Materials and Methods	**3.1.2.1 Stability** CuSO4: see carcinogenicity study report summarized in document IIIA 6.7.1., Annex IV of study report **3.3.4.2 Concentration** 0, 100, 300, 1000, 3000 ppm Cu-HDO approximately equivalent to 0, 5, 16, 54, 161 (males) and 0, 7, 20, 67, 205 (females) mg/kg bw/day 1350ppm $CuSO_4$ approximately equivalent to 82mg/kg bw or 33mg/kg bw/day Cu^{2+}
Results and discussion	Agree with applicant's version **4.2 Body weight gain** was significantly reduced in males of group 1-4 and in group 4 of females till day 7 **4.6.5 Other deviations** For clarity it should read: Furthermore, several statistically significant differences were seen in results of test group 4 (3000ppm) when compared with those of test group 5 (1350ppm $CuSO_4$). These deviations are generally not assessed as being substance-related because most of the statistically significant differences seen in this comparison are due to ~~changes in the results~~ **effects** within of test group 5, **that were not seen in test group 4** ~~and not to an effect of the test compound in the~~ **(highest dose group with test compound)**, or they are based on the different statistical test used in this evaluation.
Conclusion	**NOAEL = 300 ppm, approx. 18 mg/kg/d Cu-HDO** based on thickening of the forestomach wall (males and females), hyperkeratosis of the forestomach mucosa (females), hyperplasia of glandular stomach mucosa (females) and swollen and pigmented Kupffer's cells in the liver (males and females) at ca. 54 mg/kg bw.
Reliability	1
Acceptability	Acceptable
Remarks	-

Table A6_5.1-1 Results of clinical chemistry, haematology, and urinalysis

	Controls Cu-HDO				Test group 1 100ppm Cu-HDO				Test group 2 300ppm Cu-HDO				Test group 3 1000ppm Cu-HDO				Test group 4 3000ppm Cu-HDO				Test group 5 1350ppm $CuSO_4$			
Parameter changed	♂	days after start of treatment	♀	days after start of treatment	♂	days after start of treatment	♀	days after start of treatment	♂	days after start of treatment	♀	days after start of treatment	♂	days after start of treatment	♀	days after start of treatment	♂	days after start of treatment	♀	days after start of treatment	♂	days after start of treatment	♀	days after start of treatment
White blood cells																	↑	92/93, 184/185						
Leukocytes																	↑	359/360						
Serum alanine aminotrans-ferase																	↑	92/93, 184/185, 359/360						
Serum aspartate aminotrans-ferase																	↑	92/93, 184/185, 359/360						
Serum bilirubin																	↑	92/93	↑	359/360	↑	92/93	↑	359/360
Serum cholesterol																	↑	92/93			↑	92/93		
Squamous epithelial cells in the urine sediment																			↑	184/185, 359/360				

Table A6_5.1-2 Results of repeated dose toxicity study

Parameter	**Control 0 ppm Cu-HDO**		**Test group 1 100 ppm Cu-HDO**		**Test group 2 200 ppm Cu-HDO**		**Test group 3 1000ppm Cu-HDO**		**Test group 4 3000ppm Cu-HDO**		**Test group 5 1350ppm $CuSO_4$**		**dose-response +/-**
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
Number of animals examined	20	20	20	20	20	20	20	20	20	20		0	-
Mortality	2	0	0	0	0	0	0	2	0	0			
Clinical signs							Discoloration of faeces	Discoloration of faeces	Discoloration of faeces	Discoloration of faeces	Discoloration of faeces	Discoloration of faeces	
Body weight													
Food consumption													
Ophthalmoscopic examination													
Clinical chemistry													
Haematology									- Increase in total bilirubin - Increase of white blood cells - Increased leukocytes - Increase in alanine aminotransferase - Increase in aspartate aminotransferase - Increase in cholesterol	Increase in total bilirubin	- Increase in total bilirubin - Increase in cholesterol	Increase in total bilirubin	
Urinalysis										Squamous epithelial cells in the urine sediment			

Table A6_5.1-2 Results of repeated dose toxicity study

Parameter	Control 0 ppm Cu-HDO		Test group 1 100 ppm Cu-HDO		Test group 2 200 ppm Cu-HDO		Test group 3 1000ppm Cu-HDO		Test group 4 3000ppm Cu-HDO		Test group 5 1350ppm $CuSO_4$		dose-response +/-
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
Kidneys													
Organ weight									Increase of relative and absolute weights				
Gross pathology													
Microscopic pathology									- Hyaline (fluorescent) droplets in renal proximal tubules - Proteinaceous casts in tubular lumina				
Liver													
Organ weight										Increase of relative weight			
Gross pathology													
Microscopic pathology							Swollen and pigmented Kupffer's cells	Swollen and pigmented Kupffer's cells	- Swollen and pigmented Kupffer's cells - Single cell necrosis	Swollen and pigmented Kupffer's cells	Swollen and pigmented Kupffer's cells	Swollen and pigmented Kupffer's cells	+
Digestive tract													
Gross pathology							Thickening of the forestomach wall	Thickening of the forestomach wall	Thickening of the forestomach wall	Thickening of the forestomach wall	Thickening of the forestomach wall	Thickening of the forestomach wall	+
Microscopic pathology								- Hyperkeratosis of the forestomach	- Hyperkeratosis and hyperplasia of the	- Hyperkeratosis and hyperplasia of the	- Hyperkeratosis in the forestomach	- Hyperkeratosis in the forestomach	+

Table A6_5.1-2 Results of repeated dose toxicity study

Parameter	Control 0 ppm Cu-HDO		Test group 1 100 ppm Cu-HDO		Test group 2 200 ppm Cu-HDO		Test group 3 1000ppm Cu-HDO		Test group 4 3000ppm Cu-HDO		Test group 5 1350ppm $CuSO_4$		dose-response +/-
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
								mucosa - Hyperplasia of glandular stomach mucosa	forestomach mucosa and edema in the submucosa - Hyperplasia of the glandular stomach mucosa - Hyperplasia of duodenal mucosa	forestomach mucosa and edema in the submucosa - Hyperplasia of glandular stomach mucosa - Hyperplasia of duodenal mucosa	- Hyperplasia in forestomach, glandular stomach, duodenum	- Hyperplasia in forestomach, glandular stomach, duodenum	

Section A 6.5.2 Chronic toxicity (non rodent)

Annex Point IIA 6.5

Specify heading, route and species

	Justification for non-submission of data	**Official use only**
Other existing data [☒] Limited exposure [☒]	Technically not feasible [☐] Scientifically unjustified [☐] Other justification [☐]	
Detailed justification: Undertaking of intended data submission [☐]	– The toxicity of Cu-HDO has been examined extensively. The available acute, subacute, and chronic studies show with the exception of a liver-toxicity, which occurs only at high doses, that no systematic toxicity has to be expected. The observed toxic effects are unequivocally based on the copper part of the Cu-HDO molecule. This property has been determined by parallel examinations with copper sulfate at appropriate dose. – The subchronic study has been performed on rodent as well as on non-rodent. No different effects were observed between the species. – Because Cu-HDO is used only in industrial area, the exposure is very limited. Testing of the chronic toxicity in a second species is therefore not necessary	X X X

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	June 2005
Evaluation of applicant's justification	Within the rat long term toxicity testing the target organs were not just the liver, but also the digestive tract and the kidneys. The effects were seen not only at high dose but also at intermediate doses of ca. 140 and 54 and 33 mg/kg bw/day within the rat 3, 12 and 24 months studies. Comparing the administration of Cu-HDO with the administration $CuSO_4$ (at dose levels resulting in a similar amount of Cu^{2+} intake), some effects on the digestive tract, the liver and the kidneys were observed in both test groups and can therefore be attributed to the Cu^{2+} ion in both substances. However several effects were only observed within the Cu-HDO group: Within the 3 months study iron pigment deposits in the tunica propria of duodenum and jejunum; within the 12 months study, the increase in white blood cell count (m), in lymphocytes (m), in alanine aminotransferase (m), in aspartate aminotransferase (m), in squamous epithelial cells in the urine sediment (f), increased liver weights in females (f), higher incidence of edema in the submucosa of the forestomach (m+f), higher incidence of single cell necrosis in the liver (m), and the higher incidence of hyaline droplets and proteinaceous casts in the kidneys (m); within the 24 months study the impaired body weight and body weight change (m), increased numbers of cysts in the liver (f), storage of iron-containing pigment in macrophages in the submucosa of the duodenum (m+f). However comparing the results of the dog and rat 90 day study the NOAELs result to be similar with 26 and 35 mg/kg bw/day respectively. Also the effects are similar, though in the dogs the liver effects were stronger including gross lesions, hepatitis and cirrhosis and as sequelae additionally edema in the gall bladder (2m,4f) and in the pancreas and mesentery (2m). No renal effects were observed in dogs. Vomiting was found only in dogs (m+f, mainly in the first week of administration), but this cannot be found in rats for physiological reasons.
Conclusion	The NOAELs from the rat and dog 3 months studies are similar and no additional toxicological targets are identified in the dog. The NOAELs from the rat 3 months studies is just slightly lower compared to the NOAEL from the 12 months study, that is 35 compared to 16 mg/kg bw/day and also the target organs liver, GI and kidney are similar. Because Cu-HDO is used only in industrial area, the exposure is limited. Therefore waiving of the chronic toxicity test on non-rodents was considered acceptable.
Remarks	-

Section A6.6.1 Genotoxicity in vitro

Annex Point IIA6.6.1 **Gene mutation in bacteria** *(Ames test)*

			Official use only
		1 REFERENCE	
1.1	**Reference**	A 6.6.1 [redacted] (1987) Report on the study of Cu-HDO in the AMES TEST, study No.: 40MO254/874050, [redacted]	
1.2	**Data protection**	Yes	
1.2.1	Data owner	BASF	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes OECD Guideline 471	
2.2	**GLP**	No Test was performed before implementation of GLP	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Cu-HDO	
3.1.1	Lot/Batch number		
3.1.2	Specification	No.: 87/254	
3.1.2.1	Description	Solid, blue	
3.1.2.2	Purity	99%	
3.1.2.3	Stability	Cu-HDO is stable at room temperatures for at least one year	
3.2	**Study Type**	Bacterial reverse mutation test	
3.2.1	Organism/cell type	S. typhimurium: TA 1535, TA 100, TA 1537, TA 98	x
3.2.2	Deficiencies / Proficiencies	- all strains have a defective excision repair system (uvrB), which prevents the repair of DNA lesions, and this deficiency results in greatly enhanced sensitivity of some mutagens. - Furthermore, all strains show a considerably reduced hydrophilic polysaccharide layer, which leads to an increase in permeability to lipophilic substances.	
3.2.3	Metabolic activation system	S9 mix	
3.2.4	Positive control	The following positive control substances are used to check the mutability of the bacteria and the activity of the S-9 mix with S-9 mix: 10 µg 2-aminoanthracene (dissolved in DMSO) for the strains TA 100, TA 98, TA 1537 and TA 1535	x

Section A6.6.1 Genotoxicity in vitro

Annex Point IIA6.6.1 **Gene mutation in bacteria** *(Ames test)*

		without S-9 mix: 5 µg N-methyl-N-nitro-N-nitroso-guanidine (MNNG) (dissolved in DMSO) for the strains TA 100 and TA 1535 10µg 4-nitro-o-phemylenediamine (dissolved in DMSO) for the strain TA 98 100µg 9-aminoacridine chloride monohydrate (dissolved in DMSO) for the strain TA 1537	
3.3	**Administration / Exposure; Application of test substance**	~~Non-entry field~~	
3.3.1	Concentrations	1.25µg – 5000µg (without S-9 mix) 3µg – 5000µg (with S-9 mix)	
3.3.2	Way of application	Test substance was diluted in DMSO	
3.3.3	Pre-incubation time	Method of Yahagi and Matsushima, 20 min	
3.3.4	Other modifications		
3.4	**Examinations**		
3.4.1	Number of cells evaluated		
		4 RESULTS AND DISCUSSION	
4.1	**Genotoxicity**	~~Non-entry field~~	
4.1.1	without metabolic activation	No	
4.1.2	with metabolic activation	No	
4.2	**Cytotoxicity**	No	X
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	SYSTEM OF TESTING: AMES TEST - Species/cell type: Strains TA 1535, TA 100, TA 1537, TA 98 - Deficiences/Proficiences: all strains have a defective excision repair system (uvrB), which prevents the repair of DNA lesions, and this deficiency results in greatly enhanced sensitivity of some mutagens. - Furthermore, all strains show a considerably reduced hydrophilic polysaccharide layer, which leads to an increase in permeability to lipophilic substances. - Metabolic activation system: S-9 fraction according to Ames ADMINISTRATION: - Dosing: 0-5000µg/plate - Number of replicates: 3 test plates per dose - Application: standard plate test with and without S-9 mix POSITIVE AND NEGATIVE CONTROL GROUPS AND	

Section A6.6.1 **Genotoxicity in vitro**

Annex Point IIA6.6.1 **Gene mutation in bacteria** *(Ames test)*

		TREATMENT: - positive and negative controls with and without S-9 mix tested with different well known mutagenic substances - Pre-incubation time: method of Yahagi and Matsushima, 20 min DESCRIPTION OF FOLLOW UP REPEAT STUDY: CRITERIA FOR EVALUATING RESULTS: - doubling of the spontaneous mutation rate (control) - dose response relationship - reproducibility of the results
5.2	**Results and discussion**	An increase in the number of his^+ revertants was not observed both in the standard plate test and in the preincubation test either without S-9 mix or after the addition of a metabolising system
5.3	**Conclusion**	According to the results of the present study, the test substance Cu-HDO is not mutagenic in the Ames test under the experimental conditions chosen here.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	June 2005
Materials and Methods	**3.2.1 Organism/ Cell type** According to the actual OECD guideline 471 at least 5 strains should be used. The test with the strains e.coli WP2 uvrA or WP2 uvrA (pKM101) or S.typhimurium TA102 is missing. Approximately 7.5% of the bacterial mutagens identified are detected by E.Coli WPuvrA but not by the standard set of 4 Salmonella strains (CPMP/IHC/1141/95). However the test was carried out before the use of these strains was compulsory. **3.2.4 Positive Control** with S-9 mix: OECD method 471 states that 2-Aminoanthracene should not be used as the sole indicator of the efficacy of the S9-mix. If 2-aminoanthracene is used, each batch of S9 should also be characterised with a mutagen that requires metabolic activation by microsomal enzymes, e.g. benzo(a)pyrene, dimethylbenzanthracene. without S-9 mix OECD method 471 recommends for TA98 2-Nitrofluorene and for TA1535 and TA100 sodium azide, but since the positive control results are strongly positive this deviations are not considered to be crucial. **4.2 Cytotoxicity** Bacterial toxicity at≥5µg per plate without S9 and≥50 with S9.
Results and discussion	Agree with applicant, but see conclusion
Conclusion	Agree with applicant, but because of not guideline conform positive control for S9 mix and just 4 strains tested and no GLP the study is considered not to be fully valid.
Reliability	2 (4 instead of 5 strains, not guideline conform positive control)
Acceptability	Acceptable with restrictions
Remarks	-

Table A6_6_1-1 Table for Gene Mutation Assay

Concentration [µg/per plate]	Number of mutant cells Strain: TA 1535		Comments *give information on cytotoxicity or other*
	— S9	+ S9	
0	16 ± 2	16 ± 2	
0.6	14 ± 3		
1.25	12 ± 3		
1.5		17 ± 2	
2.5	17 ± 1		
3		16 ± 1	
5	10 ± 5		
6		15 ± 3	
10	-		Reduced his^- background
12		17 ± 3	
25		13 ± 3	

Concentration [µg/per plate]	Number of mutant cells Strain: TA 100		Comments *give information on cytotoxicity or other*
	— S9	+ S9	
0	108 ± 9	111 ± 1	
0.6	100 ± 5		
1.25	118 ± 11		
1.5		110 ± 2	
2.5	117 ± 5		
3		122 ± 6	
5	113 ± 5		
6		105 ± 6	
10	-		Reduced his^- background
12		106 ± 4	
20			
25		98 ± 7	

Concentration [µg/per plate]	Number of mutant cells Strain: TA 1573		Comments *give information on cytotoxicity or other*
	— S9	+ S9	
0	12 ± 3	11 ± 1	
0.6	14 ± 1		
1.25	16 ± 6		
1.5		15 ± 4	
2.5	11 ± 3		
3		12 ± 1	
5	-		Reduced his^- background
6		12 ± 3	
10	0		
12		12 ± 4	
20			
25		11 ± 2	

Concentration [µg/per plate]	Number of mutant cells Strain: TA 98		Comments *give information on cytotoxicity or other*
	— S9	+ S9	
0	20 ± 3	32 ± 2	
0.6	22 ± 2		
1.25	21 ± 1		
1.5		31 ± 4	
2.5	17 ± 3		
3		30 ± 3	
5	-		Reduced his^- background
6		32 ± 2	
10	0		
12		31 ± 1	
20			
25		34 ± 5	

Section A6.6.2

Annex Point II6.6.2

Genotoxicity in vitro

Cytogenicity in mammalian cells

	Justification for non-submission of data	Official use only
Other existing data [☒]	Technically not feasible [☐] Scientifically unjustified [☒]	
Limited exposure [☐]	Other justification [☐]	
Detailed justification:	Waiving: Database on genotoxicity in vitro	X

Cu-HDO has been investigated for mutagenicity in bacteria (Ames test; OECD 471) and for unscheduled DNA synthesis (UDS) in primary rat hepatocytes (in which UDS activity was evaluated from H^3 TdR exposed slides via autoradiography; OECD 482). Both tests have shown negative results. Furthermore, the assay types are ideal tools of investigation for the following reasons:

The organic HDO moiety with its nitronium compounds and its potentially positive and/or negative electric charges should be investigated either in a system where metabolic activation and the target of genotoxic activity are located within the same cell (such as the rat liver UDS assay) or in a bacterial system with permeable cell membranes that are easily crossed by bulky and/or charged compounds (such as the Salmonella strains employed for the Ames assay). These procedures allow the best trapping of potential genotoxic metabolites resulting from aromatic nitrogen compounds which frequently have short half-life times and bear electric charges that prevent them from crossing the plasma membranes of established mammalian cell lines (such as CHO or V79 cells employed for gene mutation and chromosome aberration assays). These cell lines have little metabolic competence in transforming such compounds to active metabolites and need to rely on extracellular metabolic activation (by S-9 mix) in the culture medium. This limited suitability for the class of organic nitrogenium compounds may lead to false negative results due to a lack in adequate intracellular bioavailability of relevant metabolite(s).

A gene mutation assay in an established mammalian cell line (such as V79, CHO or mouse lymphoma cells; OECD 476) is therefore considered as unnecessary. For the same reasons, also a chromosome aberration study for clastogenic effects in established cell lines (OECD 473) is not considered as meaningful and should be omitted. If a result on those endpoints was positive, the consequence would be the execution of an in vivo study. This, however, has already been done by the mouse micronucleus assay (OECD 474), which showed a negative result.

The genotoxicity of Cu-ions is well investigated and, on balance, typically negative. The chemical structure alone of the HDO moiety does not preclude a mutagenic potential since it may appear to be quite closely related to phenylhydrazine or a hydroxamic acid (though on the other hand, no dermal sensitisation has been detected for this material as one may have expected for such compounds). The formation of a nitrosamine from the HDO moiety is excluded since the material is a primary (and not secondary) amine and has no α-oxidizable alkyl group linked to the nitrogen. Moreover, mutagenic nitrosamines are detected in the Ames and UDS assays.

Section A6.6.2 Genotoxicity in vitro

Annex Point II6.6.2 **Cytogenicity in mammalian cells**

	Short-term in vitro assays for mutagenicity and clastogenicity may be regarded as screening tools for potential carcinogenicity. It is quite unlikely that a mutagen does not cause carcinogenicity, if adequately tested. Hence, a non-carcinogen is also unlikely to be a mutagen. Cu-HDO has been adequately tested and shown to be non-carcinogenic. This is a further indication that no genotoxic potential for Cu-HDO is to be expected. In addition, other Cu compounds are not suspected as genotoxic agents. **Refinement of expert judgment to UDS test:** Mechanistically, the UDS assay is an indirect test for genotoxic events. Its results coincide to a high extent with the results of mammalian cell gene mutagenicity assays. In contrast to the bacterial Ames assay (and also to clastogenicity assays in mammalian cells) the sensitivity of mammalian gene mutation assays as well as the rat hepatocyte UDS assay is comparably low, but their specificity is high. From unpublished experiences in our own lab we see a trend that molecules bearing electric charges such as aromatic nitrogen compounds and their metabolites are less likely to cross the plasma membranes of mammalian cells and may go undetected in mammalian cell gene mutation assays. The UDS assay which unifies metabolic transformation and genotoxic endpoint within the same cell is therefore considered as more appropriate.
Undertaking of intended data submission [☐]	

Evaluation by Competent Authorities	
EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	June 2005
Evaluation of applicant's justification	The in vitro cytogenicity test is considered to mechanistically correspond to the in vivo micronucleus test, which is submitted as document A6.6.4.
Conclusion	The in vitro cytogenicity test is not required.
Remarks	The arguments supplied by the applicant are relevant for the justification of submitting the in vitro UDS test instead of the in vitro gene mutation test in mammalian cells (see document A6.6.3)

Section A6.6.3 Genotoxicity in vitro

Annex Point II6.6.3

Gene mutation in mammalian cells

Rat hepatocyte DNA repair assay [UDS] in vitro

	Justification for non-submission of data	Official use only
Other existing data [☒]	**Technically not feasible [☐]** **Scientifically unjustified [☐]**	
Limited exposure [☐]	**Other justification [☒]**	
Detailed justification:	**Waiving: Database on genotoxicity in vitro**	X

Cu-HDO has been investigated for mutagenicity in bacteria (Ames test; OECD 471) and for unscheduled DNA synthesis (UDS) in primary rat hepatocytes (in which UDS activity was evaluated from H^3 TdR exposed slides via autoradiography; OECD 482). Both tests have shown negative results. Furthermore, the assay types are ideal tools of investigation for the following reasons:

The organic HDO moiety with its nitronium compounds and its potentially positive and/or negative electric charges should be investigated either in a system where metabolic activation and the target of genotoxic activity are located within the same cell (such as the rat liver UDS assay) or in a bacterial system with permeable cell membranes that are easily crossed by bulky and/or charged compounds (such as the Salmonella strains employed for the Ames assay). These procedures allow the best trapping of potential genotoxic metabolites resulting from aromatic nitrogen compounds which frequently have short half-life times and bear electric charges that prevent them from crossing the plasma membranes of established mammalian cell lines (such as CHO or V79 cells employed for gene mutation and chromosome aberration assays). These cell lines have little metabolic competence in transforming such compounds to active metabolites and need to rely on extracellular metabolic activation (by S-9 mix) in the culture medium. This limited suitability for the class of organic nitrogenium compounds may lead to false negative results due to a lack in adequate intracellular bioavailability of relevant metabolite(s).

A gene mutation assay in an established mammalian cell line (such as V79, CHO or mouse lymphoma cells; OECD 476) is therefore considered as unnecessary. For the same reasons, also a chromosome aberration study for clastogenic effects in established cell lines (OECD 473) is not considered as meaningful and should be omitted. If a result on those endpoints was positive, the consequence would be the execution of an in vivo study. This, however, has already been done by the mouse micronucleus assay (OECD 474), which showed a negative result.

The genotoxicity of Cu-ions is well investigated and, on balance, typically negative. The chemical structure alone of the HDO moiety does not preclude a mutagenic potential since it may appear to be quite closely related to phenylhydrazine or a hydroxamic acid (though on the other hand, no dermal sensitization has been detected for this material as one may have expected for such compounds). The formation of a nitrosamine from the HDO moiety is excluded since the material is a primary (and not secondary) amine and has no α-oxidizable alkyl group linked to the nitrogen. Moreover, mutagenic nitrosamines are detected in the Ames and UDS assays.

Short-term in vitro assays for mutagenicity and clastogenicity may be

	regarded as screening tools for potential carcinogenicity. It is quite unlikely that a mutagen does not cause carcinogenicity, if adequately tested. Hence, a non-carcinogen is also unlikely to be a mutagen. Cu-HDO has been adequately tested and shown to be non-carcinogenic. This is a further indication that no genotoxic potential for Cu-HDO is to be expected. Also other Cu compounds are not suspected as genotoxic agents. **Refinement of expert judgment to UDS test:** Mechanistically, the UDS assay is an indirect test for genotoxic events. Its results coincide to a high extent with the results of mammalian cell gene mutagenicity assays. In contrast to the bacterial Ames assay (and also to clastogenicity assays in mammalian cells) the sensitivity of mammalian gene mutation assays as well as the rat hepatocyte UDS assay is comparably low, but their specificity is high. From unpublished experiences in our own lab we see a trend that molecules bearing electric charges such as aromatic nitrogen compounds and their metabolites are less likely to cross the plasma membranes of mammalian cells and may go undetected in mammalian cell gene mutation assays. The UDS assay which unifies metabolic transformation and genotoxic endpoint within the same cell is therefore considered as more appropriate.	
Undertaking of intended data submission [☐]		

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	27.06.2005
Evaluation of applicant's justification	**The applicants arguments are summarised as follows:** The in vitro UDS was preferred to the in vitro gene mutation assay in mammalian cells since: 1. Mechanistically, the UDS assay is an indirect test for genotoxic events. Its results coincide to a high extent with the results of mammalian cell gene mutagenicity assays. In contrast to the bacterial Ames assay (and also to clastogenicity assays in mammalian cells) the sensitivity of mammalian gene mutation assays as well as the rat hepatocyte UDS assay is comparably low, but their specificity is high. 2. In contrast to the recommended cells for the B17 test it allows to use primary metabolically active cells. The aromatic nitrogen compounds (generated by metabolism) frequently have short half-life times and bear electric charges that prevent them from crossing the plasma membranes of established mammalian cell lines (experience within their lab, no further reference) 3. If a result on those endpoints was positive, the consequence would be the execution of an in vivo study. This, however, has already been done by the mouse micronucleus assay (OECD 474), which showed a negative result. (Our observation: However in case of a positive in vitro and a negative in vivo bone marrow test another non bone marrow in vivo test should be carried out –this has not been done) 4. A negative carcinogenicity study is available. 5. The genotoxicity of Cu-ions is well investigated and, on balance, typically negative. 6. The chemical structure alone of the HDO moiety does not preclude a mutagenic potential since it may appear to be quite closely related to phenylhydrazine or a hydroxamic acid (though on the other hand, no dermal sensitization has been detected for this material as one may have expected for such compounds). The formation of a nitrosamine from the HDO moiety is excluded since the material is a primary (and not secondary) amine and has no α-oxidizable alkyl group linked to the nitrogen. Moreover, mutagenic nitrosamines are detected in the Ames and UDS assays.
Conclusion	The justification for submitting the in vitro UDS instead of the in vitro gene mutation assay in mammalian cells is acceptable
Remarks	**Our observations:** 1. A potential additional and positive in vitro test could not overrule the negative in vivo mutation test and the negative carcinogenicity study. 2. A negative in vitro gene mutation test was submitted for K-HDO, which supports that the HDO moiety is non mutagenic in vitro.

Section A6.6.3	**Genotoxicity in vitro**
Annex Point II6.6.3	**Gene mutation in mammalian cells** Rat hepatocyte DNA repair assay [UDS] in vitro

			Official use only
		1 REFERENCE	
1.1	**Reference**	A 6.6.3 [redacted] (1992) Rat hepatocyte DNA repair assay [UDS] in vitro: 81MO679/894495, [redacted]	
1.2	**Data protection**	Yes	
1.2.1	Data owner	BASF AG	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes OECD Guideline 482	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	
		3 MATERIALS AND METHODS	
3.1	**Test material**	Cu-HDO	
3.1.1	Lot/Batch number	Reu E 7360	
3.1.2	Specification		
3.1.2.1	Description	Slightly bluish crystals	
3.1.2.2	Purity	Content of active ingredient 89.0%; molar purity 99.7 Mol%; checked via analysis	
3.1.2.3	Stability	Reanalysis after completion of all studies	
3.2	**Study Type**	Unscheduled DNA synthesis in mammalian cells in vitro	
3.2.1	Organism/cell type	Primary rat hepatocytes	
3.2.2	Deficiencies / Proficiencies		
3.2.3	Metabolic activation system	Metabolic activation and genetic endpoint are located within the same cell	
3.2.4	Positive control	2-Acetaminofluorence (2-AAF); 10µM	
3.3	**Administration / Exposure; Application of test substance**	Non-entry field	
3.3.1	Concentrations	0.1, 0.033, 0.01, 0.01, 0.0033, 0.001, 0.00033µg/ml	
3.3.2	Way of application	The test substance was dissolved in DMSO then diluted with DMSO and then given directly to Williams E medium covering the cells.	
3.3.3	Pre-incubation time		X
3.3.4	Other modifications		

3.4	**Examinations**	- the net nuclear grain count of each cell (= nuclear count minus cytoplasmic count) - the mean nuclear count - the mean cytoplasmic count - mean net nuclear grain count - the percentage of cells in repair (cells showing net grain count>5 and>0)	X
3.4.1	Number of cells evaluated		X
		4 RESULTS AND DISCUSSION	
4.1	**Genotoxicity**	Non-entry field	
4.1.1	without metabolic activation		
4.1.2	with metabolic activation	Metabolic activation system: metabolic activation and genetic endpoint are located within the same cell	
4.2	**Cytotoxicity**	No cytotoxic concentration: >0.1µg/mL	X
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and methods**	- Guideline: OECD Guideline 482 - Species/cell type: wistar rat/ hepatocytes - Metabolic activation system: metabolic activation and genetic endpoint are located within the same cell - No. of metaphases analyzed: ADMINISTRATION: - Dosing: 0.1µg/L, 0.033µg/L, 0.01µg/L, 0.0033µg/L, 0.001µg/L, 0.0003µg/mL - Application: the test chemical was added to freshly isolated rat hepatocytes attached to coverslips after an approximately 2 hours attachment phase in FCS-supplemented Williams E medium. [^{3}H]-thymidine was added during the 18 hours exposure time. The cells were washed and fixed and subsequently examined for UDS by autoradiography. The experimental details of the assay system used in this laboratory have been published [Butterworth et al., 1987] POSITIVE AND NEGATIVE CONTROL GROUPS AND TREATMENT: - positive control with 2-acetaminofluorence (2-AAF) - negative control with 0-control DESCRIPTION OF FOLLOW UP REPEAT STUDY: CRITERIA FOR EVALUATING RESULTS: test result is considered as positive if at least in one dose the mean net grain is>5 and the percentage of cells in repair (with net grains>5) is>20%.	
5.2	**Results and discussion**	GENOTOXIC EFFECTS: - With metabolic activation: metabolic activation and genetic endpoint are located within the same cell: negative - CYTOTOXIC CONCENTRATION: >0.1µg/mL - STATISTICAL RESULTS: not genotoxic under the test conditions employed	X

5.3	**Conclusion**	In none of the concentrations employed the test chemical showed net grain values of>0. The average net grains values were consistently in the range of –3 to –4. This was also the range of the solvent and blank controls. The percentage of cells in repair was unchanged in relation to the controls (<2% for net grain values of>5 and<22% for net grain values of>0). The positive control showed a mean net grain value of 11.56 ± 18.96; percentage of cells with net grains>5 was 43% and with net grains>0 was 76%. The conclusion from the study is that the test material did not show genotoxic activity under the test conditions employed.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

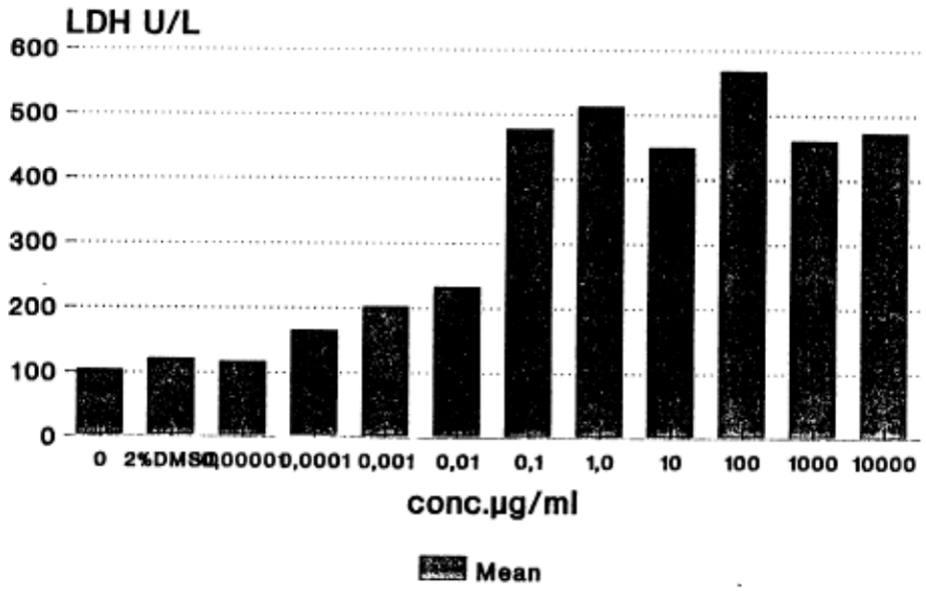
	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	December 2005
Materials and Methods	**3.3.3 Pre-incubation time** The attachment phase for the cells without test substance was 2 hours. **3.4 Examinations** In total 3 slides per test-concentration were read, resulting in about 100 cells per test concentration. This meets the OECD recommended amount of 2x 50 cells per slide.
Results and discussion	**4.2 Cytotoxicity and 5.2 Results and discussion** The highest applied concentration of 0.1µg/ml is cytotoxic. The not cytotoxic concentration is<0.1µg/ml. The range of tested concentrations meets the recommendation of cytotoxic effects in the highest concentration. **Table A6._6._1.-1Table for in vitro UDS test: Summary data from 18 hours exposure** "Percentage of cells in repair>5" and "Percentage of cells in repair>0" means percentage of cells with net grains>5 and>0, respectively. A nucleus with a net grain of>5 was considered to represent a positive result (nucleus in repair). Evaluation of nuclei with a net grain of>0 was considered as additional criterion for doubtful cases.
Conclusion	Agree with applicant's version
Reliability	1
Acceptability	Acceptable
Remarks	-

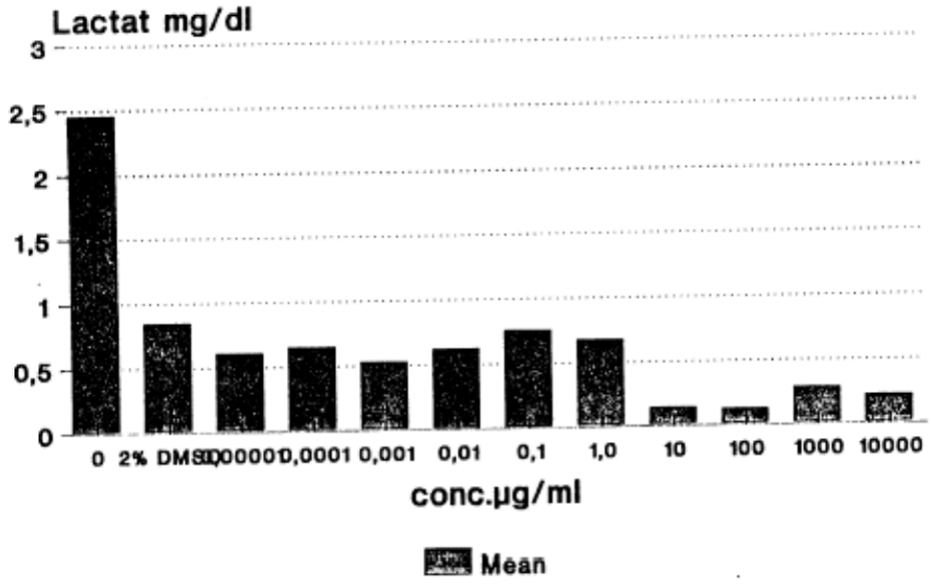
Table A6_6_1-1Table for in vitro UDS test: Summary data from 18 hours exposure

Test Article: Cu-HDO Treatment Details	Mean Nuc Count (± SD)	Mean Cyt Count (± SD)	Mean Net Grain Count (± SD)	Percentage of cells in Repair > 0	Percentage of cells in Repair > 5
0-Kontrolle	13.81 ± 3.96	17.84 ± 4.04	-4.03 ± 3.41	14	0
5% DMSO	14.99 ± 4.43	18.22 ± 4.65	-3.23 ± 3.00	18	0
0.0003 µg/ml	14.49 ± 4.59	17.86 ± 4.85	-3.37 ± 3.45	15	1
0.001 µg/ml	10.81 ± 5.91	13.70 ± 6.40	-2.89 ± 2.58	17	0
0.0033 µg/ml	9.26 ± 6.22	12.50 ± 7.64	-3.24 ± 3.52	23	0
0.01 µg/ml	10.68 ± 4.04	13.94 ± 3.75	-3.26 ± 3.65	18	2
0.033 µg/ml	9.62 ± 3.67	13.51 ± 4.36	-3.89 ± 3.40	13	0
0.1 µg/ml	11.59 ± 4.09	16.14 ± 4.42	-4.55 ± 3.74	11	0
10 µM 2-AAF	19.37 ± 23.96	7.81 ± 6.49	11.56 ± 18.96	76	43

Section A6.6.3	**Genotoxicity in vitro**
Annex Point II6.6.3	**Gene mutation in mammalian cells** Rat hepatocyte DNA repair assay [UDS] in vitro

Cytotoxicity graphs:





Section A6.6.4 **Genotoxicity in vivo**
Annex Point IIA6.6.4 / 6.6.5 / 6.6.6 *Micronucleus test*

			Official use only
		1 REFERENCE	
1.1	**Reference**	A 6.6.4 [redacted] (1990) Micronucleus assay in bone marrow cells of the mouse with Bis-(N-Cyclohexyldiazeniumdioxy)-Kupfer: 26M0679/899010, [redacted]	
1.2	**Data protection**	Yes	
1.2.1	Data owner	BASF	
1.2.2	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA Guidelines and Quality Assurance	
1.3	**Guideline study**	Yes - OECD Guideline 474 "Genetic Toxicology: Micronucleus Test" - EEC Directive 84/449, L 251, B 12, p. 137 – 139 - Environmental Protection Agency, Code of Federal Regulations, Title 40, Subpart F-Genetic Toxicity, Revision July 1, 1986, "In vivo mammalian bone marrow cytogenetics tests: Micronucleus assay"	
1.4	**GLP**	Yes	
1.5	**Deviations**	No	
		2 MATERIALS AND METHODS	
2.1	**Test material**	Cu-HDO	
2.1.1	Lot/Batch number	Reu E 7360	
2.1.2	Specification		
2.1.2.1	Description	Solid, blue	
2.1.2.2	Purity	99.7 Mol-%	X
2.1.2.3	Stability	Cu-HDO is stable at room temperatures for at least one year In vehicle: the stability of the test substance in polyethylene glycol 400 was determined analytically.	
2.1.2.4	Maximum tolerable dose	500mg/kg bw	
2.2	**Test Animals**	Non-entry field	
2.2.1	Species	mouse	
2.2.2	Strain	NMRI	
2.2.3	Source	[redacted]	
2.2.4	Sex	male and female	
2.2.5	Age/weight at study initiation	Approx. 30g	
2.2.6	Number of animals per	5m + 5f per test group	

	group	
2.2.7	Control animals	Yes
2.3	**Administration / Exposure**	Oral
2.3.1	Number of applications	1
2.3.2	Interval between applications	
2.3.3	Postexposure period	
		Oral
2.3.4	Type	
2.3.5	Concentration	24 h preparation interval: 50, 170 and 500mg/kg bw 48 h preparation interval: 500mg/kg bw 72 h preparation interval: 500mg/kg bw
2.3.6	Vehicle	Polyethylene glycol 400
2.3.7	Concentration in vehicle	24 h preparation interval: 50, 170 and 500mg/kg bw 48 h preparation interval: 500mg/kg bw 72 h preparation interval: 500mg/kg bw
2.3.8	Total volume applied	10ml/kg bw
2.3.9	Controls	Negative control: Vehicle (PEG) Positive control: reference mutagen (cyclophosphamide)
2.4	**Examinations**	
2.4.1	Clinical signs	No
2.4.2	Tissue	bone marrow
		Number of animals: all animals
		Number of cells: 20, 100, 200, 1000, 2000 or other
		Time points: 24, 48, 72 h after treatment or other
		Type of cells erythrocytes in bone marrow
		Parameters: polychromatic/normochromatic erythrocytes ratio
2.5	**Further remarks**	

Section A6.6.4	Genotoxicity in vivo
Annex Point IIA6.6.4 / 6.6.5 / 6.6.6	*Micronucleus test*

		3 RESULTS AND DISCUSSION	
3.1	**Clinical signs**	—	X
3.2	**Haematology / Tissue examination**	—	
3.3	**Genotoxicity**	No	
3.4	**Other**		X
		4 APPLICANT'S SUMMARY AND CONCLUSION	
4.1	**Materials and methods**	TEST ORGANISMS: mouse, strain NMRI - Age: minimum 10 weeks - Weight at study initiation: approximately 30g - No. of animals per dose: six males and six females ADMINISTRATION: - Vehicle: polyethylene glycol 400 - Duration of test: 24 h, 48 h, 72 h post-treatment - Frequency of treatment: once - Sampling times and number of samples: 24 h, 48 h, 72 h, 108 animals - Control groups and treatment: negative control group fed with vehicle, 24 h treatment, 48 h treatment 72 h treatment, positive control: fed with CPA, singly - Criteria for evaluating results: evaluation of the slides was performed using Nikon microscopes with 100x oil immersion objective. 1000 polychromatic erythrocytes were analysed per animal for micronuclei. To describe a cytotoxic effect the ratio between polychromatic and normo-chromatic erythrocytes were determined in the same sample and expressed as normo-chromatic erythrocytes per 1000 PCEs.	
4.2	**Results and discussion**	During the study and under the experimental conditions reported, the test article did not induce micronuclei at any preparation interval after treatment with the test article and with any dose level used. STATISTICAL RESULTS: The result is confirmed by the nonparametric Mann-Whitney test	X
4.3	**Conclusion**	Cu-HDO is considered to be non-mutagenic in this micronucleus assay.	
4.3.1	Reliability	1	
4.3.2	Deficiencies	No	

Table A6_6_4-1 Table for Micronucleus Test In Vivo

		control group 0mg/kg bw			**low dose 50mg/kg bw**			**mid dose 170mg/kg bw**			**high dose 500mg/kg bw**		
Number of cells evaluated		1000 polychromatic erythrocytes per animal			1000 polychromatic erythrocytes per animal			1000 polychromatic erythrocytes per animal			1000 polychromatic erythrocytes per animal		
Sampling time (h)		24	48	72	24	48	72	24	48	72	24	48	72
Number of erythrocytes	**Normochromatic**												
	Polychromatic												
	PCEs with micronuclei (%)	0.09	0.03	0.01	0.11			0.02			0.03	0.05	0.05
Ratio of erythrocytes	**polychromatic / normochromatic**	1000/ 718	1000/ 837	1000/ 789	1000/ 854			1000/ 732			1000/ 879	1000/ 1074	1000/ 760
	range	0-2	0-1	0-1	0-3			0-1			0-1	0-2	0-2

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	June 2005
Materials and Methods	**2.1.2.2 Purity** The study report states that this corresponds to 89% to 91% (w/w) purity.
Results and discussion	Agree with the applicant's version
Conclusion	Agree with applicant's version
Reliability	1
Acceptability	Acceptable
Remarks	**3.1 Clinical signs** 1000mg/kg bw (2 males, 2 females) reduction in spontaneous activity: 2m, 2f abdominal position: 1m eyelid closure: 1m, 1f apathy: 1m, 1f death: 1m (48h after treatment) 750mg/kg bw (2 males, 2 females) reduction in spontaneous activity: 2m, 2f eyelid closure: 2m, 1f apathy: 2m, 1f death: 1m (6h after treatment) 500mg/kg bw (2males, 2 females) reduction in spontaneous activity: 2m, 2f eyelid closure: 2m, 1f apathy: 2m, 1f On the basis of these results 500mg/kg bw was estimated to the maximum tolerated dose. **3.4 Other (compare to table 6.6.4.1 above)** The positive control cyclophosphamide (40mg/kg bw) induced at the sampling time of 24h 0.85% PCEs with micronuclei with a PCE/NCE of 1000/828. **4.2 Results** A slight cytotoxicity indicated by a slight decrease of the ratio of immature polychromatic to mature normochromatic erythrocytes was observed in the high dose group with the 48 hours sampling time point. This provides some evidence that the Cu-HDO dose reached the bone marrow and thus the absence of micronucleated polychromatic erythrocytes can be considered to be a reliable indicator for the absence of genotoxicity.

Section A6.7	**Carcinogenicity**	
Annex Point IIA6.7	*Rat*	
	1 REFERENCE	**Official use only**
1.1 **Reference**	A 6.7 [redacted] (1996) Carcinogenicity study with Bis-(N-cyclohexyl-diazeniumdioxy)-copper in Wistar rats Administration in the diet for 24 months: 70C0679/89113, [redacted]	
1.2 **Data protection**	Yes	
1.2.1 Data owner	BASF AG	
1.2.2 Companies with letter of access	No company has the right to use these data on behalf of the data owner	
1.2.3 Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA	
	2 GUIDELINES AND QUALITY ASSURANCE	
2.1 **Guideline study**	Yes	X
2.2 **GLP**	Yes	
2.3 **Deviations**	No	
	3 MATERIALS AND METHODS	
3.1 **Test material**	1. Cu-HDO 2. $Cu\text{-}SO_4$	
3.1.1 Lot/Batch number	1. Reu E 7360 2. 133A583191	
3.1.2 Specification		
3.1.2.1 Description	1. Solid, metallic violet 2. Solid, grey	
3.1.2.2 Purity	1. Pro analysi 2. pro analysi	X
3.1.2.3 Stability	The stability of Cu-HDO and Cu-SO4 was proven by reanalysis after in the in life phase of the study	
3.2 **Test Animals**	Non-entry field	
3.2.1 Species	Rats	
3.2.2 Strain	Chbb: THOM (SPF)	
3.2.3 Source	[redacted]	
3.2.4 Sex	Male and female	
3.2.5 Age/weight at study initiation	42 days males: mean 179 (163 – 195) g females: mean 142 (123 . 159) g	
3.2.6 Number of animals per group	50 male and 50 female	
3.2.6.1 at interim sacrifice		
3.2.6.2 at terminal sacrifice		
3.2.7 Control animals	Yes	

Section A6.7		**Carcinogenicity**	
Annex Point IIA6.7		*Rat*	
3.3	**Administration/ Exposure**	Oral	
3.3.1	Duration of treatment	24 months	
3.3.2	Interim sacrifice(s)		
3.3.3	Final sacrifice		
3.3.4	Frequency of exposure	daily	
3.3.5	Post-exposure period	16 – 20 hours	
		Oral	
3.3.6	Type	in food	
3.3.7	Concentration	Food: / 0 / 100/ 600 / 3000ppm Food consumption per day: ad libitum	X
3.3.8	Vehicle	Diet	
3.3.9	Concentration in vehicle	0 / 100/ 600 / 3000ppm	
3.3.10	Total volume applied		
3.3.11	Controls	Vehicle (plain diet)	
3.4	**Examinations**		
3.4.1	Body weight	Yes The body weigh was determined for the randomisation of the animals before the start of the administration period. Then body weigh was determined once a week during the first 13 weeks of the study and thereafter at 4-week intervals.	
3.4.2	Food consumption	Yes The food consumption (over a period of one week) was determined once a week during the first 13 weeks of the study and thereafter at 4-week intervals. The values were calculated as food consumption per animal per day.	
3.4.3	Food efficiency	Yes Food efficiency (group means) was calculated based upon individual values for body weight and food consumption.	
3.4.4	Intake of test substance	Yes The mean daily intake of the test substance (group means) was calculated based upon individual values for body weight and food consumption.	
3.4.5	Water consumption	No	X
3.4.6	Clinical signs	Yes Parameters and time points: Examinations concerning dead or moribund animals or evident signs of toxicity were carried out twice a day (Mondays to Fridays) and once a day (Saturdays, Sundays and on public holidays). Additional comprehensive clinical examinations and palpations of the animals were carried out al least once a week until start of necropsy. Thereafter, the remaining animals were examined individually depending on the health status.	

Section A6.7		**Carcinogenicity**		
Annex Point IIA6.7		*Rat*		
3.4.7	Macroscopic investigations	Yes		
3.4.8	Ophthalmoscopic examination	No		X
3.4.9	Haematology	Yes		
		Number of animals:	Only the blood smears of the control groups, the highest dose groups and the $CuSO_4$ group were evaluated according to the study protocol from Dec. 27, 1991	
		Time points:	About 12, 18, 24 months after the beginning of the administration period	
		Parameters:	Differential blood count	
3.4.10	Clinical Chemistry	No		X
3.4.11	Urinalysis	No		X
3.4.12	Pathology	Yes All surviving animals and animals which died intercurrent were assessed by gross pathology		
3.4.12.1	Organ Weights	Yes		
		From:	All animals sacrificed at scheduled dates	
		Organs:	Liver, kidneys, brain, adrenals, adrenal glands; testes	
3.4.12.2	Histopathology	Yes		
		From:	All surviving animals and animals which died intercurrent	
		Organs:	Brain, pituitary gland, thyroid and parathyroid glands, thymus, trachea, lungs, aorta, heart, salivary glands, liver, spleen, kidneys, adrenal glands, esophagus, stomach, duodenum, jejunum and ileum, cecum, colon and rectum, uterus and vagina, urinary bladder, lymph nodes, pancreas, testes/ovaries, oviduct, accessory genital organs, female mammary gland, skin, skeletal muscle, sciatic nerve, spinal cord, sternum with bone marrow, bone marrow (femur), femur with knee joint, eyes	
3.4.13	Other examinations	Mortality		
3.5	**Statistics**	Statistical evaluation was carried out on the computer system of the department of toxicology (e.g. variance was done via the F-test, comparisons between the groups were performed via DUNNETT's test, a comparison of the body weight data was carried out using the STUDENT's test, calculation of means, standard deviation)		
3.6	**Further remarks**			

4 RESULTS AND DISCUSSION

4.1 Body weight

3000 ppm Cu-HDO (about 169mg/kg bw)

- impairment of body weight in males, resulting in reduced values of about 10% after 24 months. No such effects were seen after administration of $CuSO_4$
- impairment of body weight change in males, resulting in reduced values of about 12% after 24 months. No such effects were seen after administration of $CuSO_4$

Section A6.7 **Carcinogenicity**

Annex Point IIA6.7 *Rat*

4.2 Food consumption

In test group 1 – 4, some statistically significant deviations (either increased or decreased values) were noted when compared to the control group. However, due to the slightness of the effect and the lack of a dose-response relationship within test groups 1-3, this was assessed as being incidental and biologically not relevant.

4.3 Food efficiency

No changes suggesting clear influence on the food efficiency were seen when comparing the individual dose groups of either sex with the corresponding control groups

4.4 Intake of test substance

The mean daily substance intake in mg/kg body weight as well as the intake of Cu^{2+} for the entire administration period is represented in the table below:

	male animals		female animals		all animals	
	test substance (mg/kg bw)	Cu^{2+} (mg/kg bw)	test substance (mg/kg bw)	Cu^{2+} (mg/kg bw)	test substance (mg/kg bw)	Cu^{2+} (mg/kg bw)
Test group 1 (100 ppm Cu-HDO)	5	1	6	1	6	1
Test group 2 (600 ppm Cu-HDO)	29	5	37	7	33	6
Test group 3 (3,000 ppm Cu-HDO)	148	27	189	34	169	31
Test group 4 (1,350 ppm $CuSO_4$)	67	27	87	35	77	31

* Calculated for $C_{12}H_{22}N_4O_4Cu$ (= Cu-HDO): ≙ 18.2% Cu^{2+} and for $CuSO_4$ ≙ 40.1% Cu^{2+}

4.5 Water consumption

—

4.6 Clinical signs

In males and females of test groups 3 (3000ppm Cu-HDO) and 4 (1350ppm $CuSO_4$), a black discoloration of the faeces was observed from day 7 onwards until the end of the administration period. This finding was observed already in earlier studies with the test substance or reference substance and was considered to represent a chemical reaction of the test substance in the digestive tract rather than being the consequence of a toxic effect.

All other clinical findings occurred sporadically in all test groups without dose-response relationship.

Thus, no substance-related adverse findings were obtained.

Mortality:

In males, the mortality until day 728 was 28% in test group 0, 18% in test group 1, 26% in test group 2, 30% in test group 3, and 32% in test group 4. In females, the mortality until day 728 was 34% in test group 0, 24% in test group 1, 28% in test group 2, and 24% in test groups 3 and 4.

Thus, no substance-related effects were seen

4.7 Macroscopic investigations

— X

4.8 Ophthalmoscopic examination

—

4.9 Haematology

- White blood cells

  After 12, 18 and 24 months of test substance administration no treatment related changes were observed in the white blood cells of the male and female rats given 3000 ppm of the test compound or 1350

Section A6.7		**Carcinogenicity**	
Annex Point IIA6.7		*Rat*	
		ppm of $CuSO_4$. - Red blood cell After 12, 18 and 24 months of test substance administration no treatment related changes were observed in the red blood cells of the male and female rats given 3000 ppm of the test compound or 1350ppm of $CuSO_4$. - Prematurely killed animals Changes in white and red blood cells were observed in few animals killed in extremis. However, these changes were considered to be spontaneous, incidental, or age-related. Accordingly, these findings are not associated with the test compound administered.	
4.10	**Clinical Chemistry**	—	
4.11	**Urinalysis**	—	
4.12	**Pathology**	Substance related findings after treatment with 3000 ppm Cu-HDO were observed in the forestomach (e.g. hyperplasia of epithelium, hyperkeratosis, submucosal edema), in the duodenum (storage of an iron-containing pigment in the macrophages), and in the liver (e.g. centrolubular liver cell vacuolation, single liver cell necrosis, copper storage in Kupffer cells and in hepatocytes, increased incidence of hepatic cysts). At 600ppm, still some of the above mentioned findings in the forestomach were seen. Comparing these findings with the findings obtained after treatment with Cu-SO4, the formation of cysts in the liver and the storage of iron-containing pigment in the macrophages of the duodenum were attributable to Cu-HDO only, but not to $CuSO_4$. There was no indication of a carcinogenic potential neither after treatment with Cu-HDO nor after treatment with $CuSO_4$.	
4.13	**Organ Weights**		X
4.14	**Histopathology**	- The graded severity of cellular hyperplasia of the forestomach's epithelium was slightly higher in males and females. This effect is comparable to the results obtained in the $CuSO_4$ group. - The number of animals affected with hyperkeratosis of the forestomach's wall as well as the graded severity of it, were increased in males and in females. This effect is comparable to the results obtained in the $CuSO_4$ group. - Increased incidences of submucosal edema in the forestomach's wall in males (39/50) and females (33/50). This effect is comparable to the results obtained in the $CuSO_4$ group. - Storage of an iron-containing pigment in macrophages in the submucosa of the duodenum of 16/50 males and 19/50 female rats. Centrolobular liver cell vacuolisation in males (26/50). This effect is comparable to the results obtained in the $CuSO_4$ group. - Single liver cell necrosis in 11/50 female rats. This effect is comparable to the results obtained in the $CuSO_4$ group. - Copper storage in Kupffer cells and in hepatocytes of female rats. This effect is comparable to the results obtained in the CuSO4 group. - Increased incidence of hepatic cysts in females (23/50).	X
4.15	**Other examinations**		

Section A6.7 **Carcinogenicity**

Annex Point IIA6.7 *Rat*

4.16 Time to tumours

There was no indication of a carcinogenic potential, as the number of animals with neoplasms, the number of animals with one or more than one primary neoplasm, as well as the number of animals with benign, malignant systemic or metastasized neoplasms, respectively, and the total number of primary neoplasms, comprising benign, malignant, systemic or metastasised primary tumours did not differ biologically from controls.

4.17 Other

No substance-related effects concerning mortality were seen.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

OECD Guidelines for Testing of Chemicals; Method No. 451: Carcinogenicity Studies: May 12, 1981

U.S. EPA, Pesticide Assessment Guidelines, Subdivision F; § 83-2: Oncogenicity Study: NTIS, Revised edition, November 1984, p. 117 – 125

EC Commission directive 87/302/EEC of November 18,1987, Part B: Methods for the determination of Toxicity

5.2 Results and discussion

Bis-(N-Cyclohexyldiazeniumdioxy)-copper (Cu-HDO) was administered to male and female Wistar rats at dietary concentrations of 0, 100, 600, and 3000 ppm for 24 months. As reference substance for Cu^{2+} toxicity, an additional group was treated with $CuSO_4$ at a concentration of 1350 ppm.

At concentrations of 3000 ppm Cu-HDO or 1350 ppm $CuSO_4$ a black discoloration of faeces was observed. This finding was observed already in earlier studies with the test substance or reference substance and was considered to represent a chemical reaction of the test substance in the digestive tract rather than being the consequence of a toxic effect.

No treatment related changes in the white and red blood cells of the differential blood count were obtained.

Pathology revealed substance-related findings after treatment with 3000 ppm Cu-HDO in the forestomach (e.g. hyperplasia of epithelium, hyperkeratosis, submucosal edema), in the duodenum (storage of an iron-containing pigment in the macrophages), and in the liver (e.g. centrilobular liver cell vacuolation, single cell necrosis, copper storage in Kupffer cells and in hepatocytes, increased incidence of hepatic cysts). At 600 ppm still some of the above mentioned findings in the forestomach were seen.

Comparing these findings with the findings obtained after treatment with $CuSO_4$, the formation of cysts in the liver and the storage of iron-containing pigment in the macrophages of the duodenum were attributable to Cu-HDO, only, but not to $CuSO_4$.

There was no indication of a carcinogenic potential neither after treatment with Cu-HDO nor after treatment with $CuSO_4$. the number of animals with neoplasms, as well as the number of animals with one or more than one primary neoplasms, as well as the number of animals with benign, malignant, systemic, or metastasized primary tumors did no differ biologically from controls.

In conclusion following substance-related adverse findings were obtained after administration of Cu-HDO (at the highest concentration with the results obtained after administration of $CuSO_4$):

3000 ppm Cu-HDO (about 169mg/kg bw)

- impairment of body weight in males, resulting in reduced values of about 10% after 24 months. No such effects were seen after administration of $CuSO_4$
- impairment of body weight change in males, resulting in reduced values of about 12% after 24 months. No such effects were seen after

Section A6.7 **Carcinogenicity**
Annex Point IIA6.7 *Rat*

administration of CuSO4

- thickening of the forestomach's mucosa at necropsy in 25/50 males and in 23/50 females, either focal (in the region of the limiting ridge/margo plicatus) or diffusely. Similar effects were seen after administration of $CuSO_4$.
- Increased numbers of cysts in the liver in female animals (18/50) at necropsy. This effect was not observed after treatment with $CuSO_4$.
- The graded severity of cellular hyperplasia of the forestomach's epithelium was slightly higher in males and females. Similar effects were seen after administration of $CuSO_4$.
- The number of animals affected with hyperkeratosis of the forestomach's wall as well as the graded severity of it were increased in males and in females. Similar effects were seen after administration of $CuSO_4$.
- Increased incidences of submucosal edema in the forestomach's wall in males (39/50) and females (33/50). Similar effects were seen after administration of $CuSO_4$ in males (36/50) but not in females (23/50)
- Storage of an iron-containing pigment in macrophages in the submucosa of the duodenum of 16/50 male and 19/50 female rats. This effect was not observed after treatment with $CuSO_4$.
- Centrilobular liver cell vacuolization in males (26/50). Similar effects were seen in principle after administration of $CuSO_4$.
- Single cell necrosis in 11/50 female rats. Similar effects were seen in principle after administration of $CuSO_4$.
- Copper storage in Kupffer cells and in hepatocytes of female rats (13 females affected with one or the other location of storage or both). Similar effects were seen in principle after administration of $CuSO_4$.
- Increased incidence of hepatic cysts in females (23/50). This effect was not observed after treatment with $CuSO_4$.

Group 2: 600ppm Cu-HDO (about 33mg/kg body weight):

- The graded severity of cellular hyperplasia of the forestomach's epithelium was slightly higher in males
- Increased number of males with hyperkeratosis of the forestomach's wall

Group 1: 100ppm cu-HDO (about 6mg/kg bodyweight):

- No substance related effects

Thus, toxic effects were seen at 3000 ppm in both sexes and to a lower extent at 600ppm in males. Target organs were forestomach, liver, and duodenum. The effects regarding body weight (males) or histopathological findings in forestomach and liver fulfil the criteria for a maximum tolerated dose (MTD). Besides the effects observed on body weight or duodenum, similar findings were seen also in the group treated with 1350 ppm $CuSO_4$; they may thus be related to the toxicity of Cu^{2+} itself.

The no observed adverse effect level (NOAEL) under the conditions of this study was 100 ppm (about 6 mg/kg body weight) in males and 600 ppm (about 33 mg/kg body weight) in females.

There was no indication of a carcinogenic potential neither after treatment with Cu-HDO not after treatment with $CuSO_4$.

Thus it can be stated that Bis-(N-Cyclohexyldiazeniumdioxy)-copper (Cu-

Section A6.7		**Carcinogenicity**
Annex Point IIA6.7		*Rat*
		HDO) is not carcinogenic to male and female Wistar rats under the conditions of this study.
5.3	**Conclusion**	There was no indication of a carcinogenic potential neither after treatment with Cu-HDO nor after treatment with $Cu\text{-}SO_4$. Thus, it can be stated that Cu-HDO is not carcinogenic to male and female Wistar rats under the conditions of this study.
5.3.1	Reliability	1
5.3.2	Deficiencies	No

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	December 2005
Materials and Methods	**2.1. Guideline study** According to OECD test guideline number 451. **3.1.2.2 Purity** According to the study report the purity of Cu-HDO is 89%. **3.3.7 Concentration** 100/ 600 / 3000 ppm corresponds to 5, 29, 148 (males) and 6, 37, 189 (females) mg/kg bw/day **3.4.5 Water Consumption** Not reported in the study, but also not explicitly recommended by OECD guideline 451 **3.4.8 Ophthalmoscopic examination and 3.4.10 Clinical chemistry and 3.4.11 Urinalysis** Potential effects were not analyzed, but these are also not explicitly recommended by OECD guideline 451
Results and discussion	**4.13 Organ weights** *Brain* Most likely due to the reduced terminal body weight, the absolute brain weight was significantly reduced in group 3 (3000 ppm Cu-HDO) , when compared to the control. But this was not regarded to be treatment related. The comparison of the brain weight of groups 3 and 4 (1350ppm $CuSO_4$) indicated a significant elevation in group 4, although the difference was only 3.7%. *Kidney* The comparison of the kidney weights of groups 3 and 4 demonstrated a significant increase (7.2%) in group 4. This weight was comparable with the control group. *Liver* The liver weights were compareable between groups 1 to 3, when compared with control group. The comparison of the liver weights of groups 3 and 4 indicated a significant decrease (-9.2%) in group 4. The comparison with the control group showed comparable weights. *Adrenal glands; testes* The mean absolute and relative weight parameters did not show significant differences when groups 1 to 3 were compared with the control group or when comparison was made between groups 3 and 4. **4.14 Histopathology** *Neoplasitic findings (Vol III p10 of report):* The mesenteric lymph nodes showed a higher incidence of hemangioma in males of group 3 (3000ppm Cu-HDO) and group 2 (600ppm Cu-HDO), when compared to the control (6-7-12-13). But the combined incidence of all vascular tumors (hemangioma, hemangiosarcoma and lymphangioma) in mesenteric lymph nodes shows a comparable incidence in all male groups (10-8-13-14) as well as in female groups (2-2-1-5). Moreover, the total number of animals with vascular tumors and the total number of vascular tumors (hemangioma, hemangiosarcoma and lymphangioma) in all organs showed no difference in groups 1,2 and 3 (100, 600 and 3 000ppm Cu.HDO), when compared to control. The same was reported in comparing group 3 (3000ppm Cu-HDO) and 4 (1350ppm CuSO4) In comparison with males of group 4 (1350ppm Cu-HDO) no difference in the incidence in group 3 (3000ppm Cu-HDO was observed (13 vs 13). The number of animals with neoplasms, with only one primary, with two or more primary neoplasms, with benign or malignant, systemic neoplasms and metastasized neoplasms –

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

was comparable in all cases between all treated groups and control group.

The total number of rats with tumors and the total number of tumors – benign and malignant- were comparable between the control group and dose groups 3 (3000ppm Cu-HDO) and 4 (1350ppm CuSO4) on the one hand and between groups 3 and 4 on the other hand.

BASF Department of Toxicology 106
PATHOLOGY REPORT 70C0679/89113
BIS-(N-CYCLOHEXYL-DIAZENIUMDIOXY)-COPPER Jan/30/1996 CEGE
24-Month Feeding Study in Rats acopat system

LIST OF TUMOR BEARING ANIMALS AND SUMMARY OF TUMORS
GROUPS 0-3 - ALL ANIMALS

Sacrifice	F1							
Sex	M				F			
Group	0	1	2	3	0	1	2	3
Animals in selected Group	50	50	50	50	50	50	50	50
Number of Animals with:								
Neoplasms	47	38	44	41	46	44	49	44
1 Primary Neoplasm	17	20	20	18	21	19	23	14
2 and > Primary Neoplasms	30	18	24	23	25	25	26	30
Number of Animals with:								
Benign Neoplasms	43	35	42	38	43	42	45	40
Benign Neoplasms only	35	28	37	28	29	31	35	25
Malignant Neoplasms	12	10	7	13	17	13	14	19
Malignant Neoplasms only	4	3	2	3	3	2	4	4
Systemic Neoplasms	2	2	1	2	.	1	1	3
Metastasized Neoplasms	1	2	2	1	1	2	2	1
Total Number of:								
Primary Neoplasms	96	62	84	79	86	82	88	92
Benign Neoplasms	82	52	77	66	67	69	70	69
Malignant Neoplasms	14	10	7	13	19	13	18	23
Systemic Neoplasms	2	2	1	2	.	1	1	3
Metastasized Neoplasms	1	2	2	1	1	2	3	1

4.7 Macroscopic Investigations

see 4.12 Pathology

Conclusion

Local oral NOAEL = 6mg/kg bw day

based on increased graded severity of cellular hyperplasia of the forestomach's epithelium in males and increased number of males with hyperkeratosis of the forestomach's wall.

Systemic NOAEL = 33mg/kg bw day

Reliability

1

Acceptability

acceptable

Remarks

Mortality rate for all groups<34%

Body weight loss for all groups<12%

Table A6_7.1-1 Table for Clinical Chemistry, Haematology and Urinalysis

affected		Control 0ppm Cu-HDO		Group 1 100ppm Cu-HDO		Group 2 600ppm Cu-HDO		Group 3 1000ppm Cu-HDO		Group 4 1350ppm $CuSO_4$	
sex	parameter	months after start of treatment									
♂	body weight							↓	24		
	body weight change							↓	24		

Table A6_7.1.-2. Results of Carcinogenicity study

Parameter	control data				Group 1 100ppm Cu-HDO		Group 2 600ppm Cu-HDO		Group 3 3000ppm Cu-HDO		Group 4 $CuSO_4$ 1350ppm		dose-response + / –	
	historical		study											
	m	f	m	f	m	f	m	f	m	f	m	f	m	f
Number of animals examined			50	50	50	50	50	50	50	50	50	50	–	–
Mortality			*28%*		*18%*		*26%*							
clinical signs									black discoloration of faeces	black discoloration of faeces	black discoloration of faeces	black discoloration of faeces		
Body weight									↓					
body weight gain									↓					
food consumption														
clinical chemistry														
haematology														
urinalysis														
Gross pathology									- thickening of the forestomach's mucosa at necropsy	- thickening of the forestomach's mucosa at necropsy	- thickening of the forestomach's mucosa at necropsy	- thickening of the forestomach's mucosa at necropsy		
Overall tumour incidence:														
No. of animals with neoplasms														
No. of animals with benign neoplasms														

Parameter	control data				Group 1 100ppm Cu-HDO		Group 2 600ppm Cu-HDO		Group 3 3000ppm Cu-HDO		Group 4 $CuSO_4$ 1350ppm		dose-response + / –	
	historical		study											
	m	f	m	f	m	f	m	f	m	f	m	f	m	f
No. of animals with malignant neoplasms														
No. of animals with > 1 neoplasm														
Organ														
tumour														
non-neoplastic changes							- cellular hyperplasia of the forestomach's epithelium		- cellular hyperplasia of the forestomach's epithelium	- cellular hyperplasia of the forestomach's epithelium	- cellular hyperplasia of the forestomach's epithelium	- cellular hyperplasia of the forestomach's epithelium		
							- hyperkeratosis of the forestomach's wall		- hyperkeratosis of the forestomach's wall	- hyperkeratosis of the forestomach's wall	- hyperkeratosis of the forestomach's wall	- hyperkeratosis of the forestomach's wall		
									- submucosal edema in the forestomach's wall	- submucosal edema in the forestomach's wall	- submucosal edema in the forestomach's wall	- submucosal edema in the forestomach's wall		
									- Storage of an iron-containing pigment in macrophages in the submucosa of the duodenum	- Storage of an iron-containing pigment in macrophages in the submucosa of the duodenum	- Storage of an iron-containing pigment in macrophages in the submucosa of the duodenum	- Storage of an iron-containing pigment in macrophages in the submucosa of the duodenum		
									- Centrilobular liver cell vacuolisation		- Centrilobular liver cell vacuolisation			
										- Single cell necrosis		Single cell necrosis		

Parameter	control data				Group 1 100ppm Cu-HDO		Group 2 600ppm Cu-HDO		Group 3 3000ppm Cu-HDO		Group 4 $CuSO_4$ 1350ppm		dose-response + / –	
	historical		study											
	m	f	m	f	m	f	m	f	m	f	m	f	m	f
										- Copper storage in Kupffer cells and in hepatocytes	-	- Copper storage in Kupffer cells and in hepatocytes		
										- Increased numbers of cysts in the liver	-	-		

Comment: The number of animals with neoplasms, the number of animals with one or more than one primary neoplasm, as well as the number of animals with benign, malignant systemic or metastasised neoplasms, respectively, and the total number of primary neoplasms, comprising benign, malignant, systemic or metastasised primary tumours did not differ biologically from controls.

Section A6.7 **Carcinogenicity**

Annex Point IIA6.7 **Mouse**

	Justification for non-submission of data	**Official use only**
Other existing data [☒] Limited exposure [☒]	Technically not feasible [☐] Scientifically unjustified [☐] Other justification [☐]	
Detailed justification: Undertaking of intended data submission [☐]	– The toxicity of Cu-HDO has been examined extensively. The available acute, subacute, and chronic studies show with the exception of a liver-toxicity, which occurs only at high doses, that no systematic toxicity has to be expected. The observed toxic effects are unequivocally based on the copper part of the Cu-HDO molecule. This property has been determined by parallel examinations with copper sulfate at appropriate dose. – The subchronic study has been performed on rodent as well as on non-rodent. No different effects were observed between the species. – Because Cu-HDO is used only in industrial area the exposure is very limited. Testing the carcinogenicity in a second species is therefore not necessary.	X X X

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	June 2005
Evaluation of applicant's justification	Within the rat long term toxicity testing the target organs were not just the liver, but also the digestive tract and the kidneys. The effects were seen not only at high dose but also at intermediate doses of ca. 140 and 54 and 33mg/kg bw/day within the rat 3, 12 and 24 months studies. Comparing the administration of Cu-HDO with the administration $CuSO_4$ (at dose levels resulting in a similar amount of Cu^{2+} intake), some effects on the digestive tract, the liver and the kidneys were observed in both test groups and can therefore be attributed to the Cu^{2+} ion in both substances. However several effects were only observed within the Cu-HDO group: Within the 3 months study iron pigment deposits in the tunica propria of duodenum and jejunum; within the 12 months study, the increase in white blood cell count (m), in lymphocytes (m), in alanine aminotransferase (m), in aspartate aminotransferase (m), in squamous epithelial cells in the urine sediment (f), increased liver weights in females (f), higher incidence of edema in the submucosa of the forestomach (m+f), higher incidence of single cell necrosis in the liver (m), and the higher incidence of hyaline droplets and proteinaceous casts in the kidneys (m); within the 24 months study the impaired body weight and body weight change (m), increased numbers of cysts in the liver (f), storage of iron-containing pigment in macrophages in the submucosa of the duodenum (m+f). However comparing the results of the dog and rat 90 day study the NOAELs result to be similar with 26 and 35 mg/kg bw/day respectively. Also the effects are similar, though in the dogs the liver effects were stronger including gross lesions, hepatitis and cirrhosis and as sequelae additionally edema in the gall bladder (2m,4f) and in the pancreas and mesentery (2m). No renal effects were observed in dogs. Vomiting was found only in dogs (m+f, mainly in the first week of administration), but this cannot be found in rats for physiological reasons.
Conclusion	The NOAELs from the rat and dog 3 months studies are similar and no additional toxicological targets are identified in the dog. The NOAELs from the rat 3, 12 and 24 months studies are within the same magnitude, that is 35 compared to 16 and 5 mg/kg bw/day and also the target organs liver, GI and kidney are similar. The genotoxicity tests (in vitro bacterial mutation test, in vitro UDS, in vivo micronucleus test) are negative. Because Cu-HDO is used only in industrial area, the exposure is limited. Therefore waiving of the mouse carcinogenic study was considered acceptable.
Remarks	-

Section A6.8.1 Teratogenicity Study

Annex Point IIA6.8.1 **Rat**

			Official use only
		1 REFERENCE	
1.1	**Reference**	A 6.8.1/01 [redacted] (1991) Study of the Prenatal Toxicity of BIS-(N-CYCLOHEXYL-DIAZENIUMDIOXY)-COPPER in rats after oral administration (gavage): 30R0679/89059, [redacted]	
1.2	**Data protection**	Yes	
1.2.1	Data owner	BASF AG	
1.2.2	Companies with letter of access	No company has the right to use these data on behalf of the data owner	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes - EC Comm. Directive 87/302/EEC of 1987; Part B, pp. 24-26 (1988) - OECD guideline for testing of chemicals No. 414, (1981)	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	X
		3 MATERIALS AND METHODS	
3.1	**Test material**	Cu-HDO	
3.1.1	Lot/Batch number	Reu E 7360	
3.1.2	Specification		
3.1.2.1	Description	Solid, metallic violet	
3.1.2.2	Purity	93%	
3.1.2.3	Stability		
3.2	**Test Animals**	Non-entry field	
3.2.1	Species	Sexually mature virgin Wistar rats	
3.2.2	Strain	Chbb: THOM (SPF)	
3.2.3	Source	[redacted]	
3.2.4	Sex	Females	
3.2.5	Age/weight at study initiation	About 68 - 77 days Mean weight: about 222g	
3.2.6	Number of animals per group	20 pregnant female rats/group	
3.2.7	Control animals	Yes, concurrent vehicle	
3.2.8	Mating period	16.00 hours to about 7.30 hours on the following day	

Section A6.8.1 Teratogenicity Study
Annex Point IIA6.8.1 **Rat**

3.3	**Administration/ Exposure**	Oral	
3.3.1	Duration of exposure		
		rat: day 6-15 post mating	
3.3.2	Postexposure period	5 days (day 16 - 20 p.c.)	X
		Oral	
3.3.3	Type	Gavage	
3.3.4	Concentration	Gavage: 10, 30 and 100mg/kg bw each day	
3.3.5	Vehicle	0.5% aqueous carboxy methyl cellulose solution	
3.3.6	Concentration in vehicle	10, 30 and 100mg/kg bw	
3.3.7	Total volume applied	Standard dose volume 10ml/kg bw	
3.3.8	Controls	Vehicle	
3.4	**Examinations**		
3.4.1	Body weight	Yes	
3.4.2	Food consumption	Yes	
3.4.3	Clinical signs	Yes	
3.4.4	Mortality	Yes	
3.4.5	Examination of uterine content	Weight of uterus Number of corpora lutea Number and distribution of implantation sites classified as: – Live foetuses – Dead implantations Calculations of conception rate and pre- and post-implantation losses	
3.4.6	Examination of foetuses	No entry field	
3.4.6.1	General	Fetal weight, sex, macroscopically examinations for any external findings, viability of the fetuses and the condition of the placenta, the umbilical cords, the fetal membranes and fluids, individual placental weights	
3.4.6.2	Skeleton	Yes	X
3.4.6.3	Soft tissue	Yes	X
3.5	**Further remarks**		
		4 RESULTS AND DISCUSSION	
4.1	**Maternal toxic Effects**	**Clinical examinations:** o Food consumption of the high-dose dams (100mg/kg bw/day) was	

Section A6.8.1 **Teratogenicity Study**
Annex Point IIA6.8.1 **Rat**

marginally reduced (about 18%) during days 6 – 8 of the treatment period, while the food consumption of the other substance treated groups (1 and 2 – 10 or 30mg/kg bw/day) does not show any differences of biological relevance if compared to the controls. Later on, food consumption of the high dose dams reached or even exceeded control values.

- Mean body weight gains of the dams of the test group 3 (100mg/kg bw/day) were marginally impaired at the beginning of the treatment period (days 6 – 8 p.c.). This corresponds to the reduced food consumption of the dams of this test group. In the subsequent time however, weight gain of the high dose dams reached or even exceeded control values.
- The results of the corrected body weight gain was marginally reduced (to about 92% of the actual control value) in the high dose group (100 mg/kg bw/day) while the other substance treated groups (1 and 2 – 10 or 30mg/kg bw/day) did not show any differences of biological relevance if compared to the controls. X
- There were no substance-related clinical findings in any of the animals

Examination of the dams at termination

Necropsy findings:

There were no substance-related observations at necropsy in any of the dams. Edema and /or marginal emphysema of the lungs were recorded for several dams of the control and the substance-treated groups without any dose-response relationship. Moreover, one animal, which did not become pregnant, showed hydrometra and typical findings after gavage error were noted for 1 – 3 rats of the substance-treated groups.

Uterus weight:

The uterus weights were not influenced by the administration of the test substance

Reproduction data of dams:

The conception rate varied between 83% (control group) and 90% (all substance treated groups).

No substance-related and/or statistically significant differences between the groups in conception rate, in the mean number of corpora lutea and implantation sites or in the values calculated for the pre- and the post-implantation losses, the number of resorptions and viable foetuses. The differences evident are considered to be incidental and within the normal range of deviations for animals of this strain and age.

4.2 Teratogenic / embryotoxic effects

No indications of any substance-induced embryo-/fetotoxicity and especially no signs of any teratogenicity up to and including the dose of 100mg/kg body weight /day

Examination of the foetuses after dissection from the uterus

Sex distribution of foetuses:

The sex distribution of the foetuses in test groups 1 – 3 (10, 30, and 100mg/kg bodyweight/day) was comparable with the control foetuses. The differences observed in comparison to the control are without any biological relevance.

Section A6.8.1 **Teratogenicity Study**
Annex Point IIA6.8.1 **Rat**

Weight of placenta:

The mean placental weights in test groups 1 – 3 (10, 30, and 100mg/kg body weight / day) were not influenced by the administration of the test substance to the dams.

Weight of foetuses:

The mean foetal weights were not influenced by the test substance administration. All values are within the range of biological variation.

External examination of the foetuses:

External mal formations were reported for 2 foetuses of test group 2 (30mg/kg body weight/day). One of these foetuses showed cheiloschisis, while the other had various malformations of the head (aglossostomia, exophthalmia and microtia). Furthermore, for one high dose foetus, a cleft palate was noted. Most of theses malformations are also present in the historical control data at a low frequency. Therefore, theses malformations are finally assessed as being of spontaneous origin. X

The external examination of the foetuses revealed no variations in any group.

One so-called unclassified observation (placenta fused) was recorded for one foetus of the control group and one foetus of test group 2 (30mg/kg body weight/day).

4.3 Other effects

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

The study was carried out in accordance with the commission directive 87/302/EEC of 18. November 1987 adapting to technical progress for the ninth time Council Directive 67/548/EEC, pp. 24 – 26 (1988) and the OECD guideline for testing of chemicals No. 414 (1981).

5.2 Results and discussion

Test group 3 (100mg/kg body weight/day):

- Slightly impaired food consumption of the dams at the beginning of the treatment period (days 6 – 8 p.c.)
- Marginally impaired body weight gain of the dams at the beginning of the treatment period (days 6 – 8 p.c.) and slightly reduced corrected body weight gain

Test group 2 (30mg/kg body weight/day):

- No substance related effects on dams or foetuses

Test group 1 (10mg/kg body weight/day):

- No substance related effects on dams or foetuses

Under the conditions of this full-scale study, Cu-HDO caused only slight signs of maternal toxicity at 100mg/kg bw/day at the beginning of the treatment period (days 6 – 8 p.c.); in the preceding range-finding study however, which was carried out under comparable study conditions (e.g. same batch, same rat strain, same treatment schedule), overt signs of maternal toxicity in the form of clearly reduced food consumption and retarded body weight gain were found at 100 and even, but less pronounced at 50mg/kg bw/day during the whole treatment period.

Section A6.8.1 Teratogenicity Study
Annex Point IIA6.8.1 **Rat**

		Therefore, the selection of 100mg/kg bw/day as the highest dose level for the present full-scale study was justified. No signs of embryo-/fetotoxicity, especially no indications of any teratogenic effect, were noted either in the range-finding or in the full-scale study up to and including the dose of 100mg/kg bw/day	
5.3	**Conclusion**	NOAEL (NOEL), LOAEL (LOEL): No signs of embryo-/fetotoxicity, especially no indications of any teratogenic effect including the dose of 100mg/kg body weight/day TOXIC RESPONSE/EFFECTS BY DOSE LEVEL: NOAEL 30mg/kg bw each day	
5.3.1	LO(A)EL maternal toxic effects		
5.3.2	NO(A)EL maternal toxic effects	30mg/kg bw	X
5.3.3	LO(A)EL embryotoxic / teratogenic effects		
5.3.4	NO(A)EL embryotoxic / teratogenic effects	100mg/kg bw	
5.3.5	Reliability	1	
5.3.6	Deficiencies	No	

Evaluation by Competent Authorities	
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	December 2005
Materials and Methods	**2.3. Deviations** see below **3.3.1. Duration of exposure** OECD guidance document Nr. 414 recommends exposure from day 5 till the end of gestation. **3.4.6.2 Skelet and 3.4.6.3 soft tissue** Half of the fetuses were analyzed for each point (guideline conform)
Results and discussion	**4.1 Maternal toxic effects** corrected body weight = terminal body weight on day 20 p.c. minus weight of the uterus before it was opened minus body weight on day 6 p.c. **4.2 Teratogenic / embryotoxic effects** The sex distribution of fetuses, the weight of placentae, the weight of fetuses were not influenced by the administration. With the exception of two specific skeletal variations in test group 1 (10mg/kg bw/day) there are no statistically significant differences between the control and the substance-treated groups concerning fetal external, soft tissue, skeletal and overall observations. The lower number of 10mg/kg fetuses with shortened 13th rib(s) and the increased number of 10mg/kg fetuses with sternebra (e) of irregular shape (both findings are skeletal variations), are assessed as being of spontaneous nature and not related to the test substance administration. All other findings appeared without a clear dose-response relationship and most of them appeared either in the actual or in the historical control group at a comparable frequency.
Conclusion	**5.3.2 NO(A)EL – maternal toxic effects** The maternal transient effect of reduced weight gain is considered to be a consequence of reduced food consumption. The value 30mg/kg bw day represents a NOEL (not a NOAEL) if only the data of this study are considered. Considering also the results of the dose finding study (which showed significantly reduced food intake and significantly reduced maternal weight gain with 50mg/kg bw) the maternal NOAEL could be set to 30 mg/kg bw. However this maternal NOAEL generated independently from the final experiment cannot be related to the developmental NOAEL defined only in the final study. We agree with the applicant, the dose selection seems too low. However considering as discussed that the acute LD50 is about 380mg/kg bw the dose of 100mg/kg bw/day was only slightly below any potentially toxicologically meaningful dose. Furthermore the NOEL of the rabbit developmental toxicity study is 10mg/kg bw/day, which means that in any case the rabbit study will be the critical one. Furthermore the NOEL of the 2 year study is 6mg/kg bw/day, which means an extra margin of safety for developmental effects on rats of at least 20x. **NOAEL maternal: 30mg/kg bw day Cu-HDO** **NOAEL embryotoxic and teratogenic: 100mg/kg bw day**
Reliability	1
Acceptability	Acceptable
Remarks	-

Table A6_8-1 Table for Teratogenic effects (separate data for all dosage groups)

Maternal effects

Modify if necessary and give historical data if available

Parameter	**control data**		**low dose**	**medium dose**	**high dose**	**dose-response**
	historical	study	**10mg/kg bw Cu-HDO**	**30mg/kg bw Cu-HDO**	**100mg/kg bw Cu-HDO**	**+ / -**
Number of dams examined		30	30	30	30	
Clinical findings during application of test substance						
Mortality of dams *state %*	0	0	3.3*	6.6*	10*	–
Abortions	0	0	0	0	0	
Body weight gain *day 0-x, day 0-y, day x-y, day 0-end of test,*					↓ days 6-8 p.c ↑ days 8-10 p.c.	+
Food consumption					↓ 18%	+
Water consumption *if test substance is applied with drinking water*						
Pregnancies *pregnancy rate or %*	92%	83%	90%	90%	90%	–
Necropsy findings in dams dead before end of test						
Lungs: edema		20%	6.7%	6.7%	6.7%	–
Lungs marginal emphysema		3.3%	0%	0%	0%	–
Particular find. on implants in dams sacr. morib./died interc.		0%	3.3%	6.7%	10%	

***The rats died accidentally on day 7 p.c. (after the second gavaging) due to the unintentional use of a faulty stomach tube**

Table A6_8-2 Table for Teratogenic effects (separate data for all dosage groups)

Litter response (Caesarean section data)

Modify if necessary and give historical data if available

Parameter	control data		low dose	medium dose	high dose	dose-response + / -
	historical	study	10mg/kg bw Cu-HDO	30mg/kg bw Cu-HDO	100mg/kg bw Cu-HDO	
Corpora lutea *state total/number of dams*	**6599/420**	**403/25**	**442/27**	**403/27**	**391/27**	–
Implantations *state total/number of dams*	**5999/420**	**344/25**	**393/27**	**367/27**	**345/27**	–
Resorptions *state total/number of dams*	**420/248**	**18/25**	**25/26**	**23/25**	**25/24**	
total number of foetuses	**5528**	**326**	**368**	**344**	**320**	
pre-implantation loss *state %*	**9.1**	**14.8**	**11.8**	**9.0**	**13.2**	
post-implantation loss *state %*	**7.9**	**5**	**6.1**	**6.0**	**7.2**	
total number of litters	**418**	**25**	**26**	**25**	**24**	
foetuses / litter	**13.2**	**13.0**	**14.2**	**13.8**	**13.3**	
live foetuses / litter *state ratio*	**5528/418**	**326/25**	**368/26**	**344/25**	**320/24**	
dead foetuses / litter *state ratio*	**0**	**0**	**0**	**0**	**0**	
foetus weight (mean) *[g]*	**3.9**	**3.8**	**3.9**	**3.9**	**4.0**	
placenta weight (mean) *[g]*	**0.43**	**0.45**	**0.46**	**0.45**	**0.45**	
crown-rump length (mean) *[mm]*						
Foetal sex ratio *[state ratio m/f]*	**2759/2769 (1 : 1.003)**	**164/162 (1 : 0.99)**	**173/195 (1 : 1.13)**	**187/157 (1 : 0.84)**	**174/146 (1 : 0.84)**	–

Table A6_8-3 Table for Teratogenic effects (separate data for all dosage groups)
Examination of the foetuses

Parameter	control data		low dose	medium dose	high dose	dose-response + / -
	historical	study				
External malformations *[%]*	**0.05**	**0**	**0**	**0.6**	**0.3**	–
External variations *[%]*	**0**	**0**	**0**	**0**	**0**	–
Skeletal malformations *[%]*	**3.6**	**6.5**	**3.2**	**5.1**	**4.3**	–
Skeletal retardations *[%]*	**40.5**	**41**	**38**	**48**	**42**	–
Skeletal variations *[%]*	**39.4**	**36**	**41**	**42**	**33**	–
Soft tissue malformations *[%]*	**0.2**	**0**	**2.2**	**1.8**	**1.9**	–
Soft tissue variations *[%]*	**33.6**	**22**	**20**	**17**	**27**	

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	**Rabbit**

			Official use only
		1 REFERENCE	
1.1	**Reference**	A 6.8.1/02 [redacted] (1994) Study of the Prenatal Toxicity of BIS-(N-CYCLOHEXYL-DIAZENIUMDIOXY)-COPPER in rabbits after oral administration (gavage)administration (gavage): 40R0141/92031, [redacted]	
1.2	**Data protection**	Yes	
1.2.1	Data owner	BASF AG	
1.2.2	Companies with letter of access	No company has the right to use these data on behalf of the data owner	
1.2.3	Criteria for data protection	Data submitted to the MS after 13 May 2000 on existing a.s. for the purpose of its entry into Annex I/IA	
		2 GUIDELINES AND QUALITY ASSURANCE	
2.1	**Guideline study**	Yes – OECD Guideline 414 "Teratogenicity" – EC Comm. Directive 87/302/EEC of 1987; Part B, pp. 24-26 (1988) – EPA and TSCA new and revised Health Effects Test Guidelines [Developmental Toxicity Study], (1984) – Testing guideline for toxicological studies, Japan/MAFF, pp. 48-49, (1985)	
2.2	**GLP**	Yes	
2.3	**Deviations**	No	X
		3 MATERIALS AND METHODS	
3.1	**Test material**	Cu-HDO	
3.1.1	Lot/Batch number	Reu E 7360 B	
3.1.2	Specification		
3.1.2.1	Description	Solid crystals, bluish	
3.1.2.2	Purity	99%	
3.1.2.3	Stability	Cu-HDO is stable at room temperatures for at least one year	
3.2	**Test Animals**	Non-entry field	
3.2.1	Species	rabbit	
3.2.2	Strain	Himalayan	
3.2.3	Source	[redacted]	
3.2.4	Sex	female	
3.2.5	Age/weight at study initiation	23 – 25 weeks mean body weight: approx. 2530g	
3.2.6	Number of animals per group	5 pregnant female rabbits / group	X
3.2.7	Control animals	Yes, concurrent vehicle	
3.2.8	Mating period	~~14 days or other~~	

Section A6.8.1		**Teratogenicity Study**	
Annex Point IIA6.8.1		**Rabbit**	
3.3	**Administration/ Exposure**	Oral	
3.3.1	Duration of exposure	13 days	
		rabbit: day 7-19 post-insemination	X
3.3.2	Post-exposure period	10 days	
		Oral	
3.3.3	Type	Gavage	
3.3.4	Concentration	Gavage: 10, 30 and 60mg/kg body weight/day	
3.3.5	Vehicle	0.5% aqueous carboxy methyl cellulose solution	
3.3.6	Concentration in vehicle	10, 30 and 60mg/kg body weight/day	
3.3.7	Total volume applied	10ml/kg bw	
3.3.8	Controls	Vehicle	
3.4	**Examinations**		
3.4.1	Body weight	Yes	
3.4.2	Food consumption	Yes	
3.4.3	Clinical signs	Yes	
3.4.4	Mortality	Yes	
3.4.5	Examination of uterine content	Weight of uterus	
		Number of corpora lutea	
		Number and distribution of implantations classified as: – Live foetuses – Dead implantations	
		Calculations of conception rate and pre- and post-implantation losses	
3.4.6	Examination of foetuses	No entry field	
3.4.6.1	General	Fetal weight, sex, macroscopically examinations for any external findings, viability of the fetuses and the condition of the placenta, the umbilical cords, the fetal membranes and fluids, individual placental weights	
3.4.6.2	Skeleton	Yes	
3.4.6.3	Soft tissue	Yes	
3.5	**Further remarks**		

4 RESULTS AND DISCUSSION

4.1 Maternal toxic Effects

Examinations of the dams

Clinical examinations:

Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice (day 29 p.i.) were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

In this study, none of the females had to be partially or totally excluded from the above-mentioned calculations because all animals used in this study became pregnant and were sacrificed as scheduled.

Food consumption:

Mean food consumption of the high dose animals was statistically significantly impaired throughout the total treatment period (days 7 – 19 p.i.) and on days 19 – 20 p.i. The food intake of the 30mg/kg does was also statistically significantly lower than that of the controls of most days of the treatment phase and between days 19 – 20 p.i. in total the high dose females consumed only about half (49.5%) and the intermediate dose animals about 76% of the respective amount of the control group during days 7 – 19 p. i. During the post-treatment period, food consumption of the 30 and 60mg/kg groups reached or even exceeded control values.

The reductions in food consumption in these two groups are clearly related to the test substance administration.

Food consumption of the low dose group (10mg/kg body weight/day) was not influenced by the test substance. All food consumption values for this group are within the range of biological variation.

Body weight data:

There were no statistically significant differences between the mean body weights of the control and those of the substance-treated groups. The mean body weight gain however was statistically significantly reduced in the high dose group (60mg/kg body weight/day). If the mean body weight gain over the whole treatment period (days 7 – 19 p.i.) is calculated the high dose females even lost body weight and the intermediate dose animals gained statistically significantly less weight than the controls.

The reduced weight gain/body weight loss of the 60 and 30mg/kg does is assessed as being substance induced.

Concerning the 60mg/kg group, the reduction in body weight gain has to be seen also in conjunction with the increased resorption rate, which appeared in this group.

Weight gain of the 10mg/kg animals was not affected by the test substance administration. Even if the weight gain of these does is insignificantly lower in comparison to the controls during the treatment period, this is considered to be of spontaneous nature, because the 10 mg/kg females gained already less weight than the controls during the pre-treatment period (days 0 – 7 p.i.).

Corrected body weight gain (net maternal body weight change)

The results of the corrected body weight gain (terminal body weight on day 29 p.i. minus weight of the uterus before it was opened minus body weight on day 7 p.i.) do not show any statistically significant differences between

the groups; all respective values are in the expected range of biological variation).

Clinical symptoms

Two does of the high dose group (60mg/kg body weight/day, which did not have any live foetuses showed clinical findings which are probably associated with the test substance administration. No defecation was observed for female No. 47 on days 10 – 13 p.i. and doe No. 53 showed blood in bedding during days 14 – 19 p.i.

There were no abnormal clinical symptoms for any other doe in the study.

Mortality:

There were no mortalities in any of the groups.

Examination of the dams at termination

Necropsy findings:

There were no substance-related observations at necropsy in any of the does. All necropsy findings which were recorded for single animals of all dose groups including the controls are of spontaneous nature. Most of these findings have to be related to the sacrifice of the animals (lungs with edema and/or marginal emphysema); moreover, animals No. 8 (control), No. 32 (30mg/kg group) and No. 47 (60mg/kg group) had a blind ending uterine horn each.

Uterus weight:

There were no substantial differences concerning the uterus weights between the controls and test groups 1 and 2 (10 and 30mg/kg body weight/day). All these values lie within the range of biological variation; however the mean gravid uterus weight of the high dose group (60mg/kg body weight/day) was clearly, but due to the high standard deviation not statistically significantly reduced; it reached only about 76% of the control value. This has to be related to the test substance administration and is in line with the higher number of resorptions and the consequently increased post-implantation loss in this group.

Reproduction data of dams

A conception rate of 100% was reached in all groups.

Concerning test groups 1 and 2 (10 and 30mg/kg body weight/day), there were no substance-related and/or statistically significant differences in conception rate, in the mean number of corpora lutea and implantation sites or in the values calculated for the pre- and the post-implementation losses, the number of resorptions and viable foetuses. The differences evinced are considered to be incidental and within the normal range of deviations for animals of this strain and age. One low dose foetus was already dead when the uterus and the foetal membranes were opened.

In test group 3 (60mg/kg body weight/day), however, the resorption rate was slightly increased, due to the fact, that 4 out of 15 pregnant does of this group had no viable foetuses at all but only (predominantly early) resorptions. As a consequence, the post-implantation loss of the 60mg/kg group was increased (31.6 5). This post-implantation loss value is outside the historical control range (3.0% - 23.1%) and assessed as being substance-induced. The mean number of live foetuses/dam, however, was not reduced

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in the remaining 11 high dose females.

4.2 Teratogenic / embryotoxic effects

Examination of the foetuses after dissection from the uterus

Sex distribution of the foetuses

The sex distribution of the foetuses in test groups 1 –3 (10, 30 and 60mg/kg body weight/day) was comparable with the control foetuses. The differences observed in comparison to the control are without any biological relevance.

Weight of placentae

The mean placental weights of the high dose group were slightly, but statistically significantly reduced as were the mean foetal body weights in this group. This is considered as being substance-related.

The mean placental weights in test groups 1 and 2 (10, and 30mg/kg body weight/day), however, were not influenced by the test substance administration. The differences observed in comparison to the control are without biological relevance and lie within the range of biological variation.

Weight of foetuses:

The mean foetal body weights were statistically significantly reduced at 60mg/kg and about 13% lower (both sexes combined) than the respective control value. This is assessed as a substance-induced effect.

The mean foetal weights of the low of the low and intermediate dose groups, however, were not influenced by the oral administration of the test substance. All values are within the range of biological variation and do not show any dose-response relationship. The slightly, but statistically significantly lower weights of all viable 10mg/kg foetuses are of spontaneous nature.

External examinations of the foetuses:

Two high dose foetuses from one litter (doe No. 59 – foetuses Nos. 7 and 8) and one 30mg/kg foetus showed external malformations. High dose foetus No. 59-7 had two supernumerary toes on the left hindlimb (polydactyly); this hindlimb appeared also thickened and shortened when examined externally. After the skeletal examination, shortened and bent tibia and fibula were identified as the cause for the thickening and shortening. The other high dose foetus 8No. 59-8) had a gastroschisis and intermediate dose foetus No. 41-5 showed shortened toes.

Gastroschisis and different malformations of the extremities occur also sporadically in control foetuses of the rabbit strain used. Therefore, the occurrence of the above described malformations in just one or two foetuses from one litter is not associated with the treatment, but assessed as being of spontaneous nature.

Only one type of external variation (peudoankylosis) was found and it was seen in 5 foetuses from 4 litters of the 10-mg/kg group and one foetus of the 30mg/kg group.

Pseudoankylosis is a rather common foetal external variation, which can be also found in control foetuses of the rabbit strain used. Therefore and because no relation to dose is given, this finding is considered random.

Necrobiotic placentae were noted for one low dose (dead) and one high dose foetus each and fused placentae occurred in another low dose female. These so-called "unclassified observations" are not substance-related.

For overall assessment of the above mentioned findings see below.

Soft tissue examination of the foetuses

The examination of the organs of the foetuses revealed several types of soft tissue malformations in single foetuses of test groups 0, 1 and 3 (0, 10, and 60mg/kg body weight/day). Hydrocephaly was recorded for one control and one high dose foetus. A septal defect was found in one foetus of the low dose group. Agnesia of gallbladder was recorded for one control and one low dose foetus, whereas abnormal position of the right kidney was seen in another high dose foetus.

All soft tissue malformations mentioned before do not show any clear relation to dosing and nearly all of them are also present at a low incidence in the historical control data. Therefore, the few soft tissue malformations seen in 2 control, 2 low and 2 high dose foetuses from 2 litters each are not associated with the test substance administration.

Variations were detected in each group including the control. Aside from a separated origin of carotids, a very common finding in the rabbit strain used, another soft tissue variation (heart with traces of intraventricular foramen/septum membranaceum) was also found quite frequently. Hypoplasia of gallbladder was recorded for one high dose foetus and dilated renal pelvis was found in one low dose foetus.

All soft tissue variations occurred without a clear dose-response relationship and/or can be found at a comparable incidence in the historical control data.

One so-called unclassified observation (focal liver necrosis) was noted for one control foetus only. Blood coagulum around the bladder was observed in two control and two low dose foetuses and one foetus of the intermediate dose group.

For overall assessment of the above mentioned findings see below.

Skeletal examinations of the foetuses:

Malformations of the foetal skeletons were noted in each group including the control. These malformations were related to the vertebral column (cervical vertebral arches fused, sacral vertebrae fused and/or of irregular shape, lumbar vertebra absent), the sternum (cleft sternum), the forelimbs (phalanges partly missing), and/or the hindlimbs (accessory toes (polydactyly), shortened and bent tibia and fibula). Skeletal malformations appeared in one or two foetuses (from one or two litters) of each group. Both high dose foetuses and one intermediate dose foetus appeared already malformed when they were examined externally; the skeletal examination of these foetuses clarified and/or completed the external findings.

Because the rate of foetuses with skeletal malformations does not show any dose-response relationship and because most of the described or very similar skeletal malformations are also present at comparable incidences in the historical control data, all skeletal malformations found in the present study are assessed as being of spontaneous nature.

The skeletal variations elicited were related to the skull (splitting of skull bones, epactal bone between nasal and frontal bones), the ribs (rudimentary or accessory 13^{th} rib(s)), the vertebral column (accessory thoracic vertebra), and the sternum (sternebra (e) of irregular shape, bipartite, fused or accessory sternebra). Most of the skeletal variation appeared without a clear relation to dosing and none of the skeletal variations showed statistically significant differences between the control group and the substance-treated groups. When all skeletal variations are summed up however, statistically significantly more 30mg/kg and 60 mg/kg litters were affected and – concerning the high dose group only- the percentage of affected foetuses per

litter is statistically significantly increased. This is predominantly die to the higher number of foetuses per litters with irregular shaped sternebra (e) and accessory 13th rib (s). For these findings, the respective foetal and/or litter incidences are at the upper limit or just outside the historical control range. Therefore, the increased rates of skeletal variations at 30 and 60mg/kg might be related to the oral administration of the test substance.

In all groups signs of retardations (incomplete or missing ossification of skull bones, hyoid bone, vertebral column, sternebra (e), and talus were found. Nearly all retardations occurred at a comparable frequency in the control and the substance-treated groups, the differences between the groups being without any biological relevance; this includes the statistically significantly higher rates of incompletely ossified skull at 10 mg/kg and of incompletely ossified sacral vertebral arch (es) at 30mg/kg.

The increased rates of high dose foetuses/litter with incomplete ossification of sacral vertebral arch (es) and/or talus however, are considered as being treatment-related, because the respective foetal/litters incidences are outside the historical control range; the delays in ossification have to be associated with the reduced foetal body weights in this group.

Abstract of foetal external soft tissue and skeletal observations and their assessment:

The morphological examinations failed to reveal significant evidence significant evidence of foetal external, soft tissue, skeletal or total malformations. The total malformation rate was low, substantially similar in all groups and did not show a clear relation to dosing. Moreover, the isolated and disparate nature of the observed malformations does not suggest any treatment-related aetiology.

The statistically significantly increased number of 30 and 60mg/kg litters and the higher percentage of high dose foetuses/litter with total skeletal variations however are assessed as embryotoxic effects representing manifestations of a non-specific stress on the does; these findings are not interpreted as the indication of a teratogenic effect of the test substance at these dose levels.

The increased occurrence of single skeletal retardations (delayed ossification of sacral vertebral arch (es) and (or talus) at 60mg/kg are in-line with the reductions in foetal body weights in this group.

There were no further statistically significant and/or biologically relevant differences between the substance-treated groups and the control in respect to external, soft tissue or skeletal findings. As already discussed with the exception of the increased rate of skeletal variations (at 30 and 60mg/k) and the increased occurrence of two skeletal retardations (at 60mg/kg) – all foetal findings are considered to be of spontaneous nature, because no dose-response relationship is given and/or the respective values are within the historical control range.

4.3 Other effects

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

The study was carried out in accordance with

- OECD Guideline 414 "Teratogenicity"
- EC Comm. Directive 87/302/EEC of 1987; Part B, pp. 24-26 (1988)
- EPA and TSCA new and revised Health Effects Test Guidelines [Developmental Toxicity Study], (1984)

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– Testing guideline for toxicological studies, Japan/MAFF, pp. 48-49, (1985)

5.2 Results and discussion

Test group 3 (60mg/kg bw/day):

- Statistically significant impaired food consumption (days 7 – 20 p.i.) [only about half of the food-intake of the controls]
- Body weight loss and/or statistically significantly impaired weight gains during the treatment period (days 7 – 19 p.i.)
- Reduced mean gravid uterus weight (only about 76% of the control value)
- One doe with blood in bedding and another female with no defecation during several treatment days
- Slightly increased resorption rate (predominantly early ones) and consequently increased post-implantation loss (31.6%) predominantly due to the fact that 4 females had no viable foetuses at all but only dead implants in uterus
- Reduced mean placental and foetal body weights
- Increased occurrence of skeletal variations and 2 skeletal retardations (incomplete ossification of sacral vertebral arch(es) and /or talus

Test group 2 (30mg/kg bw/day):

- Reduced food consumption on days 7 – 20 p.i. (with statistical significance on most of these days)
- Statistically significant impaired body weight gain (if the weight gain over the total treatment period is calculated)
- Statistically significantly increased numbers of litters with skeletal variations

Test group 1 (10mg/kg bw/day):

No substance related effects on does or foetuses

Under the conditions of this study, Cu-HDO caused overt signs of toxicity at 60mg/kg bw/day (e.g. impaired food intake, body weight loss, reduced uterus weight, adverse clinical symptoms in two of the animals, 4 does without any viable foetuses). 30mg/kg bw/day were still slightly toxic to the does (reduced food intake and impaired body weight gain); at the lowest dose 10mg/kg bw/day however, no substance-related maternally toxic effects occurred.

Clear signs of developmental toxicity were observed at the highest dose level (60mg/kg bw/day) and were substantiated by a slightly higher rate of embryo-lethality, caused by a high number of (mainly early) resorptions, a consequently increased post-implantation loss and statistically significantly lower mean placental and foetal body weights. Furthermore, in the intermediate and high dose foetuses an increased litter incidence of skeletal variations was seen. Exclusively at 60mg/kg, the rate for two skeletal retardations was statistically significantly increased.

Because of the significant reductions in food consumption and weight loos/reduction in body weight gain, the developmental toxicity effects are considered to be a consequence of maternal toxicity. X

Overall, no substance-induced teratogenic effects were observed up to and including the dose of 60mg/kg bw/day.

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	**Rabbit**

5.3	**Conclusion**	Cu-HDO does not cause developmental effects in the rabbit	X
5.3.1	LO(A)EL maternal toxic effects		
5.3.2	NO(A)EL maternal toxic effects	10mg/kg bw	
5.3.3	LO(A)EL embryotoxic / teratogenic effects		
5.3.4	NO(A)EL embryotoxic / teratogenic effects	10mg/kg bw	
5.3.5	Reliability	1	
5.3.6	Deficiencies	No	
5.3.7		–	

Section A6.8.1	**Teratogenicity Study**
Annex Point IIA6.8.1	**Rabbit**

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	December 2005
Materials and Methods	**2.3. Deviations** see below **3.3.1. Duration of exposure** OECD guidance document Nr. 414 recommends exposure from day 5 till the end of gestation. **3.2.6 Number of animals/group** 5: = Typing error; 15 animals per group were used
Results and discussion	**5.2 Results and Discussion and 5.3 Conclusion** Within the rabbit developmental toxicity study the primary maternal effect seems to be reduced food consumption during the treatment phase. This resulted in a reduced body weight gain in the medium dose group (30mg/kg bw day), which seems to produce a (not statistically significant) maternal net weight reduction without effects on uterus weight and fetal weight. In contrast in the high dose group (60mg/kg bw) the drastically reduced food consumption resulted in a body weight loss due to resorptions, subsequent litter loss and reduced uterus weight. Also the one dam that did not show defecation for several treatment days can be explained by the drastically reduced food consumption, as well as the one female with blood in bedding due to litter loss. The adverse effects on the fetuses, which were skeletal variations in the medium dose group (30mg/kg bw day), and additionally skeletal retardations and embryolethality in the high dose group (60 mg/kg bw day) could be considered as secondary to maternal effects. Furthermore the skeletal variations (not affecting survival or health) were also found occasionally in control fetuses. Thus the data available do not indicate a concern for specific developmental toxicity.
Conclusion	Agree with applicant's version **NOAEL maternal = 10 mg/kg bw day** **NOAEL embryotoxic and teratogenic = 10 mg/kg bw day**
Reliability	1
Acceptability	Acceptable
Remarks	-

Table A6_8-1 **Table for Teratogenic effects (separate data for all dosage groups)**
Maternal effects

Parameter	control data					
	historical	study **Group 0** **0mg/kg bw**	**Group 1** **10mg/kg bw**	**Group 2** **30mg/kg bw**	**Group 3** **60mg/kg bw**	**dose-response + / -**
Number of dams examined		**15**	**15**	**15**	**15**	**15**
Clinical findings during application of test substance					**No defecation on days 10 –13 p i. (1 animal)** **Blood in bedding during days 14 – 19 p i.**	
Mortality of dams *state %*		**0**	**0**	**0**	**0**	**0**
Abortions		**0**	**0**	**0**	**0**	**0**
Body weight gain *day 0-x, day 0-y, day x-y, day 0-end of test,*				**Reduced body weight gain**	**Reduced body weight gain**	+
Food consumption				**Reduced food consumption**	**Reduced food consumption**	+
Water consumption *if test substance is applied with drinking water*						
Pregnancies *pregnancy rate or %*		**100%**	**100%**	**100%**	**100%**	**100%**
Necropsy findings in dams dead before end of test		—	—	—	—	

Table A6_8-2 **Table for Teratogenic effects (separate data for all dosage groups)**

Litter response (Caesarean section data)

Modify if necessary and give historical data if available

Parameter	**control data**		**low dose**	**medium dose**	**high dose**	**dose-response + / -**
	historical	study				
Corpora lutea *state total/number of dams*	**8.0**	**111/15 (7.4)**	**112/15 (7.5)**	**116/15 (7.7)**	**112/15 (7.5)**	
Implantations *state total/number of dams*	**6.8**	**91/15 (6.1)**	**97/15 (6.5)**	**93/15 (6.2)**	**94/15 (6.3)**	
Resorptions *state total/number of dams*	**0.7**	**7/15 (0.47)**	**11/15 (0.73)**	**8/15 (0.53)**	**23/15 (1.5)**	
total number of foetuses	**2425**	**84**	**85**	**85**	**71**	
pre-implantation loss *state %*	**14.0**	**19.2**	**14.2**	**19.8**	**14.0**	
post-implantation loss *state %*	**11.2**	**12.4**	**11.2**	**8.2**	**31.6**	
total number of litters	**394**	**14**	**15**	**15**	**11**	
foetuses / litter	**6.08**	**84/14 (6)**	**86/15 (5.7)**	**85/15 (5.7)**	**71/11 (6.5)**	
live foetuses / litter *state ratio*	**6.1**	**84/14 (6:1)**	**85/15 (5.7:1)**	**85/15 (5.7:1)**	**71/11 (6.5:1)**	
dead foetuses / litter *state ratio*	**0.005**	**0**	**1/15 (0.07:1)**	**0**	**0**	
foetus weight (mean) *[g]*	**41.1**	**41.8**	**38.6**	**41.8**	**36.5**	
placenta weight (mean) *[g]*	**4.62**	**4.9**	**4.4**	**4.7**	**4.2**	
crown-rump length (mean) *[mm]*	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	
Foetal sex ratio *[state ratio m/f]*	**1109:1314 (1 : 1.2)**	**42:42 (1 : 1)**	**48:37 (1 : 0.77)**	**45:40 (1 : 0.89)**	**35:36 (1 : 0.97)**	

n.d. = not determined

Table A6_8-3 **Table for Teratogenic effects (separate data for all dosage groups)**
Examination of the foetuses

Parameter	control data		low dose	medium dose	high dose	dose-response + / -
	historical	study				
External malformations *[%]*		**0**	**0**	**1.2**	**2.8**	
External variations *[%]*		**0**	**5.8**	**1.2**	**0**	–
Skeletal malformations *[%]*		**2.4**	**1.2**	**1.2**	**2.8**	
Skeletal variations *[%]*		**13**	**17**	**20**	**30**	
Skeletal retardations *[%]*		**65**	**58**	**47**	**69**	–
Soft tissue malformations *[%]*		**2.4**	**2.3**	**0**	**2.8**	
Soft tissue variations *[%]*		**27**	**21**	**25**	**23**	

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2

	Justification for non-submission of data	Official use only
Other existing data [☒]	Technically not feasible [☐] Scientifically unjustified [☐]	
Limited exposure [☐]	Other justification [☐]	

Detailed justification:

Waiving : K-HDO/Cu-HDO

An assessment of reproductive toxicity on the basis of the present data

So far, no 2-generation study or other studies specifically tailored for the endpoints reproductive toxicity/fertility and essential reproductive functions have been undertaken.

It is postulated that on the basis of the **(sub)chronic** and **developmental** studies of CuHDO and the data available for other Cu compounds a reasonably valid conclusion can be derived that CuHDO does not bear a reproductive risk:

The toxicology of Cu-HDO is closely related to the toxicology of other copper compounds and mediated by the intracellular bioavailability of copper in the 3 only target organs showing histopathologically detectable effects at high doses: liver, kidney, intestinal tract. Cu-HDO is resorbed from the intestine and appears to be readily excreted from the body with no signs of accumulation. Some liver and kidney toxicity and intestinal irritation may be typically seen also with other copper compounds.

In the course of **subacute**, **subchronic**, and **chronic toxicity** studies, Cu-HDO has never shown effects on male or female reproductive organs even in the top dose ranges. The absence of gross-pathological or histopathological effects in the reproductive organs also indicates that they are not secondarily affected. No testicular tubular atrophy was observed nor any alterations in sperm density or in ovarian follicular appearance and numbers. In addition, hormonal effects (estro- or androgenicity or antagonistic properties) would have shown up in classical subchronic studies, where they are easily detectable via their impact on organ weights and morphology, at least qualitatively as high dose phenomena. In there quantitative terms, of course, 2-generation studies are needed in order to define the no adverse effect levels for reproductive functions and specific susceptibilities during the early life phases.

CuHDO did not show effects neither on the thyroid nor on the CNS. Thyroidal effects may interfere with the hypothalamic hormone axes and CNS effects may affect reproductive functions such as mating and lactation behavior, milk production, and hormonal balances. Not only have such effects not been seen with Cu-HDO but also not with other copper compounds.

From the chemical structure alone, a hormonal effect should not principally be excluded. On the other hand, the chemical structure of the HDO moiety does not contain structural patterns or alerts that would point to such an effect. Furthermore, the HDO moiety has no long biological half-life time, which otherwise is a frequent feature of agents with hormonal activity under environmental exposure.

Prenatal toxicity studies were carried out with CuHDO in rats and rabbits with entirely negative results and no adverse effects on sex relation and sexual development at the end of gestation. In addition, other copper compounds are not developmentally active and have not shown specific reproductive risks.

The table below lists the aforementioned toxicity studies and the respective reference-number in the Cu-HDO dossier.

Section A6.8.2 Multigeneration Reproduction Toxicity Study

Annex Point IIA6.8.2

Overview:

Study	*Endpoint*	*Ref. No. in Doc. III A Cu-HDO*
Biokinetic in rats 1	- Rapid excretion mainly via the urine.	A 6.2/01
Biokinetic in rats 2	- Rapid excretion mainly via the urine.	A 6.2/02
Subacute toxicity rats	- The toxicology of Cu-HDO is closely related to the toxicology of other copper compounds and mediated by the intracellular bioavailability of copper in the 3 only target organs showing histopathologically detectable effects at high doses: liver, kidney, intestinal tract.	A 6.3.1
Subchronic toxicity rats	- No gross-pathological or histopathological effects in the reproductive organs were seen - No testicular tubular atrophy was observed nor any alterations in sperm density or in ovarian follicular appearance and numbers - No hormonal effects (no impact on organ weights and morphology)	A 6.4.1/01
Subchronic toxicity dogs	- No gross-pathological or histopathological effects in the reproductive organs were seen - No testicular tubular atrophy was observed nor any alterations in sperm density or in ovarian follicular appearance and numbers	A 6.4.1/02
Chronic toxicity rats	- No gross-pathological or histopathological effects in the reproductive organs were seen - No testicular tubular atrophy was observed nor any alterations in sperm density or in ovarian follicular appearance and numbers	A 6.5

Study	*Endpoint*	*Ref. No. in Doc. III A Cu-HDO*
Carcinogenicity	- No gross-pathological or histopathological effects in the reproductive organs were seen - No testicular tubular atrophy was observed nor any alterations in sperm density or in ovarian follicular appearance and numbers	A 6.7
Prenatal toxicity rat	- No adverse effects on sex relation and sexual development at the end of gestation.	A 6.8.1/01
Prenatal toxicity rabbit	- No adverse effects on sex relation and sexual development at the end of gestation.	A 6.8.1/02
Biological half-life time	- short biological half-life time	A 7.1.1.1.2

Conclusion:

For the reasons outlined above there are no indications to suspect Cu-HDO respectively K-HDO as a material with an impact on reproductive organs or functions. A 2-generation fertility study with this material may therefore be omitted.

Undertaking of intended data submission [☐]

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	16.12.2005
Evaluation of applicant's justification	The arguments of the applicant are consistent with the results presented in the dossier of Cu-HDO.
Our observations:	The negative results for gross-pathology and histopathology of the reproductive organs shown for Cu-HDO in the subchronic toxicity studies were also found within the 90 day gavage study with K-HDO (see CA report K-HDO doc IIIA.6.4.1.). The application of Cu-HDO will be restricted to industrial use within closed systems. Also because of the low critical medium and long term NOAEL of 10 mg/kg bw/day, the application of a safety factor of 300 resulting in an acceptable exposure of maximal 2mg Cu-HDO per 60kg worker and due to the acute severe eye damaging and skin corrosive effects of Wolmanit CX the exposure has to be negligible.
Conclusion	In case the risk assessment results in a sufficiently large margin of safety to the available critical NOAEL from the 2-year study the waiving of the 2-generation study can be accepted.
Remarks	For further discussion of the waiving arguments see Doc II-A6.8.2

Section A6.9 Delayed Neurotoxicity

Annex Point III A6.1

	Justification for non-submission of data	Official use only
Other existing data [☒]	Technically not feasible [☐] Scientifically unjustified [☐]	
Limited exposure [☐]	Other justification [☐]	
Detailed justification:	Cu-HDO was well investigated in subacute, subchronic (rats and dogs) and chronic oral administration regimens and in prenatal toxicity studies. In no case, the results indicated a neurotoxic effect of this material or the brain as target organ. Furthermore, within the frame of a subacute toxicity study in rats (Ref. A 6.3.1), also neurotoxicity investigations along a functional observation battery (FOB) were carried out. They showed no effect on the central nerve system. Hence, the question of the neurotoxicity of Cu-HDO has already been experimentally well addressed. A new neurotoxicity study is therefore not considered necessary.	
Undertaking of intended data submission [☐]		

Evaluation by Competent Authorities	
EVALUATION BY RAPPORTEUR MEMBER STATE	
Date	June 2005
Evaluation of applicant's justification	Agree with applicant's version
Conclusion	Agree with applicant
Remarks	-

Section A6.12.7
Annex Point IIA6.9

Specific treatment in case of an accident or poisoning: fire aid measures, antidotes and medical treatment, if know

			Official use only
	General advice:	Remove contaminated clothing	
	If inhaled:	If difficulties occur after inhalation: fresh air, summon physician	
	On skin contact:	Wash thoroughly with soap and water	X
	On contact with eyes:	Wash for at least 15 minutes under running water with eyelids held open, consult an eye specialist	X
	On ingestion:	Rinse mouth immediately and drink plenty of water, summon medical aid.	
	No specific antidote known.		X
Undertaking of intended data submission [☐]			

Evaluation by Competent Authorities

EVALUATION BY RAPPORTEUR MEMBER STATE

Date	June 2005	
Evaluation of applicant's proposal	On contact with skin washing with polyethylenglycol is recommended, if immediately available otherwise soap and water is recommended. This is because the substance is poorly water soluble. On contact with eyes it is important to immediately remove contact lenses and wash with very low water pressure to avoid mechanical injuries and if available an eye washing bottle is preferred. On ingestion rinsing immediately with water and drink not plenty but some water is recommended. Drinking plenty of water might induce vomiting which is not recommended, since it could cause inhalation of the substance.	
Conclusion	General advice:	Remove contaminated clothing
	If inhaled:	If difficulties occur after inhalation: fresh air, summon physician
	On skin contact:	Wash thoroughly with soap and water or if immediately available with polyethylenglycol
	On contact with eyes:	In case contact lenses are in the eye remove them immediately; wash for 10 to 15 minutes under running (no pressure) and warm water with eyelids held open or preferably if available with an eye washing bottle, consult an eye specialist.
	On ingestion:	Rinse mouth immediately with water and drink some water, summon medical aid.
	No specific antidote known.	
Remarks	-	

Section A6.12.8 Prognosis following poisoning

Annex Point IIA6.9

		Official use only
Detailed prognosis:	Irritation of the eyes may occur	X
Undertaking of intended data submission [☐]		

	Evaluation by Competent Authorities
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	June 2005
Evaluation of applicant's prognosis	Severe damage of eyes may occur upon acute exposure. Long term exposure can lead depending on the route of exposure predominantly to irritating effects on skin or GI tract and effects on liver (see document IIA, hazard assessment).
Conclusion	See above
Remarks	-