ANNEX B

Ziram

B.6 Toxicology and metabolism
Addendum February 2004

B.6.6.2.1 Teratogenicity test by the oral route in the rat (Annex IIA 5.6.2)

During the ECCO meeting round 7 in 1999, an acute reference dose of 0.04-mg/kg bw/d was derived for ziram. The ARfD was calculated on the basis of a NOAEL in the teratogenicity study carried out in rat, 4 mg/kg bw/d, based on a slight increase in incidence of diaphragmatic thinning observed at 16 mg/kg bw/d.

A new study was provided in February 2004. The objective of this study was to investigate the influence of ziram on the development of the diaphragm in the foetus, with specific reference to diaphragmatic thinning and diaphragmatic hernia recorded in foetuses in the developmental rat study reported in the monograph, point B.5.6.2.1: developmental gavage rat study, at 1, 4, 16 and 64 mg/kg bw/d, gestation days 6-15 (Smith et al, 1990, Task Force Study).
In order to monitor the subsequent progression of this particular lesion postnatal, similar observations were made on culled offspring at day 4 of age and on weanling offspring from females allowed to litter. Treatment of parental females was restricted to the period of organogenesis (Day 6-15 post coitum) to match the period of treatment in the original study.

Ziram: supplementary study to report zir 15/24/8913721 investigation of the incidence of diaphragmatic thinning and diaphragmatic hernia in the foetuses and offspring of CD rats treated by gavage at 8, 16, 64 mg/kg bw/d during organogenesis (Willoughby et al, 2004, Task Force Study)

Findings:

Mortality: one female at 16-mg/kg bw/d was killed for human reasons. The female showed rales before dosing and was killed after dosing because of marked respiratory distress, reduced activity and piloerection. No macroscopic changes were seen at necropsy.
Clinical signs: at 64 mg/kg bw/d, females showed a reaction on the first hours after dosing characterized by reduced activity, piloerection, reduced body temperature and partially closed eyelids. Sporadic incidences of increased salivation and rales were recorded at later stages through the dosing period. At 16 mg/ kg bw/d, approximately 25% of females were judged to be cold on the first day of dosing.
Body weight: at 16 and 64 mg/kg bw/d, females showed marked body weight loss following the first day of treatment and there was a short period of body weight stasis at 8 mg/kg bw/d. Weight gain (day 6-16) were reduced at all dose levels. The effects at 8-mg/kg bw/d were considered to be of minimal biological significance. After treatment ceased, weight gain improved except for top dose females. When body weight gain was adjusted for the weight of gravid uterus, it was clear that reduction of maternal body weight gain was a direct effect on the dam. Body weight of females at start of lactation was lower at 16 and 64 mg/kg bw/d but there was a degree of recovery towards the end of lactation period.
Food consumption: was reduced in 16- and 64-mg/kg bw/d groups. At 8-mg/kg bw/d, reduction was seen for the first week of treatment.
Necropsy findings: at day 20 of gestation or day 21 of lactation, there were no significant observations recorded at necropsy. Two females (1 control and 1 low dose group) had thinning of the diaphragm. These were clearly unrelated to treatment and suggest that the background incidence of this anomaly in adults is approximately 1% in the current rat strain.
Reproductive parameters and litter response:
For females killed on day 20 of gestation: At 64-mg/kg bw/d, number of implantations was slightly reduced and at 16 and 64-mg/kg bw/d number live young was decreased. Post implantation loss was increased at 64-mg/kg bw/d. The biological significance of these findings is uncertain, as these results were not confirmed by the second phase of the study in which females were allowed to litter. Litter and foetal weight was reduced at top dose
Females allowed littering: gestation length was biologically and statistically significantly increased at top dose. At 8 and 16-mg/kg bw/d, the performance of females was intermediate between control and top dose suggesting that there was a dose-related delay in parturition at these levels. The number of implantations, litter size at birth and survival were similar in all groups. There was no effect of treatment on the sex ratio.
Necropsy findings and diaphragm evaluations: there is a clear suggestion in the current study that the incidence of diaphragmatic thinning with protrusion of the liver is increased in foetuses in females which had been treated with 64 mg/kg bw/d during organogenesis. This would be consistent with the findings from the original study.

There was no evidence, however, of any increase in the incidence of diaphragmatic thinning at 8 and 16 mg/kg bw/d.

Table B.6.6.2.1-1: Females data during gestation and lactation

Endpoints/dose	**0**	**8**	**16**	**64 mg/kg bw/d**
Mortality			1 D11	
Clinical signs: n° affected rats				
Closed eyelids				15
Reduced activity				44
Piloerection			1	36
Body temperature↓			12	44
Salivation			4	11
Rales			1	5
Body weight				
Killed GD20	428	413	395(↓7%)	361(↓15%)
Bw changes GD6-20	137	126(↓8%)	109(↓20%)	77(↓43%)
Gravid uterine weight	95	93	85(↓10%)	73(↓23%)
Adjusted GD20 bw	335	319(↓4%)	310(↓7%)	287(↓14%)
Adjusted bw change GD6-20	42	33(↓21%)	24(↓43%)	3(↓92%)
Body weight during lactation		No compound related effect		
Food consumption During gestation		↓(6.7%) D9-12	↓(28%) D6-8 ↓(17%)D9-12 ↓(13%) D13-15	↓(48%) D6-8 ↓(30%)D9-12 ↓(32%)D13-15 ↓(14%) D16-19
Dams allowed to litter				
Gestation length:				
22 days	68%	32%	33%	9%
23 days	5%	18%	14%	59%
Historical control data	65.3% 22 days 13.1% 23 days			
N° Live litter born	22	22	21	22
Gestation index %	100	100	100	100

(%) Statistically significantly different from control p<0.01
GD or D: gestation day

Table B.6.6.2.1-2: Litter data

Endpoints/dose	**0**	**8**	**16**	**64 mg/kg bw/d**
Females killed GD 20				
Mean n° implantations	16.1±1.8	16±1.2	15.2±1.6	15.1±1.2*
Mean total live young	15.7±1.8	15.4±1.2	14.5±1.7*	13.9±2.2**
Post implantation loss %	2.4	3.6	4.9	8.1*
Litter/overall foetal weight	60.04/3.83	59.76/3.88	55.31*/3.83	47.46**/3.42**
Dams allowed to litter				
Gestation length:				
22 days	68%	32%	33%	9%
23 days	5%	18%	14%	59%
Historical control data	65.3% 22 days; 13.1% 23 days			
N° Live litter born	22	22	21	22
N° pregnant rats	22	22	21	22
Mean n°implantations	15.8±1.5	15.8±1.8	15.6±2.1	15.9±1.5
Litter size D1	15.3±1.6	14.2±2.5	14.8±2.3	14.7±2.0
Viability index D4 %	100	99	99	99
Incidence diaphragmatic anomalies in foetuses/litter GD 20				

N° examined foetuses /litter	346/22	339/22	318/21	306/22
Total normal diaphragm	288/22	288/22	261/21	213/22
Total thinning w/o protrusion	0/0	4/3	2/2	2/2
Total thinning with protrusion	58/17 **(16.8/77.3%)**	47/15 **(13.9/68.2%)**	55/13 **(17.3/59.1%)**	91*/17 **(29.7*/77.3%)**
Hernia	0/0	0/0	0/0	0/0
Incidence diaphragmatic anomalies in offspring/litter LD 4				
N° examined offspring's /litter	156/22	131/21	136/21	137/22
Total Normal diaphragm	155/22	129/22	136/21	126/22
Total thinning w/o protrusion	0/0	0/0	0/0	0/0
Total thinning with protrusion (%)	1/1 **(0.6/4.5%)**	2/2 **(1.5/9.5%)**	0/0 **(0/0%)**	12*/9* **(8.8**/40.9*%)**
Total hernia	0/0	0/0	0/0	0/0
Incidence diaphragmatic anomalies in offspring/litter D 21				
N° examined offspring's /litter	173/22	174/22	167/21	176/22
Total Normal diaphragm	168/22	164/22	159/21	161/22
Total thinning w/o protrusion	0/0	0/0	0/0	0/0
Total thinning with protrusion (%)	5/4 **(2.9/18.2%)**	9/5 **(5.2/22.7%)**	8/6 **(4.8/28.6%)**	15*/8 **(8.5*/36.4%)**
Total hernia	0/0	1/1	0/0	0/0

Statistically significantly different from control ** $p<0.01$; * $p<0.05$

Conclusion: treatment of pregnant rats with ziram at levels of 16 and 64 mg/kg bw/d has marked toxic effects on the mothers expressed by clinical signs of toxicity and reduction in body weight and food intake during treatment. NOAEL maternal tox= 8 mg/kg bw/d
Fetal weight was reduced at top dose and there was evidence of an increased incidence of the thinning of the diaphragm with protrusion of the liver into the thorax. The incidence of diaphragmatic changes was lower in the offspring than in foetus and although this was still apparent at weaning there was no evidence that diaphragmatic thinning developed into diaphragmatic hernia after birth or that growth or survival of the offspring at risk was compromised.
NOAEL developmental tox = 16 mg/kg bw/d.

GLP status: the study was performed following GLP principals but is not intended to stand alone as a regulatory study.
Material and methods:
Ziram (batch n° G120012467; 99.6% purity) was administered during the organogenesis phase (day 6-15) of pregnancy to Crl: CD IGS BR rats. The suspension was prepared daily, stored at 4°C and used within 24 h of preparation. Three groups of 44 mated female rats received ziram by gavage at dosages 8, 16 or 64-mg/kg bw/d. A similarly constituted control group received the vehicle (1% w/v methyl cellulose in water) at the same volume-dosage throughout the same period. 22 females in each group were killed on day 20 after mating for foetal examination and the remaining females were allowed to litter to permit examination of offspring on day 4 and 21 of age.
Statistical analysis: Barlett's test was first applied to test homogeneity of variance between groups. Analysis of gestation length was performed by an exact Linear-by-linear test with step down, to test trend. Analysis of incidence of diaphragmatic thinning with protrusion was performed using a generalized linear model with logit function with the dam as a random effect. Each treated group was compared to control using a Wald chi-square test.

Conclusion:

From the new study, the applicant proposes to use a NOAEL development= 16-mg/kg bw/d that is, according to the reviewer, acceptable in that reported study.

The results of the study of Smith et al (1990) that were reported in the draft monograph of ziram (May, 1998) are shown in the table B.6.6.2.1-3 with somewhat more details for more easy comparison and for final decision.

From these latest results, it can be seen that the dose of 16-mg/kg bw/d cannot be considered as a developmental NOAEL: at this dose thinning of diaphragm with protrusion of the liver is biologically significantly increased at 16-mg/kg bw/d onwards when compared with the concurrent control values and also when compared with the historical control data provided by the company.

Table B.6.6.2.1-3: Results of the developmental rat study (ziram monograph, 1998)

Endpoints/dose	0	1	4	16	64 mg/kg bw/d
Females with live young	23	24	22	23	24
N°fetuses/n°litter	261/23	255/24	253/22	266/23	272/24
Mean n° implantations	11.9	11.4	11.9	12.2	11.8
Mean total live young	11.3	10.6	11.5	11.6	11.3
Post implantation loss %	4.7	7.6	3.4	5.1	3.7
Litter weight	37.84	35.02	37.63	36.90	33.78*
Mean fetal weight	3.35	3.29	3.28	3.20	2.98**
Incidence diaphragmatic anomalies in foetuses/litter					
N° examined foetuses /litter	126/23	125/24	123/22	133/20	134/24
Total thinning with protrusion liver(%)	0/0 (0%)	0/0 (0%)	1/1 (0.8/4.5%)	5/4 (3.76/20%)	5/5 (3.73/21%)
Diaphragmatic hernia	1/1	0/0	1/1	0/0	0/0
Historical control for thin diaphragm 1996-2002					
Number examined foetus/litter	9007/1249				
Thin diaphragm	268/205 3%/16.4%				

B.6.10.3 Proposal for a new Acute Reference dose (ARfD) (Annex IIA B.5.10)

During ECCO 78, it was proposed to derive an ARfD for ziram from the rat developmental study reported in table B.6.6.2.1-3. However, spacing between the NOAEL and LOAEL in this study led the applicant to perform a new study in which this gap was filled in. Based on the results reported in the new developmental rat study, an overall NOAEL development rat = 8 mg/kg bw is acceptable. Applying an assessment factor of 100 gives a

ARfD= 0.08 mg/kg bw/d.

This proposal takes into account the current toxicology package which can be used for selection of appropriate endpoint and NOAEL for setting an acute RfD, which can be, in the case of ziram, acute and subchronic neurotoxicity studies and developmental studies.