Benfluralin

Position on proposed CLH classification for reproductive toxicity

Expert Statement

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Executive Summary

This document discusses with particular respect to maternal toxicity, data which form the basis for a classification and labelling harmonization (CLH) proposal to classify benfluralin in the European Union for reproductive and developmental toxicity.

Decreased litter size in a 2-generation study occurred at a dose level associated with marked maternal toxicity clearly exceeding the toxicological target dose (maximum tolerated dose MTD, defined as a reduction of 10% of body weight gain compared to control); weight gains of F0 dams were 35% less than controls up to the point of birth, with body weight loss during the initial lactation periods. Food intake was also impaired throughout the study, and a significant degree of anaemia (a demonstrated effect of benfluralin) must be expected based on comparison with studies investigating hematology after exposure to Benfluralin at comparable doses and durations. This marked deficit undoubtedly affected the ability of the dams to maintain pregnancy to the same level of controls. This same marked toxicological impairment present throughout gestation and lactation, accounts for increased pup loss early in lactation and a decreased weaning index. As these effects are direct maternal toxicity and not related to a developmental mechanism, they should not be subject to classification for reproductive effects.

Pup loss seen in late lactation was more likely attributable to direct benfluralin intake from diet, in the absence of any maternal process, and thus does not represent a reproductive effect subject to classification. This is clearly demonstrated by mortality in post-weaning pups at 21-28 days of age, where intake from diet will be particularly elevated as a consequence of the high food intake to body weight ratio at this developmental stage. In such young animals, pup organ systems which are pivotal in metabolism and elimination are still immature, leading to elevated systemic exposure through reduced clearance. There is no evidence to postulate any transfer in milk for the toxicity seen in the weanlings.

In the developmental toxicity study in rats, an increased incidence of vertebral centrum variants in foetuses clearly correlates with decreases in corrected maternal weight gain, and is a secondary consequence of effects on the dam.

Introduction

Benfluralin is currently undergoing evaluation for hazard classification under Regulation (EC) No 1272/2008 (CLP Regulation), based on the CLH Report (Norwegian Food Safety Authority, October 2019). Among other classifications, the CLH Report proposes classification for developmental and reproductive toxicity.

The purpose of this document is to demonstrate that all findings of developmental and reproductive toxicity, and for effects on or via lactation, are findings secondary to overt maternal toxicity.

Basis of proposed classification as stated in the CLH Report

The CLH Report proposes classification as Repr Cat 2 based on (10.10.6, "Comparison with the CLP Criteria", pp73-74):

- Decreased litter size (number of pups born) in both generations of the 2-generation rat study;
- Decreased body weight and survival of offspring (weaning index) during the lactation phase of the 2-generation rat study;
- Increased incidence of vertebral centra change (reduced ossification) in fetuses of the developmental toxicity study in rats;

The CLH report argues that data are insufficient to demonstrate that these findings are a direct consequence of maternal toxicity, and in the absence of this demonstration then classification is appropriate.

Correlation with maternal toxicity

Decreased litter size (number of pups born) in the 2-generation study:

The top dose of benfluralin was maternally toxic through all study phases, causing marked impairment of body weight and food consumption (see Table 1 below). From the start of the study until LD0 (after birth), top dose F0 dams gained 35% less weight than controls (92g vs 141g). After birth (Lactation day – LD - 0), body weights of F0 dams at the top dose were 16% below those of controls (241g vs 287g). The LD0 value is considered particularly relevant since this is the weight of the "mother that has sustained pregnancy", excluding the weight of the litter, clearly demonstrating direct maternal body weight effects. Food consumption during F0 gestation had been 21% lower than that of the controls (17.9 vs 22.4 g/rat/day). Food consumption did not appear attributable to palatability since there was no marked first-week refusal to eat nor initial loss of weight; the pattern of reduced weight gain and food consumption remained approximately constant throughout the study; the effect on food consumption and body weight must be attributed to toxicity.

The top dose was therefore a group subject to severe toxicity; the decrement in maternal body weight gain until birth (35%) exceeded the level of growth impairment that would be found excessive.

The F1 generation started the premating phase with a mean body weight of 48.6g, a deficit of 43% from their controls (starting body weight 85g). These animals were clearly different to the controls. Though there was some recovery in the deficit by the start of gestation, top dose females still weighed 20% less than controls immediately after birth (LD0), having

gained 35% less weight from the start of the F1 phase, further confirming direct, excessive maternal toxicity.

Table 1: Comparison of maternal body weight and body weight change

Group	Group mean bwt (g) and (% difference from control(			Group mean bwt change (g) from start and (% difference from control)	
	Start	GD0	LD0	GD0	LD0
F0					
Control	145.2	254.5	286.6	109.3	141.4
2	145.6 (0.3)	262.3 (3.1)	294.5 (2.8)	116.7 (6.8)	148.9 (5.3)
3	147.5 (1.6)	246.3 (-6.1)	279.1 (-2.6)	98.8(-15.3)	131.6 (-6.9)
4	149.3 (2.8)	225.9 (-8.3)	241.4 (-15.8)	76.6 **(-22.5)**	92.1 **(-34.9)**
F1					
Control	85.0	264.8	297.9	179.8	212.9
2	85.7 (0.8)	275.8 (4.2)	313.4 (5.2)	190.1 (5.7)	227.7 (7.0)
3	77.0 (-9.4)	262.8 (-0.8)	291.5 (-2.1)	185.8 (3.3)	214.5 (0.8)
4	48.6 (-42.8)	219.4 **(-17.1)**	239.3 **(-19.7)**	170.8 (-5.0)	190.7 (-10.4)

(Values taken from group mean tables; no statistics applied)

Growth and food consumption of maternal F0 animals was markedly impaired both before and during pregnancy, to an extent clearly affecting their ability to maintain pregnancy as well as controls. Body weight and food consumption of F1 dams were also significantly impaired throughout the study; variation from their start weight may not have been so great but their starting baseline was lower.

In addition, Benfluralin is associated with decreased erythrocyte mass in repeat-dose studies with rats, so adult animals across both generations of the 2-generation study may reasonably be expected to be anaemic, increasing the overall maternal toxicity burden during this more vulnerable period. Table 2 shows the deficits in blood haemoglobin levels (selected, so far as possible, at 13 weeks of treatment; bearing in mind that the premating phase of the 2-generation study is at least 10 weeks).

Table 2: Haemoglobin deficit in females from rat repeat-dose studies with benfluralin

Study	ppm in diet	Benfluralin intake (mg/kg bw/day)	Haemoglobin level (% difference from control)	Reference in the CLH report
90-day rat (1996)	1100[@]	88	-3%	CLH report, Table 57
	5000	395	-8%	
	7500	604	-10%	
Carcinogenicity rat (1996)	100	6.8	-4%	CLH report, Table 23 (26-week timepoint)
	2500	168	-11%	
	5000	331	-15%	
2-Generation study (1995)				
Premating	100	11.2 - 7.4[#]		CLH report, Table 33
	1000	97 - 70		
	5000	502 - 334		
Gestation	100	7.3 - 6.3		
	1000	73.9 - 65.3		
	5000	339 - 311		
Lactation	100	9.1 - 19.9		
	1000	90.8 - 198		
	5000	394 - 854		

[@] Lower dose groups omitted, for brevity

[#] Range of intakes expressed, as taken from 2018 RAR

These data are drawn from studies described in the CLH report, but the haemoglobin deficits can be further supported by studies considered supplemental in the 2018 RAR. Particularly during premating and lactation, benfluralin intakes are at a level associated with at least a 10% haemoglobin deficit.

There was a proportion of litters with the number of pups (litter size) born being below the control range of the F0 generation (all F0 control litters having 11 pups or more: see Table 43 of the CHL report.). All F0 litters with 10 or fewer pups were born to dams with less than the mean control bodyweight at LD0 (i.e. the first post-partum maternal body weight independent of litter weight). This demonstrates the relationship between maternal toxicity (manifesting as impaired maternal weight gain) and litter size. An increase in resorptions, which might explain lower litter size, could not be determined since the study did not count uterine implantation sites. However, it is noteworthy that the prenatal development study in rats which had less maternal toxicity after dosing up to 1000 mg/kg/day (approximately 7 fold higher than the 2-generational study) had no effect on embryo-foetal survival at any dose level (which would translate postnatally to smaller litter sizes). This lack of consistency in effect across studies decreases the concern for a direct effect on the maintenance of the pregnancy and provides further evidence that the changes in litter size are secondary to marked maternal toxicity.

Further, the degree of toxicity may have been near-lethal, as may be seen later when “alternate F1 generation” mortality is discussed.

Decreased body weight and survival of offspring (weaning index) during the lactation phase of the 2-generation rat study.

Lower pup body weights and survival are well recognised as a common consequence of poor maternal nurturing and care as a secondary consequence of excessive maternal toxicity. It has already been demonstrated that the body weights and food consumption of dams at the top dose was severely impacted by treatment, constituting excessive maternal toxicity. The effect on food consumption and body weight affected all dams in the group; none appeared individually affected.

A low weaning index (survival of pups to weaning) included early pup loss within the first 4 post-natal days; control litters showed a similar early attrition although this was a lesser incidence than the high-dose litters. The increase in loss of high-dose offspring in the initial days of lactation is considered attributable to the greater stress on the dams, as demonstrated by maternal body weight loss (rather than reduced gain) in this vulnerable period, furthermore, this correlates with a greater than doubling in maternal compound consumption and resultant exposure during this period (note there was no adjustment in dietary concentration to account for the known increase in food demand during the lactation period).

There was increased pup mortality in the later part of weaning (postnatal days 4-21), which the study report considered attributable (in addition to the stress on dams) to the pups starting to consume treated diet. Due to the higher food intake to body weight ratio for small animals compared to adults, the mg/kg/day intake of test material would be higher in the pups than the adults. Toxicity was particularly evident among additional weaned pups retained for a week (days 21-28 of age) after weaning as “potential alternate” F1 animals in case of mortality in the selected F1 generation (discussed in the study report as a ”rest phase”). There was marked mortality among alternate high-dose pups in this phase, demonstrating the toxicity of benfluralin-treated diet. This correlates too with the widely

published increase in sensitivity to adverse effects at this age due to increase systemic exposure compared with adult counterparts; until 5-6 weeks of age in the rat, organ systems involved in metabolism and elimination (largely the hepatic and renal systems) are not fully mature, resulting in higher systemic exposure and propensity for adverse toxicity.

Table 3: Pup loss during lactation

Group	Control	2	3	4
Lactation period days:				
F0				
Days 0-4	10	7	21	29
Days 5-21	3	1	9	13
Alternate F1 generation, days 22-28	0	2	0	19

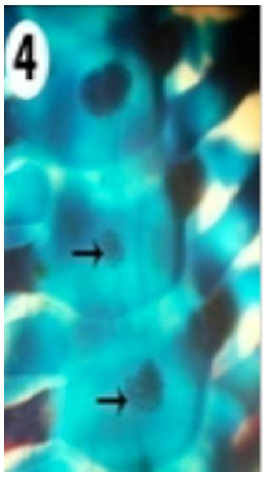
The mortality in post-weaning (alternate F1) pups demonstrates that direct toxicity to pups from diet during weaning is more plausible than toxicity of benfluralin in milk, therefore arguing against classification as “effects on lactation”. The lethality among these offspring, a consequence of the food intake to body weight ratio and poor xenobiotic clearance mechanisms increasing systemic exposure, further indicates that the toxicity seen among high-dose dams in the F0 generation was probably approaching lethal levels.

<u>Increased incidence of vertebral centra change (reduced ossification) in fetuses of the developmental toxicity study in rats</u>

The CLH Report cites an increased incidence of vertebral centra changes (reduced ossification) in foetuses of the rat developmental toxicity study as one of the findings contributory to the proposal for classification. The CLH report wording appears imprecise; vertebral centra change is indicated as “reduced ossification” while the study report terms these variants as “constricted centra” or “bipartite centra”.

Figure 1: Fetal Rat Vertebral Centra (courtesy of Dr [redacted], Exponent)

Control



Reduced Ossification
(at arrows)

(Figure 1 examples are not taken from the benfluralin study)

Gowan however finds these in this case to correlate clearly with maternal toxicity. Maternal toxicity in this study is seen as a retarded weight gain; high-dose females gained less weight

than controls. Table 4 shows, for the control and high dose groups, the individual litter findings of vertebral centra change together with reduced ossification if noted in the litter, and corrected maternal weight gain (i.e., weight gain of the female without the uterus). Group mean corrected maternal weights are included for comparison.

Table 4: Listing of litters showing vertebral centrum variations, with dam weight change

Dam identity	Corrected body weight change (g)	Litter findings (number of foetuses affected; these specific findings only)
Control group (Group 1)		
C28208	62	1 v.centrum
209	65	1 v.centrum
211	78	2 reduced ossification, 1 v.centrum
212	74	1 v.centrum
214	85	4 reduced ossification, 1 v.centrum
215	62	2 reduced ossification, 2 v.centrum
217	65	2 reduced ossification, 1 v.centra
225	49	1 v.centra
Group mean weight change	75	
Top dose (Group 5, 1000 mg/kg bw/day)		
C28302	76	3 reduced ossification, 2 v.centrum
304	62	3 v.centrum
305	47	2 reduced ossification, 1 v.centrum
306	48	1 reduced ossification, 1 v.centrum
310	68	1 reduced ossification, 1 v.centrum
312	72	6 reduced ossification,1 v.centrum
313	46	1 reduced ossification, 1 v.centrum
317	70	3 reduced ossification, 1 v.centrum
319	55	2 reduced ossification, 2 v.centrum
320	71	6 reduced ossification, 5 v.centrum
323	59	2 reduced ossification, 2 v.centrum
325	40	3 reduced ossification, 4 v.centrum
Group mean weight change	64	
Highlight: below control mean weight change; or below top dose mean weight change		

Foetuses showing reduced ossification or vertebral centra change at the top dose came predominantly from females gaining less weight than the mean of the group; and all except one gained less weight than the mean of controls. Even in controls, the vertebral centra observations were predominantly in animals which gained less weight than the mean.

Impaired maternal weight gain influenced the occurrence of variants of the vertebral centra. The increased incidence of vertebral centra variants at the top dose is therefore a secondary consequence of maternal toxicity.