BENALAXYL

First draft prepared by
C. Vleminckx[1] and Vicki Dellarco[2]

[1] *Scientific Institute of Public Health, Division Toxicology, Brussels, Belgium; and*
[2] *United States Environmental Protection Agency, Office of Pesticide Programs, Health Effects Division, Washington, DC, USA*

Explanation

Benalaxyl, the International Organization for Standardization (ISO) approved name for methyl *N*-(2,6-dimethylphenyl)-*N*-(phenylacetyl)-DL-alaninate (a racemic mixture), is a broad-spectrum phenylamide fungicide that inhibits mycelial growth of fungi and germination of zoospores. Benalaxyl was first evaluated by the JMPR in 1987 (Annex 1, reference 52), when an acceptable daily intake (ADI) of 0–0.05 mg/kg bw was established on the basis of a no-observed-effect level (NOEL) of 5.0 mg/kg bw per day for hepatic enlargement in a 13-week dietary study in rats and a safety factor of 100.

Benalaxyl was considered by the present Meeting within the periodic review programme of the Codex Committee on Pesticide Residues. The Meeting reviewed new data on benalaxyl (studies of toxicokinetics, metabolism, acute toxicity after inhalation, eye irritation, mutagenesis

and several studies of toxicity with the two main soil metabolites) that had not been reviewed previously, as well as relevant data from the previous evaluation.

All pivotal studies with benalaxyl were certified as complying with good laboratory practice (GLP).

Evaluation for acceptable daily intake

Several studies performed with benalaxyl were finalized before the Organisation for Economic Co-operation and Development (OECD) guidelines and regulations of GLP were enacted. Still, all relevant studies were subjected to quality assurance (QA) and with only minimal exceptions their protocols comply with today's guideline requirements.

1. Biochemical aspects

Studies of the degradation of benalaxyl in albino rats were evaluated by the 1986 JMPR (Residues) and showed that the substance is rapidly metabolized and excreted in rats (Annex 1, reference 48). This older data set was not summarized here because it had been superseded by newer studies; a study of biliary excretion in rats and studies of metabolism (after administration of single and repeated doses).

All the studies of metabolism were conducted with benalaxyl that was uniformly labelled with ^{14}C on the aniline ring.

1.1 Absorption, distribution and excretion

Rats

The absorption, distribution and excretion of [^{14}C]benalaxyl (radiochemical purity, 99.17%) were studied in a group of fifteen Sprague-Dawley Crl:CD(SD)Br male rats treated by gavage with a single dose of [^{14}C]benalaxyl at 10 mg/kg bw (41 µCi/kg, or 1.517 MBq/kg) and 15 animals with a single dose at 100 mg/kg bw (49 µCi/kg, 1.813 MBq/kg). Whole blood was analysed for radioactivity at 10 time-points after administration (10 min, 30 min, 1, 2, 4, 6, 8, 24, 48 and 72 h), drawing from the caudal vein of each animal (five rats per dose). For the animals of the mass balance group, urine and faeces were collected up to 1 week after administration, and expired air was collected up to 24 h after administration. Tissues were collected at sacrifice from five animals per dose at 0.5 h (t_{max}), 72 and 168 h after administration. The carcasses of all the animals were also analysed for residual radioactivity. The study complied with the principles of GLP (with QA certificate) and the protocol was in accordance with OECD test guideline 417 (1984) or TM B36 from Annex V of Directive 87/302/EEC.

The mean blood profiles at both doses were characterized by a peak level at the first sampling time (10 min) and a gradual decline in the concentrations of radioactivity according to an elimination half-life of about 30 h (33 ± 10 h for the group at the lower dose and 27 ± 4 for the group at the higher dose). Mean peak levels (C_{max}) were 776 ± 243 ng equivalents/ml and 6471 ± 2060 ng equivalents/ml for the two groups, respectively. The mean areas under the curves (AUC) for blood concentration versus time extrapolated to infinity were $9.77 \pm 0.77 \times 10^3$ and $84.93 \pm 7.20 \times 10^3$ h ng equivalents/ml, respectively. The ratios between doses and peak levels and AUCs indicate dose-proportional absorption of the test article. Mean mean residence times were 34 ± 1 h and 29 ± 5 h for the two doses, respectively. Some animals showed a secondary (minor) peak of radioactivity at 6–8 h after administration.

Mean excretion of radioactivity in urine of the group at the higher dose corresponded to 7.96% of the administered dose. Together with 0.43% of the dose found in cage-wash solutions, the total urinary excretion amounted to 8.4% of the administered dose. Most urinary excretion

occurred within 24 h (about 90%). Faecal excretion corresponded to 88.36% of the dose, this excretion being mainly distributed over the first 48 h after administration. Excretion in expired air was negligible (< 0.1% of the administered dose). The total balance of excretion at this dose accounted for 96.84% of the administered dose (Table 1). In the group at the higher dose, the radioactivity recovered in urine and cage-wash solutions accounted for 7.72% and 0.82% respectively. Total urinary excretion of radioactivity was therefore 8.5%. Compared with the excretion pattern in the group at the lower dose, urinary excretion during the collection interval 24–48 h was more substantial (about 20% of total urinary excretion). Total faecal excretion accounted for 85.05% of the administered dose. This excretion occurred mainly within 48 h, except for one animal, which excreted most of the administered dose in the 48–72 h interval. Also at this dose, excretion of radioactivity in expired air was negligible. The total balance of excretion accounted for 93.65% of the dose (Table 2).

Based on the blood profiles, the 0.5 h sampling time was chosen for determination of the tissue distribution at t_{max}. Apart from the stomach wall, where high concentrations were expected (owing to the oral route of administration), high concentrations were found in liver, kidneys and lungs.

At 10 mg/kg bw, the concentration of radioactivity in the liver was 18.3×10^3 ng equivalents/g corresponding to tissue : blood ratios of radioactivity of 82, in the kidneys was 2.7×10^3 ng equivalents/g (tissue : blood ratio, 12) and in the lungs the concentration was 2.4×10^3 ng equivalents/g (tissue : blood ratio, 8.5). The quantity of radioactivity in the liver corresponds to about 7% of the administered dose (Table 3).

At 100 mg/kg bw, the mean concentrations of radioactivity were proportionally higher in respect to the group at the lower dose, and tissue : blood ratios were similar for liver, kidneys and lungs (Table 4). Concerning the other tissues analysed, the concentrations of radioactivity measured were similar to those in the blood. The only difference in tissue distribution between the groups at the two doses was in the brown fat, where the mean tissue : blood ratio was about two times higher for the group at the lower dose. At 72 h after administration, the highest radioactivity concentrations were found in the liver (0.7×10^3 and 6.1×10^3 ng equivalents/g at the lower and higher doses, respectively), large intestine (0.2×10^3 and 1.1×10^3 ng equivalents/g in the groups at the lower and higher doses, respectively) and the kidneys (only for the group at the lower dose,

Table 1. Balance of radioactivity (percentage of total administered dose) in rats given benalaxyl as a single oral dose at 10 mg/kg bw

Collection interval (h)		Radioactivity present in excreta (cumulative)				Total excreted	
Partial	Cumulative	Urine	Faeces	Expired air	Cage wash	Partial	Cumulative
0–4	0–4	—	—	0.04	—	0.04	0.04
4–8	0–8	2.95	—	0.07	—	2.98	3.03
8–24	0–24	7.18	48.59	0.10	0.33	53.17	56.20
24–48	0–48	7.84	84.30	—	0.39	36.43	92.63
48–72	0–72	7.88	86.92	—	0.40	2.67	95.30
72–96	0–96	7.90	87.36	—	0.41	0.47	95.77
96–120	0–120	7.93	88.06	—	0.42	0.74	96.51
120–144	0–144	7.95	88.24	—	0.43	0.20	96.71
144–168	0–168	7.96	88.36	—	0.43	0.13	96.84

From Triolo (1996a)

Table 2. Balance of radioactivity (as percentage of total administered dose) in rats given benalaxyl as a single oral dose at 100 mg/kg bw

Collection interval (h)		Radioactivity present in excreta (cumulative)				Total excreted	
Partial	Cumulative	Urine	Faeces	Expired air	Cage wash	Partial	Cumulative
0–4	0–4	—	—	0.03	—	0.03	0.03
4–8	0–8	1.87	—	0.05	—	1.90	1.92
8–24	0–24	5.67	27.26	0.07	0.63	31.71	33.62
24–48	0–48	7.30	62.30	—	0.73	36.76	70.39
48–72	0–72	7.52	82.88	—	0.75	20.82	91.21
72–96	0–96	7.58	83.73	—	0.76	0.94	92.15
96–120	0–120	7.65	84.17	—	0.79	0.53	92.68
120–144	0–144	7.69	84.65	—	0.80	0.58	93.21
144–168	0–168	7.72	85.05	—	0.82	0.44	93.65

From Triolo (1996a)

Table 3. Recovery of radioactivity in tissues of rats given benalaxyl as a single oral dose at 10 mg/kg bw

Tissue	Time of sacrifice, 0.5 h			Time of sacrifice, 72 h		
	ng equiv./g[a,b]	Percentage of administered dose	Tissue/blood	ng equiv./g[b]	Percentage of administered dose	Tissue/blood
Blood	238	—	—	28	—	—
Liver	1 8305	6.72	82.28	691	0.36	27.59
Kidneys	2 736	0.27	11.95	101	0.01	3.78
Lungs	2 401	0.11	8.49	36	0.00	1.08
Stomach wall	99 417	7.47	483.31	49	0.00	2.23
Intestine wall	497	0.05	2.22	184	0.01	6.87
Brain	139	0.01	0.62	54	0.00	1.63
Spleen	395	0.01	1.82	31	0.00	0.94
Muscle	299	—	1.34	50	—	1.49
Brown fat	605	—	2.66	17	—	0.58
Bone (femur)	185	—	0.79	44	—	1.37
Testes	204	0.02	0.91	51	0.00	1.55
Carcass	947	25.96	—	0	0.00	—

From Triolo (1996a)

[a] When 40 mg of tissue was analysed, the limit of quantification was about 60 ng/g.

[b] Units are ng equivalents/ml for blood and digested carcass.

0.1×10^3 ng equivalents/g) (Tables 3 and 4). At both doses, mean concentrations in the liver corresponded to about 27 times those in the blood (0.03×10^3 and 0.2×10^3 ng equivalents/ml at the lower and higher dose, respectively). The amount of radioactivity in the wall of the intestine exceeded that in the blood by about four to eight times in all animals at each dose. For the group at the lower dose, the concentrations of radioactivity in the kidneys corresponded to about three to five times those in the blood. At 168 h, in the group at the lower dose, quantifiable levels were present only in the blood (three animals, 11 ng equivalents/ml), liver (all animals, 333 ng equivalents/g) and kidneys (two animals, 32 ng equivalents/g). In the group at the higher dose, quantifiable levels were present only in the blood (all animals, 146 ng equivalents/ml) and in liver

Table 4. Recovery of radioactivity in tissues of rats given benalaxyl as a single oral dose at 100 mg/kg bw

Tissue	Time of sacrifice, 0.5 h			Time of sacrifice, 72 h		
	ng equiv./g[a,b]	Percentage of administered dose	Tissue/blood	ng equiv./g [b]	Percentage of administered dose	Tissue/blood
Blood	4 791	—	—	230	—	—
Liver	297 671	9.84	67.92	6 141	0.34	27.19
Kidneys	54 174	0.47	11.80	149	0.00	0.55
Lungs	14 231	0.07	3.25	0	0.00	0.00
Stomach wall	1 038 349	8.89	261.88	375	0.00	1.85
Intestine wall	7 579	0.07	1.64	1 053	0.01	4.50
Brain	2 161	0.02	0.49	0	0.00	0.00
Spleen	6 246	0.01	1.38	0	0.00	0.00
Muscle	4 975	—	1.06	0	—	0.00
Brown fat	6 576	—	1.43	0	—	0.00
Bone (femur)	3 286	—	0.71	0	—	0.00
Testes	3 095	0.03	0.69	0	0.00	0.00
Carcass	9 928	28.28	—	0	0.00	—

From Triolo (1996a)

[a] When 40 mg of tissue was analysed, the limit of quantification was about 600 ng/g.

[b] ng equivalents/ml for blood and digested carcass.

(all animals, 1868 ng equivalents/g). Carcasses of animals sacrificed at 0.5 h retained about 30% of the administered dose, while from 72 h onwards, levels of radioactivity retained were not quantifiable.

[^{14}C]Benalaxyl is rapidly absorbed from the gastrointestinal tract, but the absorbed quantities are small in respect to the administered dose, as shown by the low peak concentrations and AUCs. The absorbed quantities are proportional to the doses administered. The half-life of elimination is about 30 h. The limited absorption is confirmed by the excretion pattern; < 10% of the dose is excreted by urinary route and about 90% is found in faeces. The absorbed amount is rapidly excreted in the urine, mainly within 24 h, though it cannot be excluded that part of the absorbed radioactivity is excreted by the biliary–faecal route. Elevated levels of radioactivity were found in the liver at 0.5 h after administration. Increased concentrations in respect to the blood levels were also found in the kidneys. At 72 h and 168 h after administration, relatively high concentrations of radioactivity were present only in the liver (Triolo, 1996a).

In a second study, the absorption, distribution and excretion of [^{14}C]benalaxyl (radiochemical purity, 99.17%) were studied in fifteen Sprague-Dawley Crl:CD(SD)Br male rats that were treated orally with unlabelled benalaxyl as 14 daily doses at 10 mg/kg bw, followed by a single dose of [^{14}C]benalaxyl at 10 mg/kg bw (51 μCi/kg). Whole blood was analysed for radioactivity at 10 times after administration (10 min, 30 min, 1, 2, 4, 6, 8, 24, 48 and 72 h), drawing from the caudal vein of each animal (five rats per dose). For the animals of the mass balance group, urine and faeces were collected up to 1 week after administration, and expired air was collected up to 24 h after administration. Tissues were collected at sacrifice from five animals

per dose at 0.5 h (t_{max}), 72 and 168 h after administration. The carcasses of all the animals were also analysed for residual radioactivity. The study complied with the principles of GLP (with QA certificate provided) and the protocol was in accordance with OECD test guideline 417 (1984) or TM B36 from Annex V of Directive 87/302/EEC.

The mean blood profile was characterized by a peak level at the second sampling time (0.5 h) and a gradual decline in concentrations of radioactivity, according to an elimination half-life of about 36 h (36 ± 13 h). The mean peak level was 390 ± 36 ng equivalents/ml. The mean AUC for blood concentration versus time extrapolated to infinity was $8.22 \pm 1.47 \times 10^3$ h.ng equivalents/ml. Mean residence time was 45 ± 13 h. Looking at individual blood profiles of radioactivity, the Meeting noted the variability in peak time: two animals showed the peak level at 0.5 h and three animals at 10 min. A secondary peak was observed in three animals, at 6 h or 8 h.

Mean excretion of radioactivity in urine at the lower dose corresponded to 7.41% of the administered dose. Together with 0.60% of the administered dose found in cage-wash solutions, the total urinary excretion amounted to 8.0% of the dose. Most of the urinary excretion occurred within 24 h (about 85%). Faecal excretion corresponded to 89.10% of the dose, this excretion being mainly distributed over the first 48 h after administration. Excretion in expired air was negligible (< 0.1% of the dose). The total balance of excretion accounted for 97.13% of the administered dose (Table 5).

Based on the blood profiles described above, the 0.5 h sampling time was chosen for determination of the tissue distribution at t_{max}. Apart from the stomach wall, where high concentrations of radioactivity were expected, considering the route of administration, high concentrations were found in liver and kidneys. In the liver, radioactivity concentrations were 21.8×10^3 ng equivalents/g corresponding to tissue : blood ratios of radioactivity of 51. The concentration in the kidneys was 3.3×10^3 ng equivalents/g (tissue : blood ratio, 8.0). The quantity of radioactivity in the liver corresponded to about 7% of the dose. Relatively high concentrations were also found in the intestine (1.7×10^3 ng equivalents/g) and the lungs (0.8×10^3 ng equivalents/ml) (Table 6). At 72 h after dosing, quantifiable concentrations of radioactivity were found in the blood (0.03×10^3 ng equivalents/ml), the liver (0.6×10^3 ng equivalents/g), the intestine wall (0.3×10^3 ng equivalents/g) and the kidneys (0.05×10^3 ng equivalents/g). In the kidneys, levels of radioactivity were quantifiable in three out of five animals (Table 6). At 168 h after dosing, blood concentrations were quantifiable in all animals (0.05×10^3 ng equivalents/ml).

Table 5. Balance of radioactivity (as percentage of total administered dose) in rats given benalaxyl as repeated oral doses at 10 mg/kg bw

Collection interval (h)		Radioactivity present in excreta (cumulative)				Total	
Partial	Cumulative	Urine	Faeces	Expired air	Cage wash	Partial	Cumulative
0–4	0–4	—	—	0.01	—	0.01	0.01
4–8	0–8	3.35	0.42	0.02	—	3.77	3.79
8–24	0–24	6.33	47.64	0.02	0.39	50.59	54.38
24–48	0–48	7.24	83.93	—	0.48	37.29	91.67
48–72	0–72	7.30	86.77	—	0.49	2.92	94.59
72–96	0–96	7.34	88.07	—	0.52	1.37	95.96
96–120	0–120	7.3	88.98	—	0.55	0.97	96.93
120–144	0–144	7.39	89.05	—	0.58	0.11	97.04
144–168	0–168	7.41	89.10	—	0.60	0.09	97.13

From Triolo (1996b)

Regarding the tissue concentrations, quantifiable levels were found in the liver of all five animals (0.30×10^3 ng equivalents/g) and in the kidneys (0.01×10^3 ng equivalents/g) of one animal. In all other tissues, concentrations of radioactivity were not quantifiable. In carcasses of animals sacrificed at 0.5 h, about 40% of the dose was found; in carcasses of animals sacrificed at 72 and 168 h radioactivity was not quantifiable.

[14C]Benalaxyl is rapidly absorbed from the gastrointestinal tract, but the absorbed quantities are small in respect to the administered dose, as is evidenced by the low peak concentrations and AUCs. The absorbed quantities are proportional to the doses. The half-life of elimination is about 36 h. The limited absorption is confirmed by the excretion pattern; < 10% of the dose is excreted by urinary route and about 90% is found in faeces. The absorbed amount is rapidly excreted in urine, mainly within 24 h, though it cannot be excluded that part of the absorbed radioactivity is excreted by the biliary–faecal route. Elevated levels were found in the liver at 0.5 h after administration. Increased concentrations in respect to the blood levels were also found in intestine wall and kidneys. At 72 h and 168 h after administration, relatively high levels of radioactivity were still present only in the liver. Compared with the previous study, after single benalaxyl administration to rats, blood profiles, excretion pattern and tissue distribution were very similar (Triolo, 1996b).

One study was performed to obtain information on the biliary excretion of [14C]benalaxyl. Two groups of rats, each consisting of at least four males and four females, were bile-duct cannulated. After cannulation and a 12–24 h post-operative recovery period, a single dose of [14C]benalaxyl (either 10 mg/kg bw or 100 mg/kg bw, radiochemical purity, > 99%) was administered by oral gavage. Bile was collected at 8, 24, 48 and 70 h after administration of the radiolabelled dose. Urine, faeces and cage wash were collected each day for 70 h after dosing, and

Table 6. Recovery of radioactivity in tissues of rats given benalaxyl as repeated oral doses at 10 mg/kg bw

Tissue	Time of sacrifice, 0.5 h			Time of sacrifice, 72 h		
	ng equiv./g [a]	Percent of administered dose	Tissue/blood	ng equiv./g [a]	Percent of administered dose	Tissue/blood
Blood	515	—	—	34	—	—
Liver	21 847	7.15	51.32	614	0.29	20.62
Kidneys	3 316	0.25	7.94	47	0.00	1.17
Lungs	826	0.04	1.92	0	0.00	0.00
Stomach wall	108 142	6.19	246.97	0	0.00	0.00
Intestine wall	1 740	0.13	3.71	330	0.03	12.53
Brain	145	0.01	0.40	0	0.00	0.00
Spleen	1 017	0.02	2.34	0	0.00	0.00
Muscle	467	—	1.08	0	—	0.00
Brown fat	640	—	1.37	0	—	0.00
Bone (femur)	280	—	0.64	0	—	0.00
Testes	203	0.02	0.47	0	0.00	0.00
Carcass	1 931	43.99	—	0	0.00	—

From Triolo (1996b)

[a] ng equivalents/ml for blood and digested carcass

[b] Cumulative volume of carcass and nitric acid in ml

blood and tissue were taken at sacrifice and prepared for analysis of residual radioactivity. The study was performed in compliance with the principles of GLP (with QA certificate provided).

After a single oral administration at 10 mg/kg bw, means of 88.93% and 82.13% of the administered dose were excreted in the bile of male and female rats, respectively over the 70 h study period. Most of this radioactivity (85.58% and 80.30% from males and females respectively) was excreted in the first 8 h after dosing. A mean total of 3.87% and 7.13% were excreted in the urine of male and females respectively, with 4.89% and 5.33% in the faeces and 0.57% and 0.29% in the cage wash (Table 7).

After a single oral administration at 100 mg/kg bw, means of 75.22% and 66.03% of the administered dose were excreted in the bile from male and female rats respectively, over the 70 h study period. Most of this radioactivity (69.56% and 60.62% from males and females, respectively) was excreted in the first 8 h after dosing. A mean total of 4.79% and 13.95% were excreted in the urine of the males and females respectively, with 17.56% and 9.18% in the faeces and 0.18% and 1.41% in the cage wash (Table 7).

At 70 h after dosing, the only tissue with levels of radioactivity higher than background was the gastrointestinal tract: mean of 0.01%/0.02% (male/female) and 0.12%/0.01% (male/female) at the lower and higher doses respectively.

Most of the administered radioactivity (60–86%) was excreted in the bile in the first 8 h after dosing at the higher and lower doses. By 24 h after dosing, between 89.45% and 96.80% of the administered radioactivity had been excreted in the urine, faeces and bile (including cage wash). Less than 0.2% remained in the carcass at 70 h. Thus, [^{14}C]benalaxyl is absorbed and excreted rapidly, mainly via the bile (Kemp, 2001).

1.2 Biotransformation

Rats

A generalized metabolic pathway for benalaxyl in rats is shown in Figure 1.

In the pharmacokinetic studies after single (10 or 100 mg/kg bw) or repeat (once a day 10 mg/kg bw for 15 consecutive days and a radioactive dose on the last administration only) oral administration of [^{14}C]benalaxyl to Sprague-Dawley Crl:CD (SD) Br male rats (Triolo, 1996a and 1996b), urine and faeces samples collected over 0–8, 8–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 h (first sampling for faeces was collected over 0–24 h) were analysed for metabolites. The study was performed in compliance with the principles of GLP (with QA certificate) and in compliance with United States EPA, Subdivision F, 85-1 and Commission Directive 94/79/EC (1994, Annex I, item 5.1).

The metabolic profile was the same after single oral (low and high) administration or multiple oral administration to rats. The urinary metabolic pathway was identical to the faecal metabolic pathway as the isolated faecal metabolites were the same as urinary bio-transformation products; the only difference was the absence of benalaxyl in urine (Tables 8, 9 and 10).

Urine was a minor route of elimination of radioactivity, therefore the percentages of each metabolite in urine were always very low. The main compounds were metabolite 4 (0.83%, 0.89% and 1.38% of administered low single, high single and multiple dose respectively), metabolite 6 (0.60%, 0.78% and 0.76%), metabolite 7 (1.02%, 0.995% and 0.82%) and metabolite 8 (0.63%, 1.005% and 0.78%). Radiolabel was mainly excreted via the faeces. The percentages of unchanged benalaxyl (compound 12) amounted to 2.73%, 1.05% and 1.42% of administered low single, high single and multiple doses respectively. The main degradation compound in the faeces was metabolite 8 (15.99%, 16.41% and 20.25%); the other significant compounds were: metabolite 6 (7.40%, 9.80% and 9.80%), metabolite 7 (6.56%, 5.80% and 4.73%), metabolite 5 (6.08%, 6.65% and 6.25%), metabolite 4 (6.24%, 5.18% and 5.82%), metabolite 2 (6.69%, 5.78%

and 5.68%), and metabolite 1 (8.59%, 8.12% and 9.96%). The amounts of metabolites 3, 9, 10 and 11 accounted for less than 5% (Tables 7, 8 and 9). Metabolite 1 was the sum of nine compounds: the amount of each one was always lower than 3.30%. Identification of metabolites 2 and 3 was not technically achievable. Metabolites 4, 5, 6, 7, 8, 9, 10, 11 and 12 were identified in the faeces by chromatographic and mass spectrometric techniques.

Table 7. Recovery of radioactivity[a] in bile-duct cannulated rats 70 h after receiving a single oral dose of [^{14}C]benalaxyl at 10 or 100 mg/kg bw

Site	Time-point	Nominal dose (mg/kg bw)			
		10 mg/ kg bw[a]		100 mg/kg bw[b]	
		Males	Females	Males	Females
Urine	24 h	3.78	7.02	4.71	13.68
	48 h	0.07	0.08	0.05	0.23
	70 h	0.02	0.03	0.03	0.05
	Total	3.87	7.13	4.79	13.95
Faeces	24 h	4.41	4.88	15.28	8.82
	48 h	0.42	0.42	1.16	0.32
	70 h	0.06	0.03	1.13	0.04
	Total	4.89	5.33	17.56	9.18
Cage wash	24 h	0.50	0.23	0.16	1.10
	4 h	0.04	0.03	0.01	0.22
	70 h	0.04	0.03	0.01	0.09
	Total	0.57	0.29	0.18	1.41
Bile	8 h	85.58	80.30	69.56	60.62
	24 h	2.53	1.63	5.45	5.23
	4 h	0.20	0.15	0.15	0.12
	70 h	0.63	0.06	0.07	0.06
	Total	88.93	82.13	75.22	66.03
Tissues	Gastrointestinal tract	ND	ND	0.00	ND
	Gastrointestinal tract contents	0.01	0.02	0.12	0.01
	Skin	ND	ND	ND	ND
	Carcass	ND	ND	ND	ND
	Total tissues	0.01	0.02	0.12	0.01
Blood	70 h	ND	0.01	ND	ND
Overall total		98.27	94.92	97.87	90.58

From Kemp (2001)

ND, results within background range; 0.00 means < 0.005%

[a] Results are mean values and are expressed as percentage of administered radiochemical dose.

[b] Achieved doses were: males, 11.55 mg/kg bw (397.95 kBq); females, 11.17 mg/kg bw (349.56 kBq).

[c] Achieved doses were: males, 107.67 mg/kg bw (403.76 kBq); females, 109.37 mg/kg bw (374.72 kBq).

Table 8. Proportion of metabolites excreted in the urine and faeces of male rats given benalaxyl as a single oral dose at 10 mg/kg bw[a]

Metabolites	Urine[b]	Faeces[c]	Urine plus faeces
1	0.68	8.59	9.27
2	0.62	6.69	7.31
3	0.46	5.81	6.27
4	0.83	6.24	7.07
5	0.37	6.08	6.45
6	0.60	7.40	8.00
7	1.02	6.56	7.58
8	0.63	15.99	16.62
9	0.26	3.21	3.47
10	0.23	2.28	2.51
11	0.10	3.98	4.08
12 (benalaxyl)	ND	2.73	2.73
[14]C-labelled, unextractable	NR	8.74	8.74
[14]C-labelled, total	5.80	84.30	90.10

From Castoldi & Pizzingrilli (1997); ND, not detected; NR, not relevant.

[a] Results are expressed as percentage of benalaxyl equivalents relative to ^{14}C administered.

[b] Mean values for rats Nos 11, 12 and 14 at 0–24 h.

[c] Mean values for rats Nos 11, 12, 13, 14 and 15 at 0–48 h.

Table 9. Proportion of metabolites excreted in the urine and faeces of male rats given benalaxyl as a single oral dose at 100 mg/kg bw[a]

Metabolites	Urine[b]	Faeces[c]	Urine plus faeces
1	0.710	8.120	8.830
2	0.685	5.780	6.465
3	0.415	4.710	5.125
4	0.890	5.180	6.070
5	0.370	6.650	7.020
6	0.780	9.880	10.660
7	0.995	5.800	6.795
8	1.005	16.410	17.415
9	0.215	2.240	2.455
10	0.180	1.480	1.660
11	0.130	1.880	2.010
12 (benalaxyl)	ND	1.050	1.050
[14]C-labelled, unextractable	NR	9.240	9.240
[14]C-labelled, total	6.375	78.420	84.795

From Castoldi & Pizzingrilli (1997); ND, not detected; NR, not relevant.

[a] Results are expressed as percentage of benalaxyl equivalents relative to 14C administered.

[b] Mean values for rats Nos 16, 17, 18 and 19 at 0–24 h.

[c] Mean values for rats Nos 16 and 17 at 24 –72 h and Nos 18, 19 and 20 at 0–48 h.

Table 10. Proportion of metabolites excreted in the urine and faeces of male rats given benalaxyl as repeated oral doses at 10 mg/kg bw[a]

Metabolites	Urine[b]	Faeces[c]	Urine plus faeces
1	0.67	9.96	10.63
2	0.58	5.68	6.26
3	0.43	4.12	4.55
4	1.38	5.82	7.20
5	0.29	6.25	6.54
6	0.76	9.80	10.56
7	0.82	4.73	5.55
8	0.78	20.25	21.03
9	0.36	2.37	2.73
10	0.21	1.48	1.69
11	0.05	2.80	2.85
12 (benalaxyl)	ND	1.42	1.42
^{14}C-labelled, unextractable	NR	8.99	8.99
^{14}C-labelled, total	6.33	83.67	90.00

From Castoldi & Pizzingrilli (1997).

ND, not detected; NR, not relevant

[a] Results are expressed as percentage of benalaxyl equivalents relative to ^{14}C administered.

[b] Mean values for rats Nos 6, 7, 8, 9 and 10 at 0–24 h.

[c] Mean values for rat No. 6 at 24–48 h and for rats Nos 7, 8, 9 and 10 at 8–48 h.

In conclusion, the new studies of metabolism in rats confirm that benalaxyl is rapidly excreted and extensively metabolized, mainly by oxidation of the methyl group of the aniline ring to hydroxymethyl group and finally to the carboxylic acid. Minor metabolic pathways were the hydroxylation of the phenyl ring and the hydrolysis of the carboxymethyl group (Figure 1) (Castoldi & Pizzingrilli, 1997).
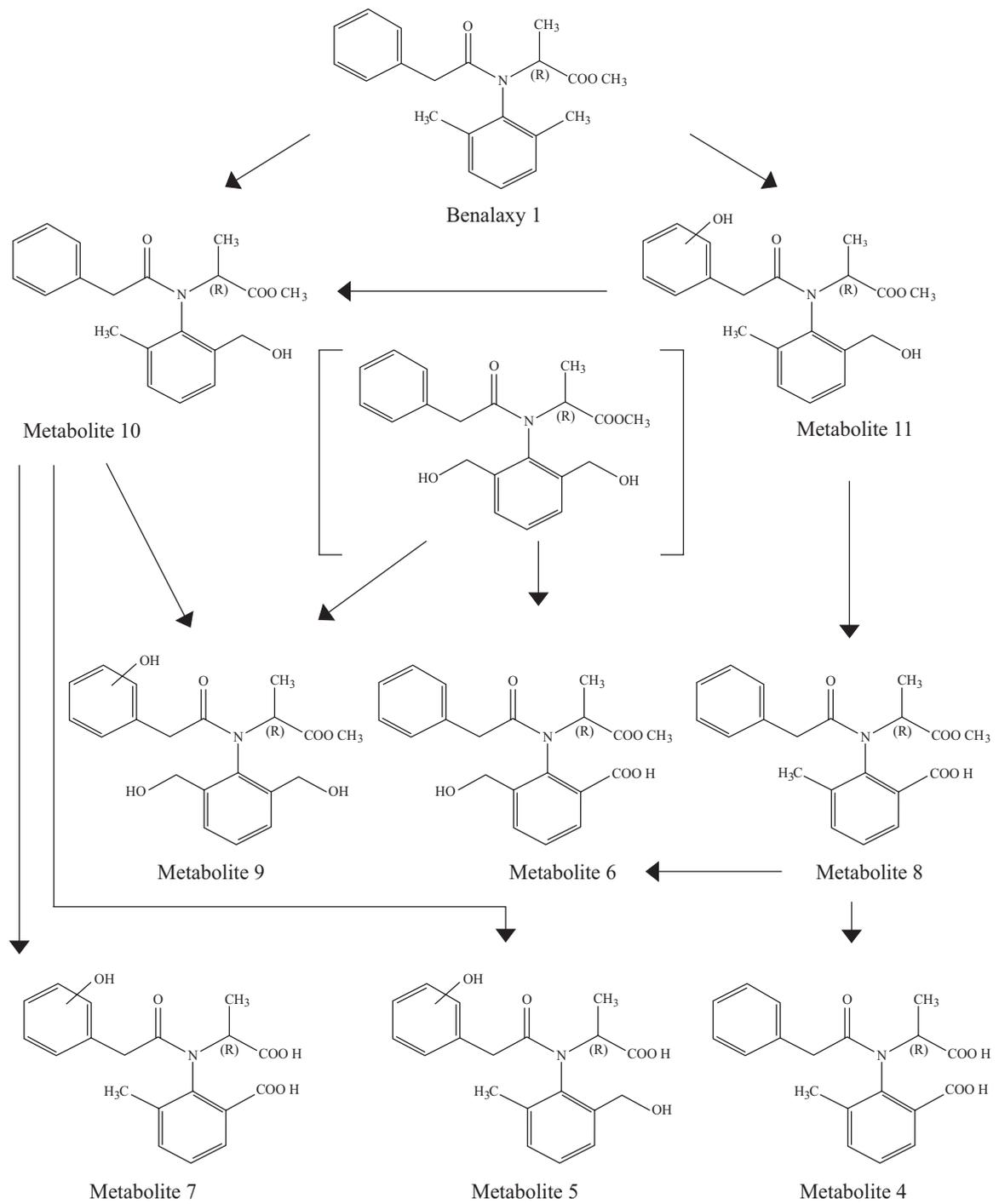
2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The acute toxicity of benalaxyl after administration by the oral, dermal, inhalation and intraperitoneal routes is summarized in Table 11.

Benalaxyl is of low acute toxicity via the oral, dermal and inhalation routes. In rats, the oral LD_{50} for males and females combined was 4200 mg/kg bw; the dermal LD_{50} was > 5000 mg/kg bw and the inhalation LC_{50} (4 h exposure) was > 4.20 mg/l air. There were no significant symptoms of intoxication in the studies of oral and dermal exposure. In mice, the oral LD_{50} was 680 mg/kg bw. Signs of intoxication included loss of sense of equilibrium, uncoordinated movements and asthenia. In rabbits, the dermal LD_{50} was > 2000 mg/kg bw.

Figure 1. Proposed metabolic pathway of benalaxyl in rats

Benalaxy 1

Metabolite 10

Metabolite 11

Metabolite 9

Metabolite 6

Metabolite 8

Metabolite 7

Metabolite 5

Metabolite 4

From Castoldi & Pizzingrilli (1997)
Benalaxyl: methyl *N*-(phenylacetyl)-*N*-(2,6-xylyl)-DL-alaninate
Metabolite 4: *N*-(phenylacetyl)-*N*-(2-carboxy-6-methylphenyl)-alanine
Metabolite 5: *N*-(hydroxyphenylacetyl)-*N*-(2-hydroxymethyl-6-methylphenyl)-alanine
Metabolite 6: methyl *N*-(phenylacetyl)-*N*-(2-carboxy-6-hydroxymethylphenyl)-alaninate
Metabolite 7: methyl *N*-(hydroxyphenylacetyl)-*N*-(2-carboxy-6-methylphenyl)-alaninate
Metabolite 8: methyl *N*-(phenylacetyl)-*N*-(2-carboxy-6-methylphenyl)alaninate
Metabolite 9: methyl *N*-(hydroxyphenylacetyl)-*N*-(2,6-dihydroxymethylphenyl)-DL-alaninate
Metabolite 10: methyl *N*-(hydroxyphenylacetyl)-*N*-(2-hydroxymethyl-6-methylphenyl)-alaninate
Metabolite 11: methyl *N*-(phenylacetyl)-*N*-(2-hydroxymethyl-6-methylphenyl)-alaninate.

(b) Dermal and ocular irritation and dermal sensitization

Benalaxyl (purity, 97.7%) is not a skin irritant to rabbit skin when applied under occlusive dressing for 24 h. No irritating reactions were observed on the intact and scarified skin during the three assessments made. No symptom of intoxication was observed (Dal Re et al., 1979c). Although, the study was performed before the implementation of GLP, it was regarded as acceptable for the purposes of evaluation.

The ocular irritation potential of benalaxyl (purity, 96.68%) was tested in three male New Zealand White rabbits given 0.1 g of test material placed in the conjunctival sac of the right eye. The other eye remained untreated to serve as a control. Ocular reactions were recorded 1, 24, 48 and 7 h after instillation. After the 24 h reading, the cornea was examined after instillation of one drop of 1% fluorescein and successive washing out with sterile saline solution. The study was performed according to GLP and the protocol applied was in compliance with TM B5 from Annex V of Directive 92/69/EEC.

Neither mortality nor adverse general clinical modifications were seen during the study. Slight (grade 1) conjunctival redness was observed in all rabbits at the readings carried out 1 h to 48 h after treatment. No eye changes were recorded at the last observation, 72 h after exposure. Negative results were obtained at the fluorescein staining performed 24 h after the test article application. Benalaxyl is not an eye irritant (Renoldi, 1999).

Benalaxyl (purity, 92.5%) is not a sensitizer when tested in guinea-pigs according to the Magnusson & Kligman method (maximization test) (Vola Gera & Vasconi, 1981).

2.2 *Short-term studies of toxicity*

Mice

In a dose range-finding study carried out for a study of carcinogenicity, groups of 20 male and 20 female Swiss mice received diets containing benalaxyl (purity, 94%) at a concentration of 0, 500, 1000, 2000, 3000 or 5000 ppm for 90 days. The doses were equal to a mean daily intake of 0, 82, 162, 318, 467 and 842 mg/kg bw per day in males and 0, 88, 177, 360, 553 and 874 mg/kg bw per day in females. Half of the mice of each group and sex were sacrificed after 40 days of treatment for interim evaluation. Observations included clinical signs, mortality (daily), body weight, food and water consumption (weekly), organ weight, gross and microscopic pathology. The test method was not specified in the report (in-house method), but detailed procedure was included in the report and complied to some extent with OECD test guideline 408 (1998). The main deviations were that no ophthalmological, haematological or biochemical exams were performed. The study is regarded as additional information for the evaluation.

No deaths occurred and there were no effects on body weight, food and water consumption. Clinical observation did not reveal any abnormality that could be related to treatment, apart from

an excessive waste of food caused by mice at 5000 ppm. At necropsy, there was a dose-related increase in absolute and relative liver weights that occurred at and above 1000 ppm at 96 days (increase in relative weight of 18.8, 39.4 and 52.8% in females at 1000, 3000 and 5000 ppm, and 32.6% in males at 5000 ppm) and at and above 2000 ppm at 42 days (increase in relative weight of 22.9, 21.8 and 27.5% in females at 2000, 3000 and 5000 ppm compared with controls), especially in female mice. Differences observed in adrenal and kidney weights were transient, inconsistent and sex limited; because of their random distribution, they were considered to be of no toxicological significance. Histopathology showed no significant differences between treated and control groups.

Table 11. Acute toxicity of benalaxyl

Species	Strain	Sex	Route	Vehicle	LD_{50} (mg/kg bw; 95% CI or range) LC_{50} (mg/l air)	Reference
Mouse	Albino, Swiss strain	M & F	Oral	30% in DMSO	680 (613–755)[a]	Dal Re et al. (1981)
Rat	Wistar	M & F	Oral	30% in DMSO	4 200 (3 500–5 040)[b]	Dal Re et al. (1979a)
Rat	Wistar (Bor:WISW SPF)	M & F	Inhalation	Aerosol, 4 h, nose-only	> 10[c]	Sterner & Pfenning (1985)
	HanIbm:WIST (SPF)	M & F	Inhalation	Aerosol, 4 h, nose-only	> 4.20 (highest attainable concentration)[d]	Dotti (2000)[e]
Rat	Wistar	M & F	Dermal	Physiological saline	> 5 000[f]	Dal Re et al. (1980)
Rabbit	NZW	M & F	Dermal	Physiological saline	> 2 000[g]	Vola Gera & Vasconi (1983)
Rat	Wistar	M & F	Intraperitoneal	30% in DMSO	1 100 (880–1 375)[h]	Dal Re et al. (1979b)

DMSO, dimethylsulfoxide; F, female; M, male

[a] Groups of five male and five female mice were treated with benalaxyl (purity, 92.5%) at a dose of 0, 400, 500, 600, 700, 800 and 900 mg/kg bw. The animals were observed for 14 days before sacrifice. The study was performed before the implementation of GLP and was based on proposed EPA Guidelines for the Registration of Pesticides in the USA (1978). Mortality occurred in one, one, two, five and five males and in zero, three, three, four and four females at 500, 600, 700, 800 and 900 respectively. Death occurred in the first 24 h after treatment and was preceded by loss of the sense of equilibrium, uncoordinated movements and asthenia.

[b] Groups of five male and five female fasted rats were treated with benalaxyl (purity, 96.5%) at doses of 0, 2500, 3750 and 4500 mg/kg bw. The animals were observed for 14 days before sacrifice. The study was performed before the implementation of GLP and was based on proposed EPA Guidelines for Registration of Pesticides in the USA (1978). The study was accepted for evaluation. Death occurred within 24 h after administration in two out of five males and four out of five females treated at 4500 mg/kg bw and two out of five males and two out of five females treated at 3750 mg/kg bw. There were no significant symptoms of intoxication during the entire observation period. No deviation from normality was found in any of the organs or tissues examined in the gross necropsy.

[c] Benalaxyl (purity, 97.1%) was applied diluted in acetone : alcohol (1 : 3) at a rate of 5% at a dose of 10 mg/l air to 10 male and 10 female rats immobilized in special tubes and connected to the inhalation chamber for 4 h, by nose only. The chamber used in this assay was a Kimmerle system; the nebulizing nozzle produced an aerosol with particle sizes of about 2–5 μm. The sample was injected at constant rate into the nebulizer by means of an automatic syringe. Mixed up with the air, an aerosol was prepared, that was uniformly distributed throughout the chamber and inhaled by the animals. The concentration of the test material in the chamber atmosphere was determined by the following formula: $A / B \times C = D$, where A is the amount of the test substance used during the exposure period (weight or volume), B is the air flow (l/min), C is the duration of exposure (min) and D is the theoretical concentration of sample in chamber atmosphere (mg or μl/l of air change). At the end of the 4 h exposure period, the animals were removed from the chamber, their heads cleaned and they were housed in individual cages. Observations were made continuously of the appearance and behaviour of the animals during the exposure period and at frequent intervals thereafter for a total of

14 days. The animals were then weighed, sacrificed and gross autopsies were performed. The study was performed before the implementation of GLP and no guidelines were mentioned. Main deviation from OECD test guidelines was that no monitoring was made of real concentration of substance in breathing zone. The study was not accepted for evaluation. During the exposure period, observations like reduced awareness, abdominal tone and skin turgor, slight diarrhoea, increased respiration rate and reduced body temperature were considered to be normal findings and not treatment related. During and several hours after the inhalation period, the animals showed a slightly reduced coordination ability as well as a slight diarrhoea: 24 h later all test animals showed a normal behaviour and looked clinically normal, reflex examinations revealed normal conditions and defecation was normal again. Body weight development was normal in males, but in females body-weight gain was slightly reduced during the 14 days of observation. No death occurred. At terminal sacrifice, no macroscopical changes in the body cavities were found.

[d] Five male and five female rats were exposed to benalaxyl (purity, 96.68%) for 4 h in a nose-only exposure system at a mean gravimetric concentration of 4.20 mg/l (highest technically achievable concentration) measured in the breathing zone. The test atmosphere was generated using a CR 3020, powder generator feeding a micronizing Jet-Mill. The aerosol generated was then discharged into the exposure chamber through a [63]Ni charge neutralizer with a target MMAD of 1 to 4 µm. The MMAD of the particles was 3.03 µm with a GSD of 2.66 µm, which ensured bronchiolar and alveolar exposure of the animals. The animals were observed for 14 days before sacrifice. Experimental protocol was in compliance with OECD test guideline 403 (1981) or TM B2 from Annex V of Directive 92/69/EEC. The study was accepted. No mortality occurred. Increased salivation was noted in all animals during the 4 h exposure period. This sign had completely disappeared thereafter. All animals showed restless behaviour and ruffled fur 5 h after start of exposure. These signs were still present by less severity on the day after exposure and disappeared completely thereafter. Two females also showed frightened behaviour 5 h after start of exposure. No effect on the body weight and body-weight gain was noted. At autopsy, no abnormalities were found.

[e] Complied with GLP and QA.

[f] Groups of five male and five female rats received dermal doses of 0 and 5000 mg/kg bw benalaxyl (purity, 96.5%). The test material was a fine powder and was applied slightly moistened in physiological saline with a spatula. The treated sites were not covered with gauze, instead, and with the same purpose to avoid lost and/or ingestion of test material, the animals were fastened on a net table with the back up and kept there for 4 h. In addition, the animals wore a collar throughout the observation period of 14 days. The study was performed before the implementation of GLP and was based on proposed EPA Guidelines for Registration of Pesticides in the USA (1978). The study was accepted for evaluation. One treated male died on day 2. No symptoms of intoxication and no local skin reactions were detected at the highest practicable dose of 5000 mg/kg bw.

[g] Groups of five male and five female rabbits received benalaxyl as dermal doses at 0 or 2000 mg/kg bw (purity, 94%). The test material was applied slightly moistened in physiological saline on the shaved skin with a spatula. Then a gauze pad was placed over the treated area, which was subsequently occluded with a strip of impermeable plaster wound around the trunk of the animal. A plastic collar was placed around the neck of each animal to prevent the premature removal of the wrappings and ingestion of the product. After 24 h the plasters and gauze were removed and the skin wiped to remove any product still remaining. The animals were observed for 14 days before sacrifice. The study was claimed to be designed to meet the requirements of EPA for Registration of Pesticides in the USA and to comply with the requirements of the FDA GLP regulations, but no statement of compliance was submitted. The in-house test method was performed according to the method of Draize, Woodward & Calvery, (1944). The study was accepted. At 2000 mg/kg bw, no mortality and no overt symptoms of toxicity or irritation were recorded. The body-weight gain was found to be normal as compared with the control group. No abnormalities, including normal findings on the treated and non treated area were recorded at the gross necropsy examination.

[h] Groups of five male and five female fasted rats were treated with benalaxyl (purity, 92.5%) at a dose of 0, 750, 1050, 1500 or 2100 mg/kg bw. The animals were observed for 14 days before sacrifice. The study was performed before the implementation of GLP and was based on proposed EPA Guidelines for Registration of Pesticides in the USA (1978). Death occurred in one, three, two and five males and in three, three, three and four females at 750, 1050, 1500 and 2100 mg/kg bw. Mortality was observed almost exclusively in the first 24 h after treatment. Obvious symptoms of intoxication were observed only at the highest dose; asthenia, prostration, uncoordinated movements.

The NOAEL was 5000 ppm (equal to 842 mg/kg bw per day in males and 874 mg/kg bw per day in females), the highest dose tested, as liver weight increases were considered to be an adaptive response and not an adverse effect (Maltoni et al., 1985).

Rats

Groups of 10 male and 10 female Wistar (BOR:WISW SPF/TNO) rats received benalaxyl (purity not reported) at a dose of 0 (control vehicle), 10, 100 or 800 mg/kg bw per day by gavage in a volume of 2 ml/100 g bw for 5 weeks. The vehicle used was Traganth 0.5%. An additional five rats of each sex were included in the control group and at the highest dose for further examination after a recovery period of 2 weeks. The originally assigned test period of 4 weeks

was prolonged to 5 weeks to achieve toxic effects at the highest dose by continuously increased dosages. After 1, 2, 3, 4 and 4.5 weeks of treatment, the highest dose was increased stepwise up to 4000 mg/kg bw per day (i.e. 1000, 1500, 2500, 3500 and 4000 mg/kg bw per day respectively in a volume of 2.5 ml/100 g bw). Daily observations included clinical symptoms, sensorial and motor behaviour, hair coat, urine and faecal excretion and mortality; body weight and food consumption were recorded weekly. Additional clinical examinations (ophthalmoscopy, hearing and reflex test) were made on weeks 0 and 5 and on animals in the recovery group on week 7. During the study, samples were taken for haematology, clinical chemistry and urine analysis. All animals were sacrificed and subjected to complete autopsy (organ weights and histopathology). Although GLP was not compulsory at the time the study was conducted, the study was accepted.

There were no deaths or clinical signs of toxicity. Body-weight gain, food consumption, food efficiency, urine, faeces and coat were also normal in all groups. At the end of the test period, coagulation time (prothrombin time) and serum cholesterol values were increased in females at the highest dose when compared with values for controls (19.5% and 55%, respectively); albumin and total protein values were significantly increased compared with those of controls, but remained within the normal range; aspartate aminotransferase (males and females, 54% and 61% of values for controls, respectively) and alkaline phosphatase (males and females, 74% and 55% of values for controls, respectively) activities were significantly reduced in males and females at the highest dose. All other haematological/biochemical parameters were normal. Urine analysis did not show treatment-related changes.

Absolute liver weights were increased in the groups at the intermediate and highest dose. Relative liver weights of the animals at the intermediate and highest dose were also increased in a dose-related manner, being highly significant in the group at the highest dose (an increase of 16.5% and 51% in males, and 12.9% and 80% in females). At the end of the recovery period, treatment-related effects were completely reversed. Histopathology performed at the end of week 5 for animals in the control group and at the highest dose revealed changes of the follicular epithelium of the thyroid in the group at the highest dose; this finding was not re-examined at the end of the 2-week recovery period. In the group at the highest dose, the number of animals with slight diffuse small droplet fatty infiltration in the liver increased, but there was no degeneration of liver cells.

The NOAEL was 100 mg/kg bw per day as effects observed at this dose were limited to slightly significant increases in relative liver weight in both sexes. Moreover, in the absence of biochemical and histopathological modifications, liver weight increases were considered to be an adaptive response and not an adverse effect (Sterner & Korn Tierarzt, 1982).

In a study that complied with the principles of GLP (with QA certificate provided), groups of 20 male and 20 female Charles River CD(SD)BR rats were fed diets containing benalaxyl (purity, 92.5%) at a concentration of 0, 10, 100, 1000 or 10 000 ppm for 13 weeks. An additional group was fed diet containing benalaxyl at 12 000 ppm for 4 weeks followed by a recovery period of 9 weeks. The doses were equal to a mean daily intake of 0, 0.56, 5.8, 59, 637 and 1052 mg/kg bw per day in males and 0, 0.66, 6.7, 72, 784 and 1277 mg/kg bw per day in females. Observations included clinical signs (daily), food consumption and body weight (weekly), haematology, blood biochemistry and urine analysis (on week 5 and 13) and organ weight, gross and microscopic pathology. Test method was not specified in the report (an in-house method was used), but detailed procedure was included in the report and complied to a great extent with OECD test guideline 408 (1998), the main deviation being that no ophthalmological examination was performed.

There were no treatment-related deaths during the study. One male at 10 000 ppm died immediately after a blood sample was taken, owing to respiratory arrest probably induced by ether anaesthesia. There were no clinical signs of toxicity after treatment. Body-weight gain was slightly decreased in rats at 10 000 and 12 000 ppm, from week 2 and 1 onward, respectively, in a

more significant way in males than in females. This decrease was also more marked in the group at the highest dose during the first 4 weeks and then disappeared during the recovery period. There were no significant differences in food intake noted between groups; however, it was pointed out that during the first weeks of the experiment, some males treated at 10 000 ppm and animals of both sexes at 12 000 ppm tended to waste part of the food (crumble the food allowing it to fall to the bottom of the cage). This abnormal behaviour made it not always possible to determine this parameter for all the animals. Slight anaemia (decrease in the number of erythrocytes, haemoglobin concentration and erythrocyte volume fraction) was seen after 4 weeks in males and females at 12 000 ppm, but values returned to normal during the recovery period. Increases in cholesterol values were found in animals of both sexes at 10 000 and 12 000 ppm, at week 5 of test. This observation was confirmed at the 13th week examination in groups at 10 000 ppm. At the highest dose, this parameter had returned to normal values after the recovery period.

Both absolute and relative liver weights were increased in males at 1000 (15.3 and 13.4%, respectively) and 10 000 ppm (41.8% and 47.8%, respectively) and in females at 10 000 (55.4% and 61.2%, respectively) and 12 000 ppm (only relative weight, 9.6%). The liver of many males at 1000 and 10 000 ppm presented lobulation. This finding was also present sporadically in other groups. In females treated at 10 000 ppm, the liver generally appeared darker than normal. In the liver, diffuse steatosis was seen in animals of both sexes treated at 1000 and 10 000 ppm, but more frequently and severely in males (males affected: 1, 0, 1, 2 and 5; females affected: 1, 0, 0, 2 and 4). The same phenomena were present sporadically in other groups in which mild perilobular steatosis was observed. In the heart, sporadic cases of necrosis limited to a few myocardial fibres have been observed in the males treated at 10 000 ppm.

The Meeting concluded that benalaxyl administered orally to rats for 13 weeks was well tolerated at dietary concentrations of up to 1000 ppm. At dietary concentrations of 10 000 ppm and greater, adverse effects were produced in the liver (diffuse steatosis) particularly in males. The effects observed in animals at 12 000 ppm for 4 weeks regressed completely after a 9-week recovery period.

The NOAEL was 1000 ppm (equal to 59 mg/kg bw per day in males and 72 mg/kg bw per day in females); as at this dose, liver effects were not considered adverse and in view of the fact that no liver toxicity was observed in the long-term study at 1000 ppm (Mondino et al., 1982a).

Dogs

In a study that complied with the principles of GLP (with QA certificate provided), groups of six male and six female beagle dogs received diets containing benalaxyl (purity, 92.5%) at a concentration of 0, 10, 200 or 800 ppm for 52 weeks. The doses were equal to a mean daily intake of 0, 0.32, 6.5 and 25 mg/kg bw per day in males and 0, 0.33, 7.0 and 28 mg/kg bw per day in females. Observations included clinical signs (daily), food consumption and body weight (weekly), ophthalmoscopic examination (before the start of the treatment and in week 52), haematology, blood biochemistry and urine analysis (before the start of the experiment and in weeks 6, 10, 15, 21, 26, 38 and 52) and organ weight, gross and microscopic pathology. Faeces collected simultaneously with the urine were tested for the presence of occult blood before the start of treatment and in weeks 26 and 52. The test method was not specified in the report (an in-house method was used), but the detailed procedure was included in the report and complied to a great extent with OECD test guideline 409 (1998).

No deaths occurred during the course of the study and no abnormalities were detected by clinical examinations or on behaviour, by ophthalmological examination, or on body-weight gain and food consumption. Haematological, clinical chemical parameters and urine analysis, investigated at regular intervals throughout the exposure, showed no consistent effects and none that were related to treatment. Examination of the faeces did show sporadic cases of occult blood.

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At necropsy, macroscopic examinations and organ weight determinations did not reveal any abnormalities. Histopathology showed atrophy of the seminiferous tubules in two males at the highest dose; this was not seen in any other group.

The NOAEL was 200 ppm (equal to 6.5 mg/kg bw per day) on the basis of findings in male dogs (Mondino et al, 1982b).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of 60 male and 60 female Swiss mice were given diets containing benalaxyl (purity, 94%) at a concentration of 0, 250, 1000 or 3000 ppm for 78 consecutive weeks. The doses were equal to a mean daily intake of 0, 45, 181 and 559 mg/kg bw per day in males and 0, 43, 174 and 522 mg/kg bw per day in females. The doses were chosen on the basis of the results of a preliminary 90-day range-finding study. Stability was determined for every batch of diet at the end of utilization and homogeneity was determined during the pelleting process. Observations for group behaviour and mortality were made at least twice daily. Measurements of individual body weight, food and water consumption were performed weekly for the first 13 weeks, and then every 2 weeks. Haematological examinations were performed before the beginning of treatment, and at weeks 52 and 78 (terminal sacrifice) in 10 animals of each sex per group. Clinical chemistry was conducted at week 78 in 10 animals of each sex per group. A complete necropsy was performed for each animal that died spontaneously or was sacrificed. Weights of brain, pituitary, thyroid, thymus with mediastinal lymph nodes, lungs, heart, liver, spleen, kidneys, adrenals, ovaries and testes were recorded. Histopathological examination was carried out on all tissues and organs taken at necropsy of animals at the highest dose and in the control group; the following tissues were examined routinely in the additional groups: all gross lesions, brain, Zymbal glands, thymus and mediastinal lymph nodes, lungs, liver, spleen, kidneys, adrenals, stomach (fore and glandular), urinary bladder, prostate, uterus, ovaries, testes and epididymis (Maltoni, 1985). The study complied with the principles of GLP (with QA certificate provided). An in-house test method was used that complied with the Proposed Chronic Health Effects Test Standard of the EPA (44 FR27334, 1979). Despite high mortality in male mice, the study was considered to be acceptable as survival was 25% at the end of the study and 65% at week 53 in the group at the highest dose.

A study on the spontaneous occurrence of amyloidosis in Swiss mice was undertaken to allow statistical evaluation on its frequency and on its fluctuations in untreated male and female mice. Seven different experiments were considered for a total of 920 male and female Swiss mice. All these experiments were carried out between 1977 and 1983 in the Institute of Oncology "F. Addarii" Bologna. In all the experiments considered, the organs in which amyloidosis is more frequently and consistently detected (i.e. liver, spleen, kidneys, and adrenals) were systematically examined, although not all the organs and tissues examined in the study with benalaxyl were submitted for histopathology (Maltoni, 1988a).

There was no effect on survival in female mice, but high levels of mortality occurred in males at 1000 and 3000 ppm. Mortality was 38.3%, 41.7%, 68.3% and 75.0% in males and 28.3%, 16.7%, 15.0% and 30.0% in females. Reduced survival was first observed after approximately 4 months of dosing (four males at week 17, 6.7%; and six males at week 21, 10%) and maximal differences (approximately 30%) between survival rates for control and treated animals occurred during the second year. Food and water consumption were not affected by treatment. Body-weight gains appeared to be affected in treated males from week 33 until the end of the study; differences between control and treated groups ranged from 5% to 10%, but there was no dose–response relationship. No abnormalities in appearance or behaviour were related to treatment. There was a significant decrease in erythrocyte count at 52 weeks in female mice of all

treated groups; however, at 76 weeks no significant difference was observed. Thus, it was concluded that there was no dose-related or toxicologically significant effects in tested parameters. Clinical chemistry parameters were not influenced by treatment.

No differences between treated and control groups were noted at necropsy. Absolute and relative liver weights of female mice exposed to benalaxyl at 3000 ppm were increased (28.0% and 26.6%, respectively). There was a statistically significant increase in the incidence of inflammatory lesions of the kidney (chronic nephritis, chronic inactive nephritis) in males at 3000 ppm (40% compared with 21.7% in control group); these disorders, which were observed in all groups, were more common in males than females and occurred at a higher rate of incidence in the animals that died.

Among the degenerative lesions, amyloidosis was frequent and widely distributed among all groups. Deposition of amyloid was found in the adrenal, kidney, liver, spleen, salivary glands, stomach and intestine, and Zymbal glands. The incidence was higher in males than in females. Because of the characteristic distribution of the lesions and because of the lack of antecedent disease, the disorder is regarded as "primary amyloidosis". In male mice who died spontaneously, a clear correlation was found between the occurrence of amyloidosis and treatment; although the association of amyloidosis and treatment was significant only at the two lowest doses (Table 12), the incidence of the lesion (as described in Table 13) was higher in the groups treated with the two higher doses, and it was considered that the differences were biologically relevant at the three doses. The association between amyloidosis and treatment was still present, although at a lesser extent, in male mice sacrificed at the end of the experiment. There thus seemed to be a correlation between amyloidosis, treatment with benalaxyl, and higher mortality rate (Maltoni, 1985).

Table 12. Categorical analysis of presence of amyloidosis and survival in male mice given diets containing benalaxyl for 78 weeks

Dietary concentration (ppm)	Animals affected by amyloidosis	Animals found dead	Surviving animals (terminal sacrifice)	$\Sigma \chi^2$	p[b]
3000	Affected[a]	30	7	1.05	NS
	Non-affected	16	7		
	Total	46	14		
1000	Affected[a]	32	5	10.44	< 0.01
	Non-affected	11	12		
	Total	43	17		
250	Affected[a]	16	8	5.38	< 0.05
	Non-affected	13	23		
	Total	29	31		
0	Affected[a]	9	5	3.26	NS
	Non-affected	17	29		
	Total	26	34		

From Maltoni (1985)

NS, non-significant

[a] No. of animals per group that were affected with amyloidosis, in any organ.

[b] Tail probability range of chi-squared.

Table 13. Distribution of regressive changes (amyloidosis) in mice given diets containing benalaxyl for 78 weeks

Dietary concentration (ppm)	Sex/No. of animals at start	Liver				Spleen		Kidneys		Adrenal glands	
		Amyloidosis		Other regressive changes							
		No.	%	No.	%	No.	%	No.	%	No.	%
3000	M/60	12	20.0	1	1.7	26**	43.3	21**	35.0	8	13.3
	F/60	1	1.7	6	10.0	0	—	2	3.3	0	—
	M & F/120	13	10.8	7	5.8	26	21.7	23	19.2	8	6.7
1000	M/60	19*	31.7	4	6.7	29**	48.3	28**	46.7	4	6.7
	F/60	6	10.0	13	21.7	6	10.0	8*	13.3	3	5.0
	M & F/120	25	20.8	17	14.2	35	29.2	36	30.0	7	5.8
250	M/60	12	20.0	3	5.0	22**	36.7	12	20.0	3	5.0
	F/60	1	1.7	7	11.7	0	—	1	1.7	0	—
	M & F/120	13	10.8	10	8.3	22	18.3	13	10.8	3	2.5
0	M/60	8	13.3	4	6.7	9	15.0	10	16.7	3	5.0
	F/60	1	1.7	7	11.7	1	1.7	0	—	1	1.7
	M & F/120	9	7.5	11	9.2	10	8.3	10	8.3	4	3.3

From Maltoni (1985)

F, females; M, males

* $p < 0.05$; ** $p < 0.01$

Comparing the incidence of amyloidosis in this experiment with that in six other experimental control groups (historical control data) it emerged that the incidence of amyloidosis in the benalaxyl-treated groups was within the range of the expected fluctuation (Table 14) (Maltoni, 1988a).

No increase in incidence or shortened latency time was detected in the treated groups when compared with the controls in term either of the most frequently occurring tumours or of the total number of tumours observed. Three lesions diagnosed as bladder transitional cell carcinomas were observed among the 60 males exposed to the highest dose. Two of these tumours were detected only on microscopic examination and were at a very early stage, and one was observed on gross examination at necropsy (Maltoni et al., 1985 and 1988b). A pathology peer review has been conducted on sections of urinary bladder tumours from three male Swiss mice used in this study of oncogenicity with benalaxyl. The sections of urinary bladder with tumour, previously examined by the study pathologist and the pathology peer reviewer, were then examined by a pathology working group panel. The results of the working group confirmed the conclusion of the pathology peer review: none of the three urinary bladder lesions were considered to be transitional cell carcinomas. There was unanimous agreement that all these three lesions were submucosal mesenchymal tumours of the mouse urinary bladder. These tumours can occur spontaneously at a high incidence (about 12% in this strain) and were not considered to be treatment-related. Moreover, this kind of lesion is non-epithelial in origin, is unique to the mouse urinary bladder, and has no counterpart in any other species, including humans (Millar, 2001a, 2001b).

In conclusion, there was no evidence of carcinogenic potential of benalaxyl when administered to Swiss mice for 78 consecutive weeks. The NOAEL was 250 ppm (equal to 45 mg/kg bw per day in males and 43 mg/kg bw per day in females) on the basis of the increased mortality observed in males at 1000 ppm and considering that the incidence of amyloidosis was well within the expected fluctuation in Swiss mice.

Table 14. Comparison of incidence of amyloidosis in historical control groups and in mice treated with benalaxyl

Experiment	Concentration	Sex/No. of animals at start	Animals bearing amyloidosis							
			Liver		Organs routinely examined in all experiments[a]		Other organs (examined only in certain experiments.)		Total	
			Incidence/No. of animals examined	%	Incidence/No. of animals examined	%	Incidence/No. of animals examined	%	Incidence/No. of animals examined	%
BT 303	0 (Controls)	M/100	41/99	41.4	71/99	71.7	0/99	—	71/99	71.7
		F/100	19/97	19.6	58/97	59.8	0/97	—	58/97	59.8
		M & F/200	60/196	30.6	129/196	65.8	0/196	—	129/196	65.8
BT 305	0 (Controls)	M/90	30/90	33.3	49/90	54.4	0/90	—	49/90	54.4
		F/90	10/89	11.2	21/89	23.6	0/89	—	21/89	23.6
		M & F/180	40/179	22.3	70/179	39.1	0/179	—	70/179	39.1
BT 606	0 (Controls)	M/60	14/57	24.6	35/58	60.3	2/58	3.4	37/58	63.8
		F/60	23/58	39.7	46/58	79.3	0/58	—	46/58	79.3
		M & F/120	37/115	32.2	81/116	69.8	2/116	1.7	83/116	71.6
BT 702	0 (Controls)	M/100	36/95	37.9	68/96	70.8	2/96	2.1	70/96	72.9
		F/100	17/96	17.7	51/96	53.1	0/96	—	51/96	53.1
		M+F/200	53/191	27.7	119/192	62.0	2/192	1.0	121/192	63.0
BT 5002	0 (Controls)	M/60	21/60	35.0	31/60	51.7	4/60	6.7	35/60	58.3
		F/60	1/60	1.7	12/60	20.0	8/60	13.3	20/60	33.3
		M & F/120	22/120	18.3	43/120	35.8	12/120	10.0	55/120	45.8
BT 5004 Maltoni et al. (1985)	3000 ppm Benalaxyl	M/60	13/60	21.7	36/60	60.0	8/60	13.3	44/60	73.3
		F/60	1/60	1.7	14/60	23.3	8/60	13.3	22/60	36.7
		M & F/120	14/120	11.7	50/120	41.7	16/120	13.3	66/120	55.0

BT 5004 Maltoni et al. (1985) cont.	1000 ppm Benalaxyl	M/60	20/60	33.3	35/60	58.3	4/60	6.7	39/60	65.0
		F/60	6/60	10.0	22/60	36.7	0/60	—	22/60	36.7
		M & F/120	26/120	21.7	57/120	47.5	4/120	3.3	61/120	50.8
	250 ppm Benalaxyl	M/60	12/60	20.0	24/60	40.0	1/60	1.7	25/60	41.7
		F/60	1/60	1.7	3/60	5.0	0/60	—	3/60	5.0
		M & F/120	13/120	10.8	27/120	22.5	1/120	0.8	28/120	23.3
	0 (Controls)	M/60	9/60	15.0	15/60	25.0	8/60	13.3	23/60	38.3
		F/60	1/60	1.7	8/60	13.3	6/60	10.0	14/60	23.3
		M & F/120	10/120	8.3	23/120	19.2	14/120	11.7	37/120	30.8
BT 5006	0 (Controls)	M/50	12/50	24.0	21/50	42.0	1/50	2.0	22/50	44.0
		F/50	6/50	12.0	6/50	12.0	1/50	2.0	7/50	14.0
		M & F/100	18/100	18.0	27/100	27.0	2/100	2.0	29/100	29.0
Total		M & F/1400	293/1381	21.2	626/1383	45.3	53/1383	3.8	679/1383	49.1

From Maltoni (1988a)

F, females; M, males

[a] Mediastinal lymph node, liver, spleen, kidneys, adrenal glands, glandular stomach, ovaries/testes

Rats

Groups of 65 male and 65 female Sprague-Dawley rats were given diets containing benalaxyl (purity, 96.8%) at a concentration of 0, 4, 100 or 1000 ppm for 104 consecutive weeks, resulting in mean intakes at 1 year of 0, 0.21, 5.2 and 52 mg/kg bw per day for males and 0, 0.26, 6.6 and 65 mg/kg bw per day for females and at 2 years of 0, 0.18, 4.4 and 44 mg/kg bw per day for males and 0, 0.23, 5.6 and 56 mg/kg bw per day for females. The doses were selected on the basis of the results obtained from the 13-week study of oral toxicity (Mondino et al., 1982a). The stability and homogeneity of the diet were determined before the start of the study and then four or five times throughout the study. Animals were given fresh diet each week.

All animals were observed daily for clinical signs of toxicity, the level of water in the bottles was observed daily and mortality was checked twice each day. Individual body weights were measured before initiation of the study, weekly for the first 13 weeks and bi-weekly thereafter; food consumption was measured at the same intervals and food conversion was calculated for the first 13 weeks (food conversion = body weight/food consumption). Ophthalmological examination was conducted on all animals before the initiation of the study and after 1, 6, 12, 18 and 24 months of dosing. Blood samples for haematology and clinical chemistry determinations were collected before dosing (10 rats of each sex) and after 3, 6, 12, 18 and 24 months (10 rats of each sex per group), and urine samples were collected after 12, 18 and 24 months from 10 rats of each sex per group. After 12 months of treatment, 10 animals of each sex per group were sacrificed and subjected to gross necropsy. All animals dying spontaneously, killed when moribund or killed at termination (after 24 months) were subjected to necropsy. The following organs were weighed: brain, heart, kidneys, liver, ovaries, testes and thymus (not weighed after 12 months). Histopathology was performed on all rats. The study was performed in compliance with the principles of GLP. Test method complied with EPA proposed guidelines for registering pesticides in the USA (Federal Register 43, No. 163, 1978).

Forty-seven to 61% of animals of each group died during the study, with mortality distribution not treatment-related. There were no signs of toxicity or behavioural abnormality related to administration of benalaxyl and the incidence and number of palpable masses appeared to be comparable between groups. There were no effects of treatment on water consumption, body weight or food consumption. Haematology, urine analysis and ophthalmoscopy were unremarkable. Clinical chemistry examination showed increased lactate dehydrogenase (LDH) activity and potassium values in male rats after 18 months of treatment, with significance being reached only in males at the highest dose (80% and 10% compared with controls, respectively). The study director considered this value still within the normal range for this age and strain of rat, this difference was not noted at any other time period so the finding was not considered biologically meaningful.

An increase in relative heart weight (10%) was noted in male rats at the highest dose at 24 months (absolute heart weight: control, 2.2 ± 0.4 g; highest dose, 2.4 ± 0.7 g). Absolute ovary weights of female rats at the lowest and intermediate doses were significantly decreased at 24 months (24% and 25% compared with controls, respectively). At necropsy, no treatment-related effects were seen in animals fed with benalaxyl at any dose for up to 104 weeks. After 12 months there was no evidence of any toxicological or pathological effects related to treatment. Again, at terminal sacrifice (104 weeks), no dose-related responses were observed. After 12 months, several neoplasms were observed microscopically, but these tumours were considered to be spontaneous, age-related and typical for this strain of rat. At terminal sacrifice, the number of benign and malignant neoplasms, and the number of primary and metastatic tumours per animal with tumours did not reveal any treatment-related effects. No difference in sensitivity was observed between male and female rats. A total of 16 primary hepatocellular tumours were observed, representing a population frequency of 3%, which is compatible with the frequency of spontaneous hepatocellular neoplasms. Although the incidence of hepatocellular neoplasm was found to be greater in females at the highest dose (1 out of 54, 3 out of 55, 2 out of 54 and 6 out of

55 in the control group, and at the lowest, intermediate and highest dose, respectively), the difference was not statistically significant; there was no dose–response relationship observed in the treated groups and no primary hepatocellular tumours were found in males at the highest dose. Therefore, the increased incidence among females at the highest dose was not considered to be treatment-related.

The maximal tolerated dose was not attained in this study because of the absence of any significant findings in either sex at the highest dose. The liver was found to be the target organ in short-term studies of toxicity in rats, with an increase in relative liver weight and steatosis. However, no evidence of hepatotoxicity was noted in this long-term study of toxicity. There was no evidence of carcinogenic potential of benalaxyl when administered to Sprague-Dawley rats for 104 weeks at doses of up to 1000 ppm.

The NOAEL was 1000 ppm (equal to 44 mg/kg bw per day in males and 56 mg/kg bw per day in females), the highest dose tested. The small changes in heart weights were not considered to be toxicologically significant and the increased levels of potassium and LDH were not considered to be treatment-related owing to the fluctuations observed at various time-points (Thompson et al., 1983).

2.4   *Genotoxicity*

The mutagenic/genotoxic potential of technical-grade benalaxyl was investigated in a battery of tests in vitro and in vivo (Table 15). All the results were negative. The Meeting considered that benalaxyl is not genotoxic.

2.5   *Reproductive toxicity*

(a)   *Multigeneration studies*

Rats

Groups of 25 male and 25 female Sprague-Dawley rats received diets containing benalaxyl (purity, 96.9%) at a concentration of 0, 100, 1000 or 5000 ppm for 16 consecutive weeks (F_0 animals); they were then allowed to mate and produce two litters, F_{1a} and F_{1b}. The concentrations used were equal to 6.0, 57 and 289 mg/kg bw per day for F_0 males and 5.3, 53 and 275 mg/kg bw per day for F_1 males and 7.9, 80 and 398 mg/kg bw per day for F_0 females and 7.9, 79 and 401 mg/kg bw per day for F_1 females. Breeding for the F_{1b} litter was initiated approximately 14 days after weaning of the last F_{1a} litter. Parents for the second generation (F_1 animals) were selected from the F_{1a} litters, fed the test diet for 15 weeks, then allowed to mate and produce two litters, F_{2a} and F_{2b}. Breeding for the F_{2b} litter was initiated approximately 14 days after weaning of the last F_{2a} litter. Both male and female F_0 animals received the test article in their diet for 16 consecutive weeks before mating, during the mating period, and continuously until the day of sacrifice. For pregnant females, this included gestation and lactation. Dosing of F_1 animals was initiated upon completion of weaning and continued for 15 consecutive weeks before mating, and then during mating, gestation and lactation. Dosing of F_1 males ended 4 weeks after the mating period to give the second litter (week 62), while dosing of F_1 females ended 1 week after weaning of the second litter (week 65).

F_0 and F_1 parents were observed for mortality (twice daily), signs of toxicity and for behavioural abnormalities (daily). Pups were observed for mortality, general appearance, gross malformation and behavioural abnormalities. Individual body weights were measured and recorded weekly (males) or before mating (females). Individual body weights of pregnant females were recorded on days 0, 6, 10, 15 and 20 of gestation and on days 0, 7, 14 and 21 of lactation. Individual pup weights were measured on days 0, 4, 14 and 21 of lactation. Food consumption was measured weekly, except during the mating period, for both males and females. All pups

(except those cannibalized) that were stillborn or died during the 21-day lactation period, were dissected and examined for gross anomalies. The sex of each pup was confirmed by gonadal inspection. No tissues were saved. After weaning of the F_{1a}, F_{1b}, F_{2a} and F_{2b} generations had been completed, five pups of each sex per group were sacrificed using CO_2 and complete necropsies performed on all animals. Fresh organ weights were obtained and organ-to-body-weight ratios were calculated from the sacrificed rats (five of each sex per group). The remaining pups in each litter were not selected for complete necropsy; they were subjected to an abbreviated necropsy (at the completion of weaning) consisting of an external examination for gross abnormalities and examination of the abdominal and thoracic cavities only. No tissues were saved. The following tissues were examined microscopically for five pups of each sex per group subjected to complete gross necropsies: adrenal glands, epididymides, heart, kidneys, liver, lungs, ovaries, prostate, seminal vesicles, spleen, testes, thymus, urinary bladder, uterus, corpus and cervix, and vagina.

After weaning of the F_{1b} and F_{2b} generations was completed, F_0 and F_1 parents were sacrificed using CO_2 and gross necropsies were performed on all animals (a gross necropsy was also performed on any animal that died during the study). Fresh organ weights were obtained and organ-to-body-weight ratios were calculated for all F_0 and F_1 animals. A complete set of tissues and organs from all animals were fixed and saved in 10% neutral buffered formalin. Microscopic examination of tissue sections was performed on the following tissues from all F_0 and F_1 parental animals: epididymides, ovaries, prostate, seminal vesicles, testes, uterus, corpus and cervix. The study complied with the principles of GLP. The test method complied with EPA proposed guidelines for registering pesticides in the USA (Federal Register 43, No. 163, 1978).

The stability, homogeneity and dietary concentration of benalaxyl remained within required limits during the study. For F_0 parents, no mortality occurred in males or females in any group fed with benalaxyl. One male in the control group died during week 30 of the study; the cause of death was not established. One F_1 female at 100 ppm died before mating for the $F2_b$ generation; the cause of death was unrelated to administration of benalaxyl. Feeding with benalaxyl had no effect on fertility, length of gestation, litter size, or number of stillborn during either the F_1 or F_2 generations. The differences of viability or lactation indices compared with those for controls were not related to treatment. Significantly reduced body weight (4–11%) before mating until terminal sacrifice was noted in F_1 males at the highest dose. Significantly reduced body weight in F_1 females at the highest dose before mating (8–9%), during gestation (10–12%) for both the F_{2a} and F_{2b} generations, during lactation for the F_{2b} generation (7.5–9%) and before terminal sacrifice (11–12%) were observed. Body-weight gain, from initiation of feeding until terminal sacrifice, was reduced by 12% and 13.9%, respectively in F_1 males and females at the highest dose. For F_1 females at the highest dose, body-weight gain was reduced before mating (9.7%) and during gestation (12–12.6%).

Intermittent statistically significant differences in food consumption were noted among one or more groups for the F_1 generation; however, no toxicologically significant effect related to the administration of benalaxyl was apparent. No effects were seen in parents of either generation to indicate any signs of toxicity, or treatment-related behaviour abnormality caused by the administration of benalaxyl. No behavioural abnormalities in mating, nesting or nursing were seen in pregnant F_0 or F_1 dams during the mating, gestation or lactation phases of the study.

A dose-dependent, treatment-related effect on liver weights was noted for males and females of both generations. For the F_0 generation parents, absolute liver weights were significantly increased in females fed with benalaxyl at 1000 and 5000 ppm, while relative liver weights were significantly increased at 1000 ppm in females (17.6%) and at 5000 ppm in males (21.2%) and females (41.2%), when compared with respective values for the control group. For the F_1 generation parents, absolute liver weights were significantly increased in females at 5000 ppm, while relative liver weights were significantly increased at 5000 ppm in males (16.7%) and females (35.5%). The liver-to-brain-weight ratio was also significantly increased in females at 5000 ppm (19.0%). No gross or microscopic lesions related to the administration of benalaxyl were observed in parent animals of the F_0 or F_1 generations.

Table 15. Results of studies of genotoxicity with benalaxyl

End-point	Test object	Concentration	Purity (%)	Results	Reference
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	1st experiment: 2–2000 μg/plate; 2nd experiment: 200–1600 μg/plate; in DMSO	98	Negative[a,b]	De Carneri (1979a)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA102	33–5000 μg/plate, in DMSO	96.49	Negative[a,c]	Wollny (2002)*°
Gene mutation	*Schizosaccharomyces pombe* P1	−S9 : 20–160 μg/ml; +S9: 100–800 μg/ml; in DMSO	98	Negative[a,d]	De Carneri (1980)
Mitotic gene-conversion	*Saccharomyces cerevisiae* D4	8–1000 μg/ml; in DMSO	98	Negative[a,e]	De Carneri (1979b)
Gene mutation	Chinese hamster, V79 cells, HPRT locus	3×10^{-7}, 1×10^{-6}, 3×10^{-6}, 1×10^{-5} and 3×10^{-5} mol/l; in ethanol	94	Negative[af]	Monaco et al (1983)
Chromosomal aberration	Human peripheral blood lymphocytes	3.3–100 μg/ml; in DMSO −S9: 26–30 h treatment, harvesting at the end of treatment +S9: 2 h treatment, harvesting 24–28 h later	NR	Negative[a,g]	Mondino et al (1980a)
Chromosomal aberration	Chinese hamster ovary (CHO) cells	1st experiment, −S9: 12.5–100 μg/ml; +S9: 25–200 μg/ml (treatment, 4 h, harvesting, 24 h) 2nd experiment, −S9: 6.3–50 μg/ml, treatment: 24 h (harvesting at the end); 18.8–50 μg/ml (treatment: 46 h, harvesting at the end); +S9: 25–200 μg/ml (treatment: 4 h, harvesting: 46 h); in acetone	96.49	Negative[a,h]	Schulz (2002a)*°
Unscheduled DNA synthesis	Rat F344 primary hepatocytes	0.5–50 μg/ml; in DMSO	94	Negative[i]	Myhr & Brusick (1983)**
Chromosomal aberrations	Chinese hamster (two males and two females per group) bone-marrow cells	Oral: 1000, 2000 and 4000 mg/kg bw in methocellulose × 2 (24 h apart); sampling time: 7 h after last treatment	NR	Negative[j]	Mondino (1980b)
Micronucleus formation	Rats (SD) (five males and five females per group), bone-marrow cells	Single intraperitoneal doses of 125, 250 and 500 mg/kg bw in 0.5% (w/v) methylcellulose Sampling times: 24 and 48 h	96.68	Negative[k]	Golzio (2000)*°

DMSO, dimethylsulfoxide; GLP, good laboratory practice; NR, not reported; °QA, quality assurance; S9, 9000 × *g* supernatant of liver of rats induced with Aroclor, except where otherwise stated; SD, Sprague-Dawley.

The studies described were accepted for evaluation, except where otherwise stated. The vehicle was used as negative control. Positive control substances were used in all assays and gave the expected results.

* The study complied with GLP. ** A compliance statement was included.

[a] With and without metabolic activation.

[b] At 1600 and 2000 μg/plate, the product precipitates in soft agar. Without S9 a strong antibacterial effect was seen at 2000 μg/plate, with disappearance of the lawn and spontaneous reverse mutants; at 1600 μg/plate, the product caused reduction of the lawn due to its toxic effect. The substances used as positive controls were highly mutagenic. No guidelines or GLP were compulsory at the time the study was performed. The study was conducted in accordance with the Ames method (1975) and the experimental protocol complied with OECD test guideline 471 (1983).

[c] S9, 9000 × g supernatant of phenobarbital/β-naphthoflavone-induced rat liver . A plate incorporation method and a preincubation method were used. Relevant toxic effects, evident as a reduction in the number of revertants of less than 0.5 times that in the corresponding solvent control, occurred with plate incorporation in strain TA98 at 2500 and 5000 μg/plate –S9 and at 5000 μg/plate + S9. In strain TA100, a relevant reduction was observed only at 5000 μg/plate –S9. A single reduction below this threshold occurred with preincubation in strain TA1537 at 5000 μg/plate +S9. The plate incubated with the test substance showed normal background growth at up to 5000 μg/plate ±S9 in all strains used. Appropriate reference mutagens were used as positive controls and showed a distinct increase in the number of induced revertant colonies. The study complied with OECD test guideline 471 (1997).

[d] Positive controls: –S9, methyl methanesulfonate; +S9, N-dimethylnitrosamine. In the first experiment, the following concentrations were tested: 0.32, 1.6, 8, 40, 200 and 1000 μg/ml. The product was found to be toxic at the highest concentrations. Both positive controls induced significant increases in gene mutation frequency. No guidelines or GLP were compulsory at the time the study was performed and an in-house method was used. The study was accepted as additional information.

[e] Positive controls: –S9, methyl methanesulfonate; +S9, cyclophosphamide. Benalaxyl precipitates at 1000 μg/ml. Both positive controls showed significant increases in gene conversion frequency. No guidelines or GLP were compulsory at the time the study was performed. Main deviations from OECD test guideline 480 (1986) were the use of four different concentrations instead of five, the growing stage (stationary or growing) of cells was not reported and the test was not repeated using stationary-phase cells to confirm negative results. The study was considered as additional information.

[f] Positive controls: –S9, ethyl methanesulfonate; +S9, dimethylnitrosamine. The toxicity test was performed with benalaxyl at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} mol/l. At the highest concentration, the test substance was not soluble in the medium, while at 10^{-4} mol/l (–S9) it was cytotoxic (no survival). The same concentration + S9 resulted in 56% cell survival. Significant increases in mutation frequency were observed for the positive controls. No guidelines or GLP were compulsory at the time the study was performed. An in-house method was used that complied to a great extent with OECD test guideline 476 (1984).

[g] Positive controls: –S9, mitomycin C; +S9, phenacetin. Benalaxyl was found to be toxic to lymphocyte cultures, especially in the absence of metabolic activation; observation of a number of metaphases equal to the control, for each dose, was not always possible. The positive controls showed statistically significant increases in the frequency (%) of chromosome aberrations, ± gaps. No guidelines or GLP were compulsory at the time the study was performed. The in-house method used complied to some extent with OECD test guideline 473 (1983). Since the purity was not reported, the study was accepted as additional information.

[h] S9, 9000 × g supernatant of rat liver induced with phenobarbital/β-naphthoflavone. Positive controls: –S9, ethyl methanesulfonate; +S9, cyclophosphamide. In a range-finding pre-test, cell numbers 24 h after start of treatment were scored as indicator for cytotoxicity. Concentrations of between 22.3 and 2850 μg/ml were applied. Clearly toxic effects were observed after 4 h treatment with ≥ 89.1 μg/ml –S9 and at ≥ 178.1 μg/ml +S9. In addition, 24 h of continuous treatment at ≥ 44.5 μg/ml –S9 induced strong toxic effects. In the pre-test, precipitation of benalaxyl in the culture medium was observed 4 h after the start of treatment at ≥ 178.1 μg/ml ±S9. In experiment 2, +S9 at 46 h, precipitation was observed after 4 h treatment at 200 μg/ml. In the cytogenetic experiments, strong toxic effects indicated by reduced cell numbers and/or mitotic indices of < 50% of control were observed in all parts of experiment 2. Also in experiment 1, distinct cytotoxicity was observed at the concentrations evaluated. Positive controls showed statistically significant increases in the frequency of cells with structural chromosome aberrations. The study complied with OECD test guideline 473 (1997).

[i] Unscheduled DNA synthesis was measured by autoradiography. 2-acetylaminofluorene was used as a positive control. At 1000 μg/ml to 250 μg/ml, benalaxyl was completely lethal to the cells and at 100 μg/ml caused excessive toxicity. The results obtained with the positive control showed the adequacy of the methodology. The test method was based on procedures described by Williams (1977, 1980) and complied to a great extent with OECD test guideline 482 (1986). The main deviation was that no independent experiment was performed to confirm the negative results.

[j] No preliminary toxicity test was performed, so the highest dose was chosen based on the LD_{50} in mice. Mitomycin C administered intraperitoneally was used as the positive control. Two animals treated with benalaxyl at 2000 mg/kg bw and one treated with mitomycin C died after the second treatment. Mitomycin C induced statistically significant increases in the frequency

of chromosomal aberrations. No guidelines or GLP were compulsory at the time the study was performed. The study did not comply with OECD test guideline 475 (1984) for the following reasons: only two animals of each sex per group were used instead of five, only one sampling time was used (7 h after the last treatment) instead of two (at 6 and 24 h) and purity was not reported. The study was not accepted.

[k] Cyclophosphamide was used as positive control. Doses were selected on basis of a preliminary assay. Three male and three female rats were given benalaxyl as a single dose at 600 and 700 mg/kg bw intraperitoneally. At 700 mg/kg bw, one female died. At 600 mg/kg bw, one male and one female died. All animals showed clinical signs on the day of treatment. The second sampling time was used only for the control group and the group treated at the highest dose. In the main experiment, five animals at 500 mg/kg bw died and were replaced. The ratio of PCE/NCE in male and female animals remained unaffected by treatment with benalaxyl, indicating that benalaxyl is not toxic to bone-marrow cells. The study complied with OECD test guideline 474 (1997).

Mean pup body weight was significantly reduced for the F_{2b} litters at 5000 ppm on days 4, 14 and 21 of lactation (10 to 19%). No physical anomalies or behavioural abnormalities related to feeding with benalaxyl at any dose were noted in any pup during either generation. For the F_{1a} generation, a statistically significant increase in both the absolute and relative (60.0%) weights of the ovaries was seen in the females at the highest dose. Since absolute and relative organ weights for females at the highest dose were similar to control values for the F_{1b} generation, the increase in ovary weights noted for the F_{1a} generation was not considered to be treatment-related. A statistically significant increase in the relative weight of the liver was observed in F_{2b} males at 1000 ppm (17.6%) and in F_{2b} males (27.5%) and females (18.5%) at 5000 ppm.

No lesions or malformations related to administration of benalaxyl were noted during postmortem examination of any F_{1a}, F_{1b}, F_{2a} or F_{2b} pups that were stillborn, died spontaneously during lactation, or were sacrificed after lactation.

Feeding Sprague-Dawley rats with benalaxyl at concentrations of 100, 1000 or 5000 ppm for 16 weeks before mating produced no toxicological significant effects upon fertility, reproductive performance or pup growth and viability for litters of the two first generations. However, a treatment-related dose-dependent increase in absolute and relative liver weights was noted for both F_0 generation males and females at terminal sacrifice. Feeding of benalaxyl to a second generation of Sprague-Dawley rats at the same doses for 15 weeks before mating resulted in the following compound-related effects: significantly reduced body weight in F_1 males and females at the highest dose, significantly reduced body weight in F_{2b} generation pups at the highest dose, significantly increased absolute liver weight in F_1 females at the highest dose and relative liver weights in both F_1 males and females at the highest dose and significantly increased relative liver weight in F_{2b} generation male pups at 1000 ppm and in F_{2b} generation male and female pups at 5000 ppm. No gross or histopathological abnormalities related to feeding with benalaxyl were noted in any organ examined in parents or pups. Since histopathological evaluation of the livers revealed no abnormalities, the increases in the liver weight noted were most likely to be related to a physiological adaptation by the liver in an attempt to metabolize the test compound.

The NOAEL was 1000 ppm (equal to 53 mg/kg bw per day for males and 79 mg/kg bw per day for females, for the F_0 generation) for general toxicity in parent animals (based on decreased body weight) and adverse effects in pups (decreased pup weight and liver weight) at 5000 ppm, although fertility and reproductive parameters were not affected by treatment up to and including the highest dietary concentration of 5000 ppm (equal to 275 mg/kg bw per day for males and 398 mg/kg bw per day for females, for the F_0 generation) (Johnson & Becci, 1983).

(b)   *Developmental toxicity*

Rats

In a study that complied with the principles of GLP (with QA certificate provided), groups of 23 gravid Charles River CD(SD)BR rats were given benalaxyl (purity, 95%) at a dose of 0, 12.5, 50 or 200 mg/kg bw per day in methylcellulose (0.5%) administered orally from day 6 to day 15 of gestation. Objective examination of the clinical symptoms and behaviour was made daily. Body weight was determined on days 0, 6, 10, 15, 18 and 20. Twenty days after mating, the female rats were sacrificed by exsanguination (resection of the aorta) under ether anaesthesia and the following parameters were determined: weight of the mother at the moment of sacrifice, weight of gravid uterus, weight of the females without gravid uterus, number of corpora lutea, number of resorptions (early and late), number of implantations, number and sex of live fetuses, and fetus and litter weights. Fetuses were examined for external, skeletal and soft tissue anomalies and developmental variations. At the time the study was performed no method was compulsory. Detailed test procedure (in-house method) was included in the report, it recalls OECD test guideline 414 (1981).

No animals died during the study. Examination of the curves relative to the body weight and to the weight gains, both in absolute and percentage terms, did not reveal any variation attributable to treatment. The index of fertility was found to be completely comparable in all the various experimental groups.

Statistical analysis performed on the data obtained for the control and treated females does not reveal any significant difference between the number of corpora lutea, implantations and resorptions, the number and weight of the fetuses, the litter weight and the distribution of the sexes. A statistical analysis reveals a significant increase ($p < 0.05$) of the percentage preimplantation losses in the group at the highest dose (4.78%, 3.29%, 7.27% and 12.10% in the control group, and at the lowest dose, intermediate dose and highest dose, respectively). According to the test laboratory this increase was not attributable to treatment, as all the individual values fall within the limits of normal variability of the species, but in the absence of historical controls this could not be confirmed and thus the increase in preimplantation losses was considered to be treatment-related.

Fetuses with major deformities were not observed in any of the experimental groups. The incidence of minor skeletal anomalies was increased and was statistically significant only in animals at 50 and 200 mg/kg bw per day (10.42%, 16.12%, 20.18% and 24.21%[1] in the control group, and at the lowest, intermediate and highest dose, respectively, see Table 16). This increase was mainly due to delayed ossification of the cranial bones (incidences of incomplete ossification were 10%, 15.58%, 18.13% and 26.14% in the control group, and at the lowest, intermediate and highest dose, respectively). The number of litters with minor abnormalities was not affected (60.00%, 57.89%, 65.00% and 63.16%). Finally, the skeletal variants were uniformly distributed among the various experimental groups and fully fell within the variability of the species. No teratogenic potential was found.

At doses of up to 200 mg/kg bw per day, benalaxyl was without any toxic action on the treated mothers and did not induce major malformations in the fetuses. The NOAEL for maternal toxicity was 200 mg/kg bw per day, the highest dose tested. The NOAEL for embryotoxicity was 50 mg/kg bw per day on the basis of the increased percentage of pre-implantation losses at 200 mg/kg bw per day. The NOAEL for developmental toxicity was 12.5 mg/kg bw per day on the basis of retarded ossification of cranial bones observed in fetuses at 50 mg/kg bw per day (Mondino et al., 1982c).

Rabbits

In a study that complied with the principles of GLP (with QA certificate provided), groups of 16 mated female New Zealand White rabbits were given benalaxyl (purity, 94%) at a dose of 0, 5, 50 or 250 mg/kg bw per day orally by gavage in methylcellulose (1%) from day 6 to day 27 (inclusive) of gestation, maintained without treatment until day 28 of gestation, then killed and examined. Twice daily, all animals were examined to detect dead or moribund animals and all females were examined at least once daily after mating for signs of ill health, toxicity or behavioural change. The body weight of each female was recorded on day 0 and on days 6 to 28, inclusive after mating. On day 28 of gestation, surviving animals were killed by cervical dislocation and necropsied. All major organs were examined macroscopically and abnormalities were recorded. At necropsy, the contents of the uterus were examined and preserved in 10% neutral buffered formalin as were the ovaries and any lesion. The following data were recorded: weight of gravid uterus, number of corpora lutea, number and intrauterine position of implantations (live fetuses, early and late intrauterine deaths), individual fetal weight, individual fetal crown–rump lengths, and sex of fetuses. Fetuses were examined for external, skeletal and soft tissue anomalies and developmental variations. At the time the study was performed no method was compulsory. The detailed test procedure (in-house method) was included in the report and resembles OECD test guideline 414 (1981).

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[1] Mean obtained from the percentage of each individual litter.

Table 16. Skeletal abnormalities reported in a study of developmental toxicity with benalaxyl in rats

Observation	Dose (mg/kg bw per day)			
	0	12.5	50	200
No. of live fetuses	241	236	238	231
No. of litters	20	19	20	19
No. of fetuses examined (diaphanized)	160	154	160	153
Minor abnormalities				
No. of fetuses	16	25	30*	40**
Mean No.	0.80	1.31	1.50	2.18
Mean %[a]	10.42	16.12	20.18	24.21
No. of litters	12	11	13	12
%	60.00	57.89	65.00	63.16
Skeletal variations				
No. of fetuses	54	73	73	65
Mean No.	32.41	47.90	43.63	39.56
Mean %[a]	3.93	4.83	6.36	6.96
No. of litters	18	19	18	17
%	90.00	100.00	90.00	89.47
Incomplete ossification of cranium:				
No. of fetuses	16	24	29	40
%	10.00	15.58	18.13*	26.14***
Atrophy of vertebral bodies:				
No. of fetuses	0	2	3	3
%	0	1.30	1.88	1.96
Poorly ossified ischium or pubes:				
No. of fetuses	0	0	6	0
%	0	0	3.75*	0

From Mondino et al. (1982c)

[a] Mean obtained from the percentage of each individual litter.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Seven of the 64 rabbits died during the study, either from intubation error or respiratory disease, but these mortalities occurred in all groups at similar numbers. There was no effect of benalaxyl on the nature or incidence of clinical changes. During the last 5 days of the dosing period (days 23 to 27), most of the animals at 250 mg/kg bw per day showed weight losses. Although the differences in weight change from the controls were not statistically significant, this was thought to be an effect of treatment and related to the smaller fetal size that was observed (Table 17). When adjusted for gravid uterus weight, weight gain from day 0 to day 28 was slightly lower in groups treated at intermediate and highest doses than in the control group (29.6% and 70.4% of value for controls, respectively). The differences were not, however, statistically significant. The incidence of pregnancy was within the normal range in all groups. In the group treated at 250 mg/kg bw per day, mean gravid uterus weight was lower than expected for the number of implantations per doe and was lower than in the control group (96.7% of value for controls). Treatment had no effect on the implantation rate or postimplantation losses. Mean number of fetuses and their sex ratios were unaffected, but mean litter weight was slightly reduced in the group at the highest dose (without attaining statistical significance, 94.2% of value for controls). Mean fetal weight (92.4% and 85.3% of value for controls, respectively) and crown–

rump lengths (97.9 and 95.1% of value for controls, respectively) were lower than controls at the intermediate and highest dose. In animals receiving 50 mg/kg bw per day the difference from the controls was slight and was a consequence of the greater number of fetuses per doe (greater intralitter competition is generally associated with smaller fetal size). In animals at 250 mg/kg bw per day the difference in fetal size from the controls was statistically significant and was greater than that expected on the basis of the larger number of fetuses per doe (8.3, 8.2, 9.3 and 9.1 for the control group, and at the lowest, intermediate and highest dose, respectively), and therefore this was considered to be an effect of treatment.

There was no consistent type of major defect observed and, as the incidence at the highest dose was comparable to that among the controls, the major defects observed were considered to be unrelated to treatment with benalaxyl. There was no effect of treatment on the incidence of fetuses with minor external/visceral defects. The number of fetuses with minor skeletal defects was higher in all treated groups than in the control group (37.6%, 47.7%, 53.9% and 58.6% of fetuses in the control group, and at the lowest, intermediate and highest dose, respectively); this increase was statistically significant at the highest dose. In all groups, including the controls, the incidence of minor skeletal defects was higher than the normal background incidence because of an unusually high proportion of fetuses with irregularities of the frontal bones. Since it was also observed in the controls, this anomaly was considered not to be associated with administration of benalaxyl. Even excluding this defect, there was still a slightly higher incidence of fetuses with minor skeletal defects at the intermediate and highest doses. Most of these minor skeletal defects were related to retardation of ossification and were considered to be a consequence of the smaller fetal size in these groups rather than a direct effect on the fetuses. The incidence of fetuses with variants of skeletal development was within the normal range in the control group, and in the groups at the lowest and intermediate doses. At the highest dose, the percentage of fetuses (82% at the highest dose compared with 60% in the control group) and the number of litters with fetuses showing variants was statistically significantly higher than in the control group and was related to a general retardation of ossification. No teratogenic potential was found.

Administration of benalaxyl orally to rabbits during organogenesis and the fetal period at a minimally maternally toxic level (250 mg/kg bw per day) was not associated with embryolethality or teratogenicity. The fetal growth retardation observed was considered a consequence of the larger litter size (at 50 mg/kg bw per day) or a consequence of the minimal maternal toxicity and not a direct effect on the fetus. The NOAEL for maternal toxicity was 50 mg/kg bw per day on the basis of weight loss during the last 5 days of treatment. The NOAEL for developmental toxicity was 50 mg/kg bw per day on the basis of reduced mean fetal weight and crown–rump length and retardation of ossification at 250 mg/kg bw per day (Irvine, 1984).

2.6   Special studies

(a)   Neurotoxicity

Except for some nonspecific symptoms observed in the studies of acute toxicity at doses at or above the median lethal dose (LD_{50}), the studies of acute toxicity and short- and long-term studies reported previously revealed neither clinical signs nor any biochemical or histopathological changes that might point to a neurotoxic potential of benalaxyl. Special studies in the field of neurotoxicity were therefore not necessary.

(b)   Studies with metabolites of benalaxyl

The degradation of benalaxyl in soil produces two major degradation products: methyl-*N*-malonyl-*N*-(2,6-xylyl)-D,L-alaninate (Metabolite A) and *N*-malonyl-*N*-(2,6-xylyl)-D,L-alanine (metabolite B). They occurred for more than 10% of the active substance added in studies of the

Table 17. Selected findings of a study of developmental toxicity with benalaxyl in rabbits

Finding	Dose (mg/kg bw per day)			
	0	5	50	250
Maternal data				
Mean body weight (kg), day 0	3.73	3.67	3.78	3.78
Mean body weight (kg), day 28	4.28	4.32	4.28	4.28
% body-weight change, day 0–28	14.7	17.7	13.2	13.2
Mean gravid uterus weight (g)	449.4	470.1	468.1	434.5
Mean adjusted body weight (kg), day 28	3.83	3.85	3.81	3.85
% body weight change (adjusted value), day 0–28	2.7	4.9	0.8	1.9
Uterine/implantation data				
No. of pregnancies	16/16	16/16	13/16	15/16
No. of pregnant animals alive, day 28	15	13	11	14
No. of corpora lutea	152	120	117	152
Mean No./doe	10.1	9.2	10.6	10.9
No. of implantations	138	113	114	142
Mean No./doe	9.2	8.7	10.4	10.1
Preimplantation loss (%)	9.2	5.8	2.6	6.6
No. of early intrauterine deaths	2	2	4	7
Mean No./doe	0.1	0.2	0.4	0.5
No. of late intrauterine deaths	11	4	8	7
Mean No./doe	0.7	0.3	0.7	0.5
Postimplantation loss (%)	9.4	5.3	10.5	9.9
Litter data				
Mean No. of fetuses/doe	8.3	8.2	9.3	9.1
Mean litter weight (g)	309.8	317.5	322.6	292.0
Mean fetal weight (g)	38.2	39.8	35.3	32.6*
Mean crown–rump length (mm)	98.6	99.9	96.5	93.8*
No. of fetuses examined	125	107	102	128
Skeletal defects				
No. of fetuses showing minor defects only (%)	47 (37.6)	51 (47.7)	55 (53.9)	75 (58.6**)
No. of litters affected	15/15	12/13	11/11	13/14
No. of fetuses showing major defects (%)	0 (0.0)	0 (0.0)	2 (2.0)	2 (1.6)
No. of litters affected	0/15	0/13	2/11	1/14
Variants				
No. (%)	75 (60.0)	74 (69.2)	70 (68.6)	105 (82.0**)
No. of litters affected	14/15	13/13	11/11	14/14
Fetal defects				
Frontals incompletely ossified[a]	0 (0.8)	4 (3.7)	4 (3.9)	5 (3.9)
Frontals fused[a]	1 (0.8)	—	—	—
Cleft in frontals[a]	28 (22.4)	30 (28.0)	34 (33.3)	48 (37.5)
Abnormal line of suture between frontals[a]	1 (0.8)	2 (1.9)	3 (2.9)	—
Small island of bone between frontals[a]	1 (0.8)	3 (2.8)	1 (1.0)	—

From Irvine (1984)

$* p < 0.05$; $** p < 0.01$ [a]Figures in parentheses denote the percentage of affected fetuses/fetuses examined

soil degradation of benalaxyl. These degradation products were not found in animals. Studies of acute toxicity, short-term studies and studies of genotoxicity were performed with both substances.

(i)   Acute toxicity

The results of studies of acute toxicity are summarized in Table 18.

The two metabolites are of low acute oral toxicity in rats. No mortality occurred at a dose of 2000 mg/kg bw.

(ii)   Genotoxicity of metabolites

The genotoxic potential of metabolites A and B was assessed in several tests in vitro (Table 19). Metabolite A gave negative results in the tests for gene mutation in bacteria and mammalian cells, but positive results have been obtained in tests for chromosomal aberrations in the absence of microsomal activation and for continuous exposure. Therefore this clastogenic potential was also assessed in vivo in a test for micronucleus formation in mice. This test does not confirm the clastogenic potential of metabolite A in vivo. Metabolite B gave negative results in the various assays in the presence or absence of metabolic activation.

(iii)   Short-term studies of oral toxicity

In a study that complied with the principles of GLP (with QA certificate provided), groups of 10 male and 10 female Wistar (HsdCpb:WU) rats were fed diets containing metabolite A (purity, 98.15%) at a concentration of 0, 300, 1000, 3000 or 10 000 ppm for 13 weeks. Two additional groups (an untreated control group and a group that received a high dose) were treated for 13 weeks followed by a recovery period of 4 weeks. The highest dose of 10 000 ppm was increased to 13 000 ppm on treatment day 43 to achieve a maximum tolerated dose. Accordingly the calculated weighted average dose for the high-dose/high-dose recovery group was 11 600 ppm. The doses were equal to a mean daily intake of 0, 23, 82, 243 and 923 mg/kg bw per day in males and 0, 27, 89, 271 and 1073 mg/kg bw per day in females.

Table 18. Acute toxicity of metabolites of benalaxyl

Metabolite	Species and strain	Sex	Vehicle	LD_{50} (mg/kg bw; 95% CI or range)	GLP or QA	Reference
Oral administration						
Metabolite A[a]	Rat (Crl:CD(SD)BR)	M & F	Deionized water	> 2000[a]	GLP & QA	Yu (1997a)
Metabolite B	Rat (Crl:CD (SD) BR)	M & F	Deionized water	> 2000[b]	GLP & QA	Yu (1997b)

F, females; GLP, good laboratory practice; M, males; QA, quality assurance

Metabolite A: methyl-*N*-malonyl-*N*-(2,6-xylyl)-D,L-alaninate (soil metabolite)

Metabolite B: *N*-malonyl-*N*-(2,6-xylyl)-D,L-alanine (soil metabolite)

[a] Groups of five male and five female rats were treated with metabolite A (purity not reported) at a dose of 2000 mg/kg bw (limit test). The animals were observed for 14 days before sacrifice. No mortality or clinical changes were observed. Normal body-weight growth was recorded for rats of each sex. No appreciable macroscopic modifications were found at terminal sacrifice. The experimental protocol complied with OECD test guideline 401 (1987).

[b] Groups of five male and five female rats were treated with metabolite B (purity not reported) at a dose of 2000 mg/kg bw (limit test). The animals were observed for 14 days before sacrifice. No mortality or clinical changes were observed. Normal body-weight growth was recorded for the rats of each sex. No appreciable macroscopic modifications were found at terminal sacrifice. The experimental protocol complied with OECD test guideline 401 (1987).

Observations included clinical signs (daily), neurological examination (12th/13th week), ophthalmological examination (start and end of treatment and recovery period), food consumption and body weight (weekly), haematology, biochemistry and urine analysis (end of treatment and recovery periods) and organ weight, gross and microscopic pathology. The protocol complies with OECD test guideline 408 (1998).

No deaths occurred during the study and no treatment-related clinical signs or neurological abnormalities were observed. Body weight and food consumption were not affected by treatment. No treatment-related changes in haematology or biochemistry were observed. There were no treatment-related changes in organ weights, or gross or histopathological findings in males or females.

The NOAEL for metabolite A was 11 600 ppm (equal to 923 mg/kg bw per day in males and 1073 mg/kg bw per day in females), which was the highest dose tested (Kumar, 2003).

In a study that complied with the principles of GLP (with QA certificate provided), groups of 10 male and 10 female Wistar (HsdCpb:WU) rats were fed diets containing metabolite B (purity, 97.44 ± 0.18%) at concentrations of 0, 300, 1000, 3000 or 10 000 ppm for 13 weeks. Two groups additional groups (untreated control and highest dose groups) were treated for 13 weeks followed by a recovery period of 4 weeks. The high dose of 10 000 ppm was increased to 13 000 ppm at treatment day 36 to achieve a maximum tolerated dose. Accordingly the calculated weighted average dose for the highest-dose/highest-dose recovery group was 11 833 ppm. The doses were equal to mean daily intakes of 0, 20, 67, 205 and 819 mg/kg bw per day in males and 0, 24, 81, 242 and 978 mg/kg bw per day in females. Observations included clinical signs (daily), neurological examination (12th/13th week), ophthalmological examination (start and end of treatment and recovery period), food consumption and body weight (weekly), haematology, biochemistry and urine analysis (end of treatment and recovery periods) and organ weight, gross and microscopic pathology. The protocol complies with OECD test guideline 408 (1998).

There were no deaths during the study and no treatment-related clinical signs were observed. No treatment-related neurological abnormality was observed. Body weight and food consumption were not affected by treatment. No treatment-related haematology or biochemical changes were observed. There were no treatment-related changes in organ weights, or gross and histopathological findings in both sexes.

The NOAEL for metabolite B was 11 833 ppm (equal to 819 mg/kg bw per day in males and 978 mg/kg bw per day in females), the highest dose tested (Stanley, 2003).

3. Observations in humans

Routine medical examinations, including anamnesis, physical examination and comprehensive blood and urine analysis of employees who handled benalaxyl in different manufacturing or formulating plants since the early 1980s have revealed no adverse effects on health and no skin sensitization/allergenicity effects (ISAGRO, medical data from manufacturing plants). No cases of skin sensitization/allergenicity were ever reported in applicators.

No cases of poisoning involving workers in the production and formulation or the field use of benalaxyl have been reported in the open literature.

Table 19. Results of studies of genotoxicity with metabolites of benalaxyl

End-point	Test object	Concentration	Purity (%)	Results	GLP or QA	Reference
Metabolite A						
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	33–5000 µg/plate, in DMSO	98.51	Negative[a,b]	GLP & QA	Wollny (2002b)
Forward mutation	Mouse lymphoma L5178Y cells, TK locus	13–2500 µg/ml, in DMSO	98.51	Negative[a,c]	GLP & QA	Wollny (2002c)
Chromosomal aberration	Human peripheral blood lymphocytes	2.5, 5 and 10 mmol/l; 1st expt ±S9: 3 h exposure, harvesting, 20 h; 2nd expt: –S9: 20 h exposure, +S9: 3 h exposure, harvesting, 20 h; in DMSO	98.51	Negative +S9 Positive –S9 (20 h)[d]	GLP & QA	Pritchard & Knights (2002)
Chromosomal aberration	Chinese hamster ovary cells (CHO)	1st expt: 625, 1250 and 2500 µg/ml ±S9 (4 h exposure, harvesting, 24 h); 2nd expt: –S9: 625, 937.5, 1250 and 1875 µg/ml (24 h exposure), 937.5 µg/ml (46 h exposure); +S9: 625, 1250 and 2500 µg/ml; 3rd expt: –S9: 1000, 1250 and 1500 µg/ml (24 h exposure); 1000 µg/ml (46 h exposure); in DMSO	98.51	Negative +S9 Positive –S9 (24 and 46 h)[e]	GLP & QA	Schulz (2002b)
Micronucleus formation	Mice (NMRI) bone-marrow cells, five males and five females	Single intraperitoneal administration, 500, 1000 and 2000 (48 h only) mg/kg bw; vehicle, corn oil; sampling times, 24 & 48 h	98.51	Negative[f]	GLP & QA	Honarvar (2002)
Metabolite B						
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	33–5000 µg/plate, in DMSO	98.48	Negative[a,g]	GLP & QA	Wollny (2002d)
Forward mutation	Mouse lymphoma L5178Y cells, TK locus	–S9: 168.8–2700 µg/ml; +S9: 168.8–2565 µg/ml; in culture medium	98.48	Negative[a,h]	GLP & QA	Wollny (2002e)
Chromosomal aberration	Chinese hamster ovary cells (CHO)	482.5, 965 and 1930 µg/ml, in DMSO; 1st expt: ±S9: 4 h exposure, harvesting at 24 h; 2nd expt: –S9: 24 h or 46 h exposure, +S9: 4 h exposure, harvesting at 46 h	98.48	Negative[a,i]	GLP & QA	Schulz (2002c)

DMSO, dimethyl sulfoxide; GLP, good laboratory practice; QA, quality assurance; S9, 9000 × g supernatant of rat liver induced with phenobarbital/β-naphthoflavone, unless otherwise stated

Solvent or vehicle was used as negative control. Positive control substances were used in all assays and gave the expected results.

Metabolite A: methyl-*N*-malonyl-*N*-(2,6-xylyl)-D,L-alaninate (soil metabolite)

Metabolite B: *N*-malonyl-*N*-(2,6-xylyl)-D,L-alanine (soil metabolite)

[a] With and without metabolic activation

[b] The first experiment was the standard plate incorporation assay and the second used the preincubation method. Slight toxic effects, evident as a reduction in the number of revertants, occurred at high concentrations in TA1537 in the first experiment −S9. The plates incubated with metabolite A showed normal background growth at up to 5000 μg/plate ±S9 in all strains used. Appropriate reference mutagens were used as positive controls and showed a distinct increase in frequency of induced revertant colonies. The experimental protocol complied with OECD test guideline 471 (1997).

[c] The assay was performed as two independent experiments, each using two parallel cultures. The first main experiment was performed ±S9 with a 4-h treatment. The second experiment was performed only −S9 with a 24-h treatment. The highest concentration applied (2500 μg/ml) in the pre-test for toxicity was chosen with regard to the solubility of metabolite A in DMSO. Positive controls: −S9, methyl methanesulfonate; +S9, 3-methylcholanthrene. No substantial, reproducible dose-dependent increase in mutant frequency and no relevant shift in the ratio of small to large colonies occurred in the first and second experiment at up to the maximum concentration tested, ±S9. Positive controls showed a distinct increase in induced total mutant colonies and an increase in the relative quantity of small versus large colonies. The experimental protocol complied with OECD test guideline 476 (1997).

[d] S9, 9000 × g supernatant of rat liver induced with Aroclor. Positive controls: −S9, mitomycin C; +S9, cyclophosphamide. In the first experiment, −S9, metabolite A at 10 mmol/l caused a reduction in mitotic index (MI) to 72% of the value for the solvent control; +S9, metabolite A failed to cause a reduction in the MI relative to the solvent control. In the second experiment, −S9, metabolite A failed to cause a reduction in the MI relative to the solvent control; + S9, metabolite A at 10 mmol/l caused a reduction in MI to 81% of the value for the solvent control. In the absence of S9, after 20 h exposure, metabolite A at 10 mmol/l caused a statistically significant and biologically significant increase in the proportion of metaphases containing chromosomal aberrations , when compared with the solvent control. No statistically significant increases in the proportion of polyploid cells were seen. Positive controls induced statistically significant increases in cells with structural chromosome aberrations. The experimental protocol complied with OECD test guideline 473 (1997).

[e] The highest concentration applied in the pre-test for toxicity (2500 μg/ml, approximately equal to 8.4 mmol/l) was chosen with regard to solubility of metabolite A in DMSO. Dose selection for the cytogenetic experiments was performed considering the toxicity data. Clear toxic effects as indicated by strongly reduced cell numbers were observed +S9 in experiment I after 4 h treatment at 2500 μg/ml. In addition, clearly reduced MIs were observed −S9 after 46 h continuous treatment at 1000 μg/ml (experiment III). In the absence of S9 in experiment II after 24 and 46 h continuous treatment, concentrations causing strong cytotoxicity were not evaluable for cytogenetic damage. Positive controls: −S9, ethyl methanesulfonate; +S9, cyclophosphamide. In the absence of S9, in experiment I after 4 h treatment, neither statistically significant nor biologically relevant increases in the number of cells carrying structural chromosome aberrations were observed. In experiments II and III −S9 after 24 and 46 h continuous treatment, reproducible statistically significant and biologically relevant increases in the number of cells carrying structural chromosomal aberrations were observed after treatment with metabolite A. No increase in the frequencies of polyploid metaphases was found after treatment with metabolite A when compared with the frequencies in the controls. Positive controls induced statistically significant increases in cells with structural chromosome aberrations. The experimental protocol complied with OECD test guideline 473 (1997).

[f] The highest dose was estimated after pre-test for toxicity (2500 μg/ml, causing marked toxic signs but no mortalities at up to 48 h after treatment. Cyclophosphamide was used as the positive control and showed a substantial increase in frequency of micronucleus formation. After treatment with metabolite A, the number of NCE was not substantially increased when compared with the mean value of NCE in the vehicle control, thus indicating that the test substance did not exert any cytotoxic effects in the bone marrow. The experimental protocol complied with OECD test guideline 474 (1997).

[g] The first experiment was performed as the standard plate incorporation assay and the second by the preincubation method. No relevant toxic effects, evident as a reduction in the number of revertants, occurred up to the maximum concentration ±S9. The plates incubated with metabolite B showed normal background growth at up to 5000 μg/plate ±S9 in all strains used. Appropriate reference mutagens were used as positive controls and showed a distinct increase in the number of induced revertant colonies. The experimental protocol complied with OECD test guideline 471 (1997).

[h] The assay was performed in two independent experiments, each using two parallel cultures. The first main experiment was performed ±S9 and with a treatment period of 4 h. The second experiment was performed only –S9, with a treatment period of 24 h. The highest concentration applied in the pre-test for toxicity (2000 µg/ml) was the limit of solubility of metabolite B in DMSO. However, precipitation in the culture medium was only observed at the highest concentration after continuous treatment. Since neither toxicity nor precipitation occurred in this pre-experiment at up to the highest concentration after 4 h treatment, the main experiments were performed with metabolite B dissolved in medium at the limit of solubility to increase the highest concentration to 2700 µg/ml (–S9) and 2565 µg/ml (+S9). Positive controls: –S9, methyl methanesulfonate; +S9, 3-methylcholanthrene. No substantial, reproducible dose-dependent increase in mutant frequency and no relevant shift in the ratio of small to large colonies occurred in the first and second experiment at up to the maximum concentration tested, ±S9. Positive controls showed a distinct increase in induced total mutant colonies and an increase in the relative quantity of small versus large colonies. The experimental protocol complied with OECD test guideline 476 (1997).

[i] The highest concentration applied in the pre-test for toxicity (1930 µg/ml, approximately equal to 6.8 mmol/l) was chosen with respect to the current OECD test guideline and with regard to the solubility of metabolite B in DMSO. The pH values of culture medium containing metabolite B at highest concentrations were adjusted with NaOH to physiological values. Dose selection for the cytogenetic experiments was performed considering the toxicity data and the occurrence of precipitation. Positive controls: –S9, ethyl methanesulfonate; +S9, cyclophosphamide. No toxic effects indicated by strongly reduced mitotic indices and/or cell numbers were observed up to the highest concentration of metabolite B except in experiment II –S9 after 46 h continuous treatment (47% of value for controls). No increase in the frequencies of polyploid metaphases was found after treatment with metabolite B when compared with the frequencies in the controls. Positive controls induced statistically significant increases in the number of cells with structural chromosome aberrations. The experimental protocol complied with OECD test guideline 473 (1997).

Comments

Biochemical aspects

Several toxicokinetic studies in rats given ^{14}C-labelled benalaxyl as single and repeated oral doses showed that the active substance is rapidly and extensively absorbed and distributed by all organs and tissues, with the greatest proportion of radioactivity remaining in the intestine and its contents, and in the liver and kidneys (minor quantities). Seven days after treatment, only approximately 0.3% of the administered radiolabelled dose remained in the rat and was distributed among organs and tissues. The half-life of elimination was about 30 h after administration of single doses and 36 h after administration of repeated doses. The pattern of elimination in the urine and faeces was also similar in all situations (administration of single and repeated oral doses) and was not sex-dependent. At 48 h after dosing, the radioactivity was mainly excreted in the faeces (at least 80%), via the bile and in the urine (approximately 8%).

The metabolites of benalaxyl that appeared in the faeces and urine were similar, irrespective of dose and type of administration (single or repeated doses). Unchanged benalaxyl was not detected in the urine. Eight metabolites were identified and corresponded to approximately 65% of the radioactivity present in the faeces and urine. The identity of three additional very polar metabolites remained unknown, but their proportions were very low compared with those of some other identified compounds. Benalaxyl undergoes extensive metabolism, mainly by oxidation of the methyl group of the aniline ring to a hydroxymethyl group, and finally to the carboxylic acid; minor metabolic pathways were the hydroxylation of the phenyl ring and hydrolysis of the carboxymethyl group.

Toxicological data

Benalaxyl has low acute oral toxicity in rats and mice (LD_{50} values were 4200 mg/kg bw and 680 mg/kg bw, respectively), low acute dermal toxicity in rats and rabbits (LD_{50} values were > 5000 mg/kg bw and > 2000 mg/kg bw, respectively) and low acute toxicity in rats exposed by inhalation (the 4-h LC_{50} value was > 4.2 mg/l, the highest achievable concentration). Although no significant clinical signs were observed in rats treated by oral or dermal administration, signs of intoxication including loss of equilibrium, uncoordinated movements and asthenia occurred in mice treated by oral administration. Benalaxyl is not an irritant to the skin and eyes of rabbits. In a maximization test in guinea-pigs, benalaxyl did not show sensitizing potential.

The toxicity of benalaxyl administered orally was investigated in short-term studies: a 90-day dose range-finding study for a long-term study of toxicity and carcinogenicity in mice, 5-week and 90-day studies in rats, and a 1-year study in dogs. The major target organs were the liver in mice and rats, and the testes in dogs. In the absence of any changes in clinical chemistry or histopathology, the Meeting considered that hepatic enlargement was an adaptive response and not an adverse effect.

In a 90-day study in Swiss mice, a dose-related increase in liver weights occurred at dietary concentrations of 1000 ppm and greater at 96 days and of 2000 ppm and greater at 42 days. There were no histopathological lesions associated with this increase in liver weight. The NOAEL was 5000 ppm, equal to 842 mg/kg bw per day, the highest dose tested.

In a 5-week study in Wistar rats treated by gavage, changes in haematological (coagulation time) and biochemical (increases in cholesterol, albumin and total protein, decreases in aspartate amino transferase and alkaline phosphatase activities) parameters were observed at the highest dose of 800 mg/kg bw per day. The relative weight of the liver was increased in groups treated with benalaxyl at doses of 100 mg/kg bw per day and greater. All these changes had returned to normal relative to values for controls by the end of the 2-week recovery period. The NOAEL was 100 mg/kg bw per day on the basis of changes in haematological and biochemical parameters.

In a study in Sprague-Dawley rats given diets containing benalaxyl at concentrations of up to 10 000 ppm for 13 weeks, or 12 000 ppm for 4 weeks followed by a 9-week recovery period, animals treated at 10 000 and 12 000 ppm had decreased body-weight gain and increased serum cholesterol values relative to those for controls. At 12 000 ppm, there were also some changes in haematological parameters (decreases in erythrocyte count, haemoglobin concentration and erythrocyte volume fraction in both sexes); all changes were reversible after a recovery period. Liver weight was reversibly increased in animals at 1000 ppm (males) and above (both sexes) and lobulation was observed in males in these groups, sometimes associated with rounded edges (this finding was also observed sporadically in other groups). The livers of females at 10 000 ppm were darker than normal, and diffuse steatosis was seen in both sexes at this dietary concentration, although the pattern was more severe in males. The NOAEL was 1000 ppm (equal to 59 mg/kg bw per day).

In a 1-year study in beagle dogs, the only finding that could be attributed to treatment was atrophy of the seminiferous tubules of the testes in two out of six males treated with benalaxyl at the highest dietary concentration of 800 ppm. The NOAEL in males was 200 ppm (equal to 6.5 mg/kg bw per day).

Long-term studies of toxicity and carcinogenicity were carried out in Swiss mice and Sprague-Dawley rats.

In a long-term study of toxicity and carcinogenicity, Swiss mice were given diets containing benalaxyl at concentrations of up to 3000 ppm for 78 consecutive weeks. While there was no effect on survival in female mice, a high incidence of mortality occurred in males at 1000 and 3000 ppm, mainly during the second year of the study. Because 25% of the males at the highest dose survived to termination, this study was considered to be acceptable. In males, body-weight gain was slightly depressed in all treated groups, particularly during the second year of treatment, without a dose-related effect. In females, there was no effect of treatment on body weight. In females at 3000 ppm, absolute and relative weights of the liver were significantly increased (as observed in the 90-day preliminary test). No increase in the incidence of tumours was observed when compared with the control group. The NOAEL was 250 ppm (equal to 43 mg/kg bw per day) on the basis of mortality in males. There was no evidence for carcinogenic potential in Swiss mice treated with benalaxyl for 78 consecutive weeks.

In rats given diets containing benalaxyl at concentrations of up to 1000 ppm for 104 weeks, there was no evidence of neoplastic or non-neoplastic effects related to administration of the test article. Although the incidence of hepatocellular neoplasms was found to be greater in females at the highest dose than in controls, the difference was not statistically significant, no dose–response relationship was observed and the frequency was compatible with that of spontaneous hepatocellular neoplasms. The NOAEL was 1000 ppm (equal to 44 mg/kg bw per day, the highest dose tested), in the absence of any significant findings in either sex.

The Meeting concluded that benalaxyl is not carcinogenic in rodents.

A comprehensive range of studies of genotoxicity in vitro and in vivo with benalaxyl gave consistently negative results. The Meeting concluded that benalaxyl is unlikely to be genotoxic.

In view of the absence of genotoxicity and the lack of carcinogenicity in mice and rats (albeit noting the limitation of the study in rats because the maximum tolerated dose was not attained), the Meeting concluded that benalaxyl is unlikely to pose a carcinogenic risk to humans at dietary doses and anticipated exposures of consumers or workers.

The reproductive toxicity of benalaxyl has been examined in a two-generation study in rats, and in studies of developmental toxicity in rats and rabbits.

In a two-generation (two litters per generation) dietary study of reproductive toxicity in rats, the NOAEL was 1000 ppm (equal to 53 mg/kg bw per day for the F_0 generation) for general toxicity in parent animals (decreased body weight) and adverse effects in pups (decreased pup weight and liver weight) at 5000 ppm, although fertility and reproductive parameters were not

affected in the F_0 generation at dietary concentrations of up to 5000 ppm (equal to 289 mg/kg bw per day, the highest dose tested).

In Sprague-Dawley female rats given benalaxyl at doses of up to 200 mg/kg bw per day by gavage from day 6 to day 15 of gestation, no toxicity was apparent in dams. Benalaxyl induced a marginal but statistically significant increase in the delay in ossification of the cranial bones at 50 and 200 mg/kg bw per day (10%, 16%, 18% and 26% of the fetuses in the control group, and at the lowest, intermediate and highest dose, respectively). In addition, in the group receiving the highest dose a statistically significant increase of the percentage of pre-implantation losses was observed. The NOAELs for maternal toxicity, embryotoxicity and developmental toxicity were 200 mg/kg bw per day (the highest dose tested), 50 mg/kg bw per day and 12.5 mg/kg bw per day, respectively.

In female New Zealand White rabbits given benalaxyl by gavage from day 6 to day 27 of gestation, minimal maternal toxicity was manifest as weight loss during late gestation and a low gravid uterus weight at a dose of 250 mg/kg bw per day. There were no treatment-related effects on implantations. No teratogenic potential was seen, but there were statistically significant effects at a dose of 250 mg/kg bw per day on fetal weight and crown–rump lengths and on the incidence of fetuses with delayed skeletal development. The NOAELs for maternal and developmental toxicity were both 50 mg/kg bw per day.

No specific studies of neurotoxicity with benalaxyl were available; however, no evidence of neurotoxicity was apparent from the available studies of toxicity.

No adverse effects were reported in personnel involved in the production and formulation of benalaxyl, or in the use of this product in the field.

The two major soil metabolites, methyl-*N*-malonyl-*N*-2,6-xylyl-D,L-alaninate (metabolite A) and *N*-maolonyl-*N*-2,6-xylyl-D,L-alanine (metabolite B) were also investigated. The results of studies of acute toxicity and 90-day studies of oral toxicity with both metabolites in rats, showed that both metabolites have very low toxicity (oral $LD_{50}s > 2000$ mg/kg bw; NOAEL in 90-day dietary studies in rats, 923/1073 and 819/978 mg/kg bw per day for metabolite A and metabolite B, respectively, the highest doses tested) and are thus less toxic than the parent.

The results of a range of studies of genotoxicity, including tests in vitro with metabolite A and metabolite B, and a test for micronucleus formation in vivo with metabolite A, indicated that neither metabolite was genotoxic.

The Meeting concluded that the existing database on benalaxyl was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting established an ADI of 0–0.07 mg/kg bw based on a NOAEL of 6.5 mg/kg bw per day for atrophy of the seminiferous tubules occurring at 25 mg/kg bw per day in a 1-year study in dogs and using a safety factor of 100.

Benalaxyl has little acute toxicity and short-term dosing produced no significant general toxicity; however, a delay in ossification of cranial bones was observed at a dose of 50 mg/kg bw per day in the absence of maternal toxicity and of other markers of developmental delay in a study of developmental toxicity in rats. Although statistically significant, this is a marginal effect, but in the absence of data on historical controls, it was considered to be treatment-related. The Meeting established a conservative ARfD of 0.1 mg/kg bw for benalaxyl for women of childbearing age on the basis of a NOAEL of 12.5 mg/kg bw per day in a study of developmental toxicity in rats, and a safety factor of 100. There is no concern regarding the acute toxicity of this compound for the rest of the population, including children.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Mouse	2-year studies of toxicity and carcinogenicity[a]	Toxicity	250 ppm, equal to 43 mg/kg bw per day	1000 ppm, equal to 174 mg/kg bw per day
		Carcinogenicity	3000 ppm, equal to 522 mg/kg bw per day[c]	—
Rat	2-year studies of toxicity and carcinogenicity[a]	Toxicity	1000 ppm, equal to 44 mg/kg bw per day[c]	—
		Carcinogenicity	1000 ppm, equal to 44 mg/kg bw per day[c]	—
	Multigeneration reproductive toxicity[a]	Parental	1000 ppm, equal to 53 mg/kg bw per day	5000 ppm, equal to 275 mg/kg bw per day
		Offspring toxicity	1000 ppm, equal to 53 mg/kg bw per day	5000 ppm, equal to 275 mg/kg bw per day
		Reproductive toxicity	5000 ppm, equal to 275 mg/kg bw per day[c]	—
	Developmental toxicity[b]	Maternal toxicity	200 mg/kg bw per day[c]	—
		Developmental toxicity	12.5 mg/kg bw per day	50 mg/kg bw per day
Rabbit	Developmental toxicity[b]	Maternal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
		Developmental toxicity	50 mg/kg bw per day	250 mg/kg bw per day
Dog	1-year study of toxicity[a]	Toxicity	200 ppm, equal to 6.5 mg/kg bw per day	80 ppm, equal to 25 mg/kg bw per day

[a] Dietary administration

[b] Gavage administration

[c] Highest dose tested

Estimate of acceptable daily intake for humans

0–0.07 mg/kg bw

Estimate of acute reference dose

0.1 mg/kg bw for women of childbearing age

Unnecessary for the rest of the population

Information that would be useful for continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposures.

Critical end-points for setting guidance values for exposure to benalaxyl

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, at least 80% based on biliary and urinary excretion
Distribution	Widely distributed
Potential for accumulation	None
Rate and extent of excretion	Rapid and extensive (> 90% within 72 h, mainly via faeces)
Metabolism in animals	Extensive metabolism, mainly by oxidation and hydroxylation
Toxicologically significant compounds (animals, plants and environment)	Parent compound
Acute toxicity	
Rat LD_{50} oral	4200 mg/kg bw
Mouse LD_{50} oral	680 mg/kg bw
Rabbit LD_{50} dermal	> 2000 mg/kg bw
Rat LC_{50} inhalation	> 4.2 mg/L air (4 h, nose only, aerosol)
Rabbit, skin irritation	Not irritating (24 h)
Rabbit, eye irritation	Not irritating
Skin sensitization (test method used)	Not sensitizing in guinea-pigs (Magnusson & Kligman)
Short-term studies of toxicity	
Target/critical effect	Liver (steatosis in rats), and testes (atrophy seminiferous tubules in dogs)
Lowest relevant oral NOAEL	59 mg/kg bw per day (90-day study in rats)
	6.5 mg/kg bw per day (1-year study in dogs)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Genotoxicity	
	Not genotoxic in vitro and in vivo
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Increased mortality (mice)
Lowest relevant NOAEL	43 mg/kg bw per day (18-month study in mice)
Carcinogenicity	No carcinogenic risk to humans
Reproductive toxicity	
Reproduction target/critical effect	Decreased body-weight gain and increased liver weight of pups at parentally toxic doses
Lowest relevant reproductive NOAEL	Parents and offspring: 53 mg/kg bw per day (rats)
	Reproductive toxicity: 275 mg/kg bw per day, highest dose tested (rats)
Developmental target/critical effect	Delay in ossification of cranial bones in absence of maternal toxicity (rats)
	Minor skeletal deviations at maternally toxic doses (rabbits)
Lowest relevant developmental NOAEL	Maternal: 50 mg/kg bw per day (rabbits)
	Developmental: 12.5 mg/kg bw per day (rats)
Neurotoxicity/delayed neurotoxicity	
	No specific study; no findings in other studies

Other toxicological studies

Toxicity of soil and groundwater metabolites

Metabolite A:	Oral LD$_{50}$, > 2000 mg/kg bw (rats)
	NOAEL in 90-day study, 923 mg/kg bw per day (rats)
	Results of studies of mutagenicity in vitro and in vivo: negative
Metabolite B:	Oral LD$_{50}$ > 2000 mg/kg bw (rats)
	NOAEL in 90-day study, 819 mg/kg bw per day (rats)
	Results of studies of mutagenicity in vitro: negative

Medical data

No adverse effects on health in manufacturing personnel

Summary

	Value	Study	Safety factor
ADI	0–0.07 mg/kg bw	Dog, 1-year study of toxicity	100
ARfD[a]	0.1 mg/kg bw	Rat, developmental toxicity	100

[a] For women of childbearing age, unnecessary for the rest of the population.

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