Parameter Evaluated	Time Point(s)
Physical examination/mortality	Daily
Body weight	pretest, 1X/ Drug Week 1, 2X/Drug Week 2-13, 1X/wk for the remainder of the study
Food consumption	2X/week for Weeks 1-13, then 2X/week q4wks
Necropsy - gastrointestinal tract	Week 53
Histopathology -esophagus, stomach, small and large intestine, cecum, all gross GI changes	Week 53

Results

Mortality - There were no treatment-related mortalities. There were 3 deaths which the Sponsor considered incidental: [1] 1 male in the 1.0 mg/kg/day group with unresolving epidermal ulceration [etiology not indicated], weight loss, and poor skin turgor; [2] 1 female at 1.0 mg/kg/day with dyspnea 2° to pneumonia [histopathology], weight loss, and mild dehydration; and [3] 1 male at 0.5 mg/kg/day with ataxia, decreased activity, oculonasal discharge and severe weight loss, etiology not determined.

Clinical signs - There were no treatment-related effects.

Body weight - There were no treatment-related effects.

Food consumption - There were no treatment-related effects.

Necropsy - There were no treatment-related effects.

Histopathology - There were no treatment-related effects.

Reviewer's Comment [Study Design and Data Presentation] - For the stated purpose, these were adequate. The rats were fed slightly less than in other studies [e.g. 16 vs. 17 g/day and 22 vs 24 g/day for females and males, respectively.]

Sponsor's Conclusions [numbered] and Reviewer's Comments

1. "MK-0966 produces no gastrointestinal effects when given orally to rats for 53 weeks at doses up to 1 mg/kg/day."

The initial Pharmacology/Toxicology Reviewer, Dr. Will Coulter, reviewed the following study. Additional comments by the current Reviewer are in italics.

4.3.10. Fifteen-Day IV Toxicity Study in Rats [Vol. 1.20; B-4477 and Vol. 1.21; B-4577]

Study: TT#95-075-0, -1
Compound: L-748,731-000R014
Formulation: Sterile solutions prepared daily in 0.08 % PEG 400 in 0.9 % NaCl injection, USP. A
Route: IV via the tail vein at approximately 2 mL/min.
Diet: Certified Rodent Diet - approximately 17 g for F and 24 g for M each day.
Dose Levels:

Group:		Volume
1	Control (0.9% sterile saline)	12 mL/kg 1/day x 14 days
2	Vehicle control (sterile 0.08% PEG 400/saline)*	12 mL/kg 1/day x 14 days
3	20 µg/Kg/day L-748,731	4 mL/kg 1/day x 14 days
4	40 µg/Kg/day L-748,731	8 mL/kg 1/day x 14 days
5	60 µg/Kg/day L-748,731	12 mL/kg 1/day x 14 days

* Due to an oversight, VC M received sterile 0.1% PEG 400 in saline, rather than 0.08% PEG 400 in saline Drug Day 1 only. Vehicle control animals received sterile 0.008% PEG 400 in saline from D3/4 - D8/9 (11/30/95 through 12/5/95), rather than 0.08% PEG 400 in saline,

Total No. of Doses: 14
Strain: Crl:CD®(SD)BR
Number: 15/sex/group, 63-64 days old, body weight M 262-344 g, F 181-225 g-
Control Treatment: Sterile 0.08 % PEG 400 in 0.9 % saline
Study Site: Merck Research Laboratories, West Point, PA
Date: November 3, 1995 to April 29, 1996
GLP/QAU: Both present with signatures.

The purpose of this study was to determine the toxicity and irritation potential of L-748,731 in rats after intravenous administration. A factor of 1.0 was used for L-748,731. The animals were observed daily. Body weights were recorded pretest and once during W 1 and W3. Food consumption was observed twice/week. Indirect and slit lamp ophthalmoscopy was conducted on all surviving control and high dose animals during W2. Serum biochemistry (total protein-glucose-creatinine-AST-ALT- alkaline phosphatase-triglycerides-K-Ca-albumin- urea nitrogen-A/G ratio-cholesterol-Na+-Cl-phosphorus), hematology (erythrocyte count-Hct-leukocyte count-differential leukocyte count-platelet count-Hb-mean corpuscular volume-mean corpuscular Hb-mean corpuscular Hb concentration) and urinalyses (protein-bilirubin-glucose-occult blood- specific gravity-microscopic examination of sediment-urobilinogen-pH-ketones-volume) were determined W2 on all surviving rats. Gross examination was performed on all rats. Organ weights (heart, spleen, brain, pituitary, kidneys, testes, prostate, thyroid, liver, adrenal, ovaries) were determined on all rats, and microscopic examination was done on 29 different tissues from all animals in the saline control and high dose group.

RESULTS AND DISCUSSION

- clinical signs: none related to drug - purple discoloration at injection in 2G4 and 1G5 *[considered by Sponsor to be related to iv procedure and not drug]* –
- mortality: none-
- body weight: no treatment effects on body weight-
- food consumption: no significant changes-
- ophthalmic examination: no treatment related effects were stated-
- hematology: platelets average value ↑G5 (19.4%)- platelets ↑ M G5 (DR in No. with individual values >1000 mm^3)-
- serum biochemistry: AST and ALT: ↑in 1 M 40 µg/kg/day (2.3x and 2.8x over PEG control)
- urinalysis: ↑ in G5 F with +1 bilirubin (10/15 vs 4/15 in saline control)-
- gross changes: no treatment changes-
- organ weights: no significant changes in average absolute or relevant weights-
- histopathology: n = 15
  - injection site: perivascular cellular infiltration/fibrosis/hemorrhage/necrosis, vascular necrosis seen in both G1 and G5-
  - liver: cholangitis 1 F G5, hemorrhage 1F G5, focal necrosis 2 F G1, 2 F G5, 3 M G5,
  - kidney: pelvis dilatation 1 M G5 - mineralization 2 FG5-
  - testis: epididymis, cellular infiltration 2G5-
  - lung: cellular infiltration 1 M G5 - focal pneumonia 1 F G5-
  - heart: focal degeneration 1 M G5-
  - lymph node: focal telangiectasis 1 M G5-
  - skeletal muscle: cellular infiltration 2 M G5-

Focal necrosis in the liver increased slightly in the high dose group, following intravenous administration of L-748,731 to rats at 20, 40, and 60 µg/kg/day for 14 days. Lesions developed at the site of injection in controls and the high dose group, with type and number of lesions similar in both groups. All lesions in the study were classified as 1 (very slight) or 2 (slight or small). When compared to the PEG control, individual values for hematology and serum biochemistry were slightly higher in the high dose group. The average value for platelets was increased 19% in M of the high dose. The IV administration of 60 µg/kg/day of parent drug may be the level at which drug changes begin to appear.

4.4 REPEAT DOSE STUDIES - MICE

4.4.1. FOURTEEN-WEEK ORAL RANGE-FINDING STUDY IN MICE:[*Vol. 1.19; p. B-3987*]

Study: TT#95-610-0
Compound: L-748,731-000R, batch # 014
Formulation: Suspensions prepared daily in 0.5% aqueous methylcellulose.
Stability: 72 hr at 0.1 mg/mL and 200 mg/mL-
Control Vehicle: 0.5% aqueous methylcellulose (400 cps)
Route: Oral, gavage at 10 mL/Kg body wt.
Dosage Groups: Group: 1 2 3 4 5 6
mg/Kg/day: 0 30 100 300 600 1000
Total No. of Doses: M 91, F 92
Strain: Crl:CD-1®(ICR)BR albino mice, 38 days old,
body weight- M 26.2 to 33.3 g, F 20.1 to 27.0 g
Number: 10/sex/group
Study Site: Laboratoires Merck Sharp&Dohme-Chibret, Centre de Recherche, Riom, France
Date of Study: 14 April 1995 to 27 October 1995
GLP/QAU Statements: Both present and signed.

The object of this study was to determine the subchronic toxicity of high dose levels of the drug in mice in order to support the dosage selection for the mouse carcinogenicity study.

Animals were examined daily. Body weight was determined pretest, Week 1, and twice/week thereafter. Food consumption was measure once/week based on a 2-3 day period. Ophthalmic examinations were done pretest on all mice and on Weeks 6 and 12 on all surviving control and high-dose groups. Hematology and serum biochemical determinations were determined on all surviving mice Drug Week 14. Necropsy was done on all surviving mice and on all mice found dead. Histopathology examination was carried out on tissues from 5 males and 5 females from the control and high-dose groups and all mice found dead from all groups.

RESULTS AND DISCUSSION

- mortality:

DRUG RELATED DEATHS					
mg/Kg/day	30	100	300	600	1000
M	-	-	1	1	3
F	-	1	1	2	1
Total	-	1	2	3	4

- incidental deaths: G3 1D17, 1D4
G4 1D14
G5 1D8, 1D10
- signs: abdominal distension, decreased activity, hypothermia, pallor, dehydration, piloerection - all signs occurred prior to death-

- body wt: irregular throughout the study- not dose related-

	Week 13 Group Mean Weight Change in Grams		Week 13 Body Weight Decrement in Percent	
	M	F	M	F
G1	10.8	8.6	-	-
G2	11.6	7.2	-	8.3
G3	10.3	7.6	1.3	3.1
G4	11.1	7.1	-	6.2
G5	9.8	6.6	2.5	7.1
G6	8.6	8.3	4.2	0.3

- food consumption: no significant changes-
- ophthalmic examination: no drug-related changes-
- hematology:
  RBC: ↓ M G5 (8.9%), G6 (9.8%)-
  Hb: ↓ M G5 (7.9%), G6 (6.5%): G6 (4.6%)-
  leukocyte count: ↑ M G6 (25.5%)-
  neutrophils (%): ↑ M G6 2.6x; ↓ F in all drug groups-
  segmented neutrophils (%):↑ M G6 2.6x-
  lymphocytes, cells/mm^3: ↑ in all drug groups (19%-41%)-

  Male (95-0046) in G3 (100 mg/Kg) had elevated leukocytes and neutrophils and was found with peritonitis at necropsy. Other group hematology parameters in the study showed variations at all evaluation times.
- serum biochem:

Increased Transaminase Activity in Males Week 14
(% of Control Mean Values)

Group	AST	ALT
100 mg/Kg	+86(2/8)*	+72(1/8)
300 mg/Kg	+55(2/8)	+97(2/8)
600 mg/Kg	+34(1/9)	+69(1/9)
1000 mg/Kg	+41(1/6)	+72(1/6)

* Parenthesis indicated the number of individual values above the 95% confidence limits of the historical control.

triglycerides: ↑ in F drug groups (10% - 65%)-
↓ in M drug groups (G6 29%)-
glucose: ↓ M G6 (13%)-
SUN: dose related ↑ M G6 (29%) -
creatinine: M G5 (animal 95-0094) ↑ 3x over control-

- organ weights: no drug related changes-
- gross and histopathologic findings:

INCIDENCE OF FINDINGS										
mg/Kg/day	30		100		300		600		1000	
Sex	M	F	M	F	M	F	M	F	M	F
Small intestine, ulcer							1			1
Large intestine, ulcer	1		1	1	2	2	2		1	2
Peritonitis	1		1	2	1	2	2	1	1	4
Inflammation, large intestine										1
Kidney: tubular basophilia	1	1			1		2			
papillary necrosis			1							
tubular dilatation	1						1			
Liver, focal necrosis							1			

spleen: some enlargement-
extramedullary hematopoiesis G3, 4, 5, 6-
lymphocytic necrosis: 1F G3, 1M G4, 1M G6-
lymph nodes: some enlargements - lymphoid reactive hyperplasia in 1-3 animals in each drug group
lymphocytic necrosis 1 each in G4, 5, and 6-
bone marrow: ↑ granulopoiesis
kidney: papillary necrosis: 1F G3(No. 95-0045, slight, and considered drug related-
tubular basophilia: 1M ,1 F G2(very slight)
1M G4 (slight)
2 M G5 (very slight to slight)
tubular dilatation: 1M G2(very slight)
1M G5 (slight)
hydronephrosis: 2 M G4(slight)
liver: focal necrosis in 1F G5(very slight)-
peritoneum: peritonitis G2(1M very slight); G3(1F marked, 2 M very slight to moderate); G4(1 F slight, 2 very slight to moderate); G5(2 F slight to moderate, 1 M slight); G6(1 F slight, 4 M very slight to slight)-

- organ weights: no drug related changes- for male 95-0046 (100 mg/Kg/day) the liver was affected by peritonitis and could not be separated and weighed (see table above)-

No drug group was free of lesions, as peritonitis and ulcerations occurred in the large or small intestine of all drug treated groups. Very slight unilateral papillary necrosis occurred in one female dosed at 100 mg/Kg/day and was considered drug related by the sponsor; however, no other group developed this lesion. At 600 mg/Kg/day and 1000 mg/Kg/day the erythroid parameters were reduced by 6.5% - 9.8% in males. The kidney and large intestine were the target organs. The doses proposed by the sponsor for the oral carcinogenicity study are 5, 10, 20, and 30 mg/Kg/day.

Additional Reviewer's Comment - The Reviewer concurs with Dr. Coulter's and the Sponsor's conclusions. In addition, the other histopathological lesions [e.g. extramedullary splenic hematopoiesis and lymphocytic necrosis; lymph node reactive hyperplasia and necrosis; thymic involution] were generally observed in animals also exhibiting intestinal ulceration and/or peritonitis and were considered secondary to the ulceration and peritonitis.

4.5 SUMMARY OF TOXICOLOGY

"The approximate lethal dose$_{50}$ for L-748,731 is >2000 mg/kg when administered as a single oral or intraperitoneal dose to female mice and rats."

Dog Studies - Toxicities observed in dogs following repeat doses of L-748,731 were minimal. The NOAEL in the 14-week repeat dose study [TT# 94-040-0] was 10 mg/kg/day [AUC = 15.67 ± 5.07 μg•hr/ml; ≈4[1]X human exposure at 25[50] mg daily]. At 50 mg/kg/day, one female exhibited reddening of the gastric mucosa. The histopathological changes at 50 mg/kg/day included intestinal ulceration in 1/4 males and females, each, and very slight renal papillary necrosis in 2/4 females. The intestinal ulceration occurred over Peyer's patches. This distribution of ulcerative lesions was also observed in the 14-day exploratory study [TT# 94-020-0] at a dose of 100 mg/kg. In the 14-week study, immunohistochemical staining for distribution of COX-1 and COX-2 in the GI tract revealed comparable staining between control and treated dogs with the exception of staining in the ulcerated areas. There was a decrease in COX-1 staining in the areas of the ulcerated jejunum. The changes in other parameters would be consistent with changes secondary to GI toxicity that included ulceration and potential blood loss. The altered parameters included [1] decreased weight gain and food consumption; [2] decreases in RBC indices (<16%); [3] increases in neutrophils [45%]; [4] increases in platelets [30%]; and [5] an increase in BUN [maximum of 1.5-2X] without a concomitant increase in creatinine. In the 53-week study with a 27-week interim

sacrifice, the NOAEL was 10 mg/kg/day. However, it does not appear that the maximum tolerated was achieved since only mild toxicity was observed. The NOAEL was based on the observation of treatment-related blood in the stool. However, there was no blood in the stool observed after Week 25 and the blood in the stool was not associated with any histopathological change. The alterations in hematology [decrease in WBC and lymphocyte counts] and serum chemistry [increases in BUN and ALT] tended to be sporadic, were of minimal magnitude, and of unknown relationship to treatment. One high dose male at the terminal sacrifice exhibited very slight to slight multifocal atrophy/necrosis of the small intestine. The relationship of this lesion to drug treatment is not known. *Slight or moderate cellulitis [skin] was observed in 2/4 males at 50 mg/kg/day.* In both the 14-week and 53-week study, there was a decrease in ovary weights. In the 14-week study, a decrease of 13-28% was observed at ≥10 mg/kg/day. In the 53-weeks study, there was a decrease of 20, 12, and 26% at 3, 10, and 30 mg/kg/day.

In the 14-week dog study, there was also an increase in the incidence of cellulitis in the high dose males [2/4 vs. 0/4 for treated vs. control dogs].

Rat Studies - Two primary target organs were identified in the repeat dose rat studies: [1] the gastrointestinal tract and [2] the kidney. The GI toxicity was characterized by small and large intestinal ulceration/perforation. In Study TT# 95-601-0 [27-week study], the Sponsor indicated that the most common site for intestinal ulceration was the jejunum, although ulcers were also observed in the cecum, colon, duodenum, ileum, and/or stomach. Mortality was associated with this lesion. The incidence of GI toxicity tended to be greater in females. Exposure to drug was greater in females than in males. At 10 mg/kg/day, drug related mortality due to GI toxicity was observed within approximately 10 weeks of initiation of dosing in Study TT #95-045-0 [53-week toxicity study]. In other studies [TT# 93-149-0 and TT# -4-615-0], doses up to 300 mg/kg/day for ≤13 weeks did not result in any drug-related mortalities and only minimal GI toxicity [e.g. 1/15 females with small intestinal ulceration in each study]. The reason for this difference was not identified. A NOAEL for GI ulceration/perforation following drug administration for 52 weeks was determined to be 1 mg/kg/day. There were several other findings in ≥1 of the studies that were considered to be secondary to the GI toxicity. These included [1] clinical signs; [2] a mild decrease in RBC indices, [3] increases in neutrophils and thrombocytes; [4] decreases in albumin and total protein; [5] extramedullary hematopoiesis in the spleen and/or liver; [6] increased bone marrow erythropoiesis and/or granulopoiesis; [7] hepatic subcapsular necrosis; [8] lymph node histiocytosis and/or reactive hyperplasia; [9] thymic atrophy, [10] adrenal cortical hypertrophy; [11] increases in BUN without a concomitant increase in creatinine; and [12] peritonitis. A comparative study between ibuprofen and L-748,731 indicated that L-748,731 resulted in minimal change in COX activity and PG levels in the intestinal tract [e.g. up to 20-30%]. Ibuprofen resulted in a decrease in COX activity and PG levels by 60-90%. These repeat dose studies in rats indicate that GI toxicity is not only a function of dose, but is also a function of the duration of exposure.

Renal tubular basophilia was observed in rats administered L-748,731 at doses of ≥10 mg/kg/day for 26 weeks and at ≥100 mg/kg/day for 13 weeks. The lesion was more common in males than females. Tubular basophilia, however, was not observed at 27 or 53 weeks in Study TT# 95-045-0 [2-10 mg/kg/day]. Tubular basophilia was reversible within 14 days following cessation of L-748,731 at 300 mg/kg/day for 14 weeks. Renal papillary necrosis, which has been associated with the administration of non-specific COX inhibitors, was only sporadically observed and generally at ≥100 mg/kg/day.

Cervical lymph node granulomas were observed at doses of ≥125 and ≥100 mg/kg/day administered for 8 and 13 weeks, respectively. Immunohistochemical staining indicated that there was an increase in COX-2 positive macrophages in the cervical lymph nodes of rats administered 300 mg/kg/day when compared to control rats. Cervical lymph node granulomatous lesions were reversible within 27 weeks following cessation of drug at a dose of 300 mg/kg/day for 14 weeks.

Centrilobular hepatocellular hypertrophy in females was observed after 14 weeks of drug administration at 300 mg/kg/day. This correlated with an increase in liver weights. Mild increases in AST and ALT primarily in the 27 and 53-week studies were also noted.

Similar findings with respect to GI and renal lesions were seen in mice administered 100-1000 mg/kg/day for 13 weeks. The NOAEL for small and large GI ulceration/perforation and peritonitis was not determined in males but was <30 mg/kg/day. The NOAEL for females was 30 mg/kg/day. Deaths occurred at ≥100 mg/kg/day. As in the rat, several findings were considered to be secondary to the GI toxicity including [1] decreases in RBC indices; [2] increases in PMNs in males; [3] extramedullary splenic hematopoiesis and lymphocytic necrosis; [4] lymph node reactive hyperplasia and necrosis; and [5] thymic involution. However, unlike the rat, the incidence of GI toxicity tended to be greater in males. Exposure to drug was greater in males than in females. AST and ALT were mildly elevated [<100% compared to controls]. Tubular basophilia was also observed in 1-2 animals at ≥30 mg/kg/day.

APPEARS THIS WAY
ON ORIGINAL

5. Genotoxicity

The previous Pharmacology/Toxicology Reviewer, Dr. Will Coulter, reviewed the following study. Additional comments by the current Reviewer are in italics.

5.1 *In Vitro* Assays

5.1.1. Mutagenicity Assays

5.1.1.i. Microbial Mutagenesis Assay *[Vol. 1.31; p. D-39]*

Study: TT #94-8024; TT #94-8026
Compound: L-748,731-000R009
Formulation: Solution in DMSO.
Concentration Range Tested: 100, 300, 1000, 3000, 6000 µg/plate *[high dose was based on limiting solubility; 48-hour exposure duration; triplicate plates]*
Strain: Salmonella typhimurium: TA1535, TA97a, TA98, TA100
E.coli: WP2, WP2 uvrA, WP2 uvrA pKM101:
Negative Control: DMSO at 100 µL/plate
Positive Control:
2-aminoanthracene (2 and 5 µg/plate or 10 and 15 µg/plate)
hydrazine sulfate (500 and 1000 µg/plate) disolved in water
sodium azide (1.5 µg/plate) disolved in water
methyl methanesulfonate (2 µL/plate)
daunomycin (5 µg/plate)
ICR-191 (1.0 µg/plate)
Study Site: Merck, West Point, PA
Date: May-August 1994
GLP/QAU Statements: Both present and signed.

Incubations were for 49 hours at 37° C. The studies were conducted without and with metabolic activation (S9). The revertant colonies were counted on the plate and averaged to compare with the appropriate control plate average. Values were considered positive if the number of revertants were at least two fold higher than the solvent control and showed a dose-related increase. With the exceptions noted above, DMSO was the solvent for the positive controls.

Results and Discussion

[Mean data and not individual replicates were submitted. Standard deviations were included.]

No two-fold increase occurred in the number of revertants per plate with the four *S. typhimurium* or three *E.coli* genotypes, in the presence or absence of S-9. A precipitate was observed on the plates at 1000, 3000, and 6000 µg L-748,731 per plate. This was said not to interfere with the scoring. The exception was TA98 without S-9, in which the precipitate prevented scoring. Bacterial lawn growth was inhibited with TA100 with S-9 at 6000 µg/plate and TA97a with and without S-9 at 3000 and 6000 µg per plate. In the repeat study the results were similar, with the exception that no inhibition in lawn growth was seen at doses up to 6000 µg/plate and precipitate did not interfere with scoring. The positive controls produced the expected increases. An exploratory study did not produce a two-fold increase in revertants, but precipitate was seen at ≥1000 µg/plate. At 10,000 µg/plate, scoring was inhibited.

The current Pharmacology/Toxicology Reviewer reviewed the following studies.

5.1.1.ii. L-748,731: V-79 Mammalian Cell Mutagenesis Assay [Vol. 1.33; p. D-484]
Study Identification: TT #95-8510 [range finding], TT #95-8506, TT #95-8501, and TT #95-8503
Site: Merck Research laboratories, West Point, PA
Study Dates: Oct. 15, 1995-June 3, 1996; Oct 31, 1995 -June 6, 1996; Jan. 18 - June 3, 1996; and Feb. 12, 1996 - June 3, 1996
Formulation and Lot No.: L-748,731-000R009; [redacted]
Certificate of Analysis Submitted: No (X) Assayed for concentration and stability that were within acceptable limits, according to the Sponsor
Final Report (X) Aug. 9, 1996
GLP and QA Statements Signed: Yes (X) Objective: "To determine if L-748,731 induced mutation, measured as resistance to 6-thioguanine, at the *hpt* locus in V-79 Chinese hamster lung cells".

Test Material/ Group Designation	Doses Scored* mM/flask		Replicates
Negative control - MEM-E	-		Duplicates
Solvent control - DMSO	-		
Positive control with activation - 3MC	0.0373		
Positive control without activation - MNU	0.1		
Test compound - L-748,731	With S9	Without S9	
	0.05	0.05	
	0.08	0.07	
	0.12	0.09	
	0.15	0.11	

V-79 Chinese hamster lung fibroblasts, 0.984 - 1.59 X 10^7 cells recovered at time of treatment
*Concentrations tested in range finding study 0.050, 0.100, 0.150, 0.200, and 0.300 mM with and without S-9, selected based on solubility results in CHO cytogenetics assay, TT #94-8626

Cells were exposed X 3hours, washed, suspended in fresh media, and incubated at 37° for 6 days for cytotoxicity.

Parameter Evaluated*	Timing
Cytotoxicity	Exposed to treatment on Day 0 and subcultured during an expression period of 9 days, stained 6 days after seeding
Plating efficiency - 3 plates - absolute [mean colony/plate + 300] and relative [mean colony/plate + mean of negative controls]	Exposed to treatment on Day 0 and subcultured during an expression period of 9 days, stained 6 days after seeding
Selection -9 plates - 6-TG resistant colonies/plate, total resistant colonies, mutant fraction	Exposed to treatment on Day 0 and subcultured during an expression period of 9 days, stained 6 days after seeding

*Criteria for a positive as defined by the Sponsor -i. a statistically significant trend "at a dose level less than or equal to the top dose tested" and

-ii. an induced mutant fraction [IMF = mean mutant fraction-mean concurrent negative control mutant fraction] outside the 95% confidence interval of historical controls.

Results -

Precipitation - With S-9 - Dose Range Finding Study: present after 3 hours at ≥0.2 mM
- Without S-9 - Dose Range Finding Study: present after 3 hours at ≥0.15 mM

Cytotoxicity [Relative Survival] - With S-9 - Ranged from a relative survival of 78-26% [TT #9-8506]; 72-55% [TT #96-8501]; 93-36% [TT #96-8503] over dose range

- Without S-9 - Ranged from a relative survival of 100-89% [TT #9-8506]; 95-62% [TT #96-8503] over dose range

The Sponsor indicates that doses were selected based on solubility limitations. According to ICH guidelines, the concentrations of drug selected for use in mammalian cell mutation tests should result, ideally, in toxicity that is at least 80%. If solubility factors limit the concentrations that can be achieved, then the lowest precipitating concentration should be used.

All dose levels were considered scorable [e.g. $\geq 10^6$ surviving cells].

Plating efficiency - **With S-9** - Dose Range Finding Study: 93-45% at 0.05 to 0.3 mM
- **Without S-9** - Dose Range Finding Study: 107-89% at 0.05 to 0.3 mM

Mutagenesis - The IMF was within the 95% confidence level for historical controls and there was no statistically significant trend towards an increase in mutagenesis for any of the studies. Although the IMF for the 3MC was significantly different from the negative controls in Study TT #9-8506, it was considered low and a repeat of the study using S-9 activation was conducted [Study TT #96-8501 and TT #96-8503]. According to the Sponsor, it was later determined that degraded 3-MC was the source of the low positive response, and, therefore, it was felt that the results for L-748,731 were valid and are included. The positive controls, with this exception, increased IMF sufficiently with statistically different values from concurrent negative controls.

Reviewer's Comment -Study Design and Data Presentation - The Sponsor indicates that doses were selected based on solubility limitations using the highest dose evaluated without precipitation. According to ICH guidelines [Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals], the concentrations of drug selected should result, ideally, in toxicity that is at least 80%. If solubility factors limit the concentrations that can be achieved, then, "if no cytotoxicity is observed, then the lowest precipitating concentration should be used as the top concentration." Neither criterion was met; however, this study was conducted prior to the issuance of the guideline in April, 1996. However, the maximum concentrations selected are only slightly less than the precipitating doses with or without metabolic activation. Cytotoxicity with metabolic activation ranged from 45-75%. Cytotoxicity for L-748,731 without metabolic activation ranged from only 11- 38%.

Sponsor's Conclusions (numbered) and Reviewer's Comments

1. L-748,731 was negative in the *in vitro* assay for mutations in Chinese hamster lung cells at all concentrations tested with and without S-9. **Reviewer's Comment** - The Reviewer concurs that under these experimental conditions, L-748,731 was negative for mutagenesis.

5.1.2 Clastogenicity

The previous Pharmacology/Toxicology Reviewer, Dr. Will Coulter, reviewed the following study. Additional comments by the current Reviewer are in italics.

5.1.2.i. Chromosomal Aberrations *in Vitro*, in Chinese Hamster Ovary Cells: [Vol. 1.32; D-267]

Study Nos.: TT #95-8626 (range finding cytotoxicity study)
TT #94-8627 (chromosomal aberration study)
Compound: L-748,731-000R-009
Cell Type: Chinese hamster ovary cells, sub clone of WBL; designated CHO-WBL, at less than 15 passages since cloning
S-9 Source: β-naphthoflavone with phenobarbital treated rat liver
Positive Control: cyclophosphamide with S-9 (solution in water)
Mitomycin C without S-9 (solution in water)
Negative Control: McCoy's 5A medium
Solvent Control: DMSO
Study Site: Merck, West Point, PA

Date: August 1994
GLP/QAU Statements: Both present and signed

The high dose was limited by its solubility and does not include dose levels that produced visible precipitates. The top dose is also limited to that dose estimated to give up to about a 50% reduction in cell growth and/or substantial reduction in mitotic index. The test was considered positive if there was a statistically significant increase ($p \leq 0.05$) over the concurrent controls in the percentages of cells with chromosomal aberrations at two separate concentrations of the drug and less than 50% cytotoxicity. If the assay is positive with or without S-9, the assay is considered positive. Two hundred cells were scored with each concentration of L-748,731.

Results

In the range finding cytotoxicity and solubility study (TT #94-8626), doses ranged from 0.625 to 100 µM with S-9 and 0.625 to 160 µM without S-9. No mitotic reduction or reduction in cell counts occurred with or without S-9 at 7 or 24 hours.

In the chromosomal aberration study (TT #94-8627) there were no significant increases in the percent of aberrant cells at 25 to 125 µM with S-9 or 25 to 100 µM without S-9 activation. The top doses were not scored due to a precipitate. Both cyclophosphamide and mitomycin C produced significant increases ($p \leq 0.05$ to ≤ 0.01), as expected.

In conclusion, this *in vitro* assay for the chromosomal aberrations in CHO cells was negative at the concentrations used and under the conditions in which the assay was conducted.

The current Pharmacology/Toxicology Reviewer reviewed the following studies.

5.1.2.ii. L-748,731: Assay for Chromosomal Aberrations *In Vitro*, in Chinese Hamster Ovary Cells [Vol. 1.32: D-327]
Study Identification: TT #96-9604
Site: Merck Research laboratories, West Point, PA
Study Dates: Jan 23, 1996-May 9, 1996
Formulation and Lot No.: L-748,731-000R009; [redacted]
Certificate of Analysis Submitted: No (X)
Final Report (X) May 29, 1996
GLP and QA Statements Signed: Yes (X)
Objective: "To determine if L-748,731 induced chromosomal aberrations in the Chinese hamster ovary cell line".

Test Material/ Group Designation	Doses Scored* µmol/flask	
Negative control - McCoy's 5A medium	-	
Solvent control - DMSO	-	
Positive control with activation - CPS	25 and 50	
Positive control without activation - mitomycin C	3.5 and 7.5	
Test compound - L-748,731	with S-9	without S-9
	75	25
	100	50
	125	100

CHO-WBL subclone 15 passages from cloning; 1.2 X 10^5 cells/10 ml McCoy's 5A medium [supplemented] on day prior to treatment
*Doses tested 12.5, 25, 50, 75, 100, 125, 150, 175, and 200 µM with S-9, 12.5, 25, 50, 100 and 200 µM without S-9

Cells were treated X 3hours, washed, suspended in fresh media, incubated at 37° for 17 hours, and then harvested. Colcemid was added to arrest mitosis app. 2-3 hours prior to harvest.

Parameter Evaluated	Positive Test [As defined by the Sponsor]
Total number of cells/flask - % of control	"Statistically significant increase over concurrent controls in the percentages of cells with chromosomal aberrations at two separate concentrations of test article, with less than 50% cytotoxicity" Considered positive if positive results are obtained with or without activation
Chromosomal aberration - Giemsa staining,	

Results -

Precipitation - With S-9 - Equivocal precipitation at 125 µM and definite precipitation at ≥150 µM.
Without S-9 - Precipitation at ≥150 µM.

Cytotoxicity - With S-9 – No concentration of drug resulted in a significant reduction in cell count or monolayer confluence.

Without S-9 - No concentration of drug resulted in a significant reduction in cell count or monolayer confluence.

Chromosomal Aberrations - The maximum concentration evaluated was limited by solubility. Positive controls significantly increased the number of aberrations observed when compared to vehicle controls. L-748,731 did not significantly alter the number of aberrations observed when compared to controls. The table below delineates the findings in this study.

20 Hour Cytotoxicity and Aberration Report Summary

Treatment	Cell Counts (% Controls)	% Aberrant Cells	Frequency Abs per 100 Cells[a]
With S-9 Activation			
Medium		1.5	1.5
DMSO		2.0	2.0
CP (2.5 µM)	73	14.0**	17.5
CP (5.0 µM)	68	45.0**	70.0
L-748,731			
12.5 µM	105	ns	
25 µM	94	ns	
50 µM	108	ns	
75 µM	107	1.5	1.5
100 µM	113	3.0	3.0
125 µM	110	1.5	1.5
150 µM	102	ns[b]	
175 µM	112	ns[b]	
200 µM	100	ns[b]	
Without S-9 Activation			
Medium		1.5	1.5
DMSO		2.0	2.5
MMC (0.35 µM)	86	6.5**	7.0
MMC (0.75 µM)	81	20.0**	24.0
L-748,731			
12.5 µM	105	ns	
25 µM	106	1.5	1.5
50 µM	106	2.5	2.5
100 µM	101	1.5	1.5
150 µM	99	ns[b]	
200 µM	98	ns[b]	

DMSO = Dimethyl sulfoxide
Cyclophosphamide (CP) and Mitomycin C (MMC) are positive controls.
a = The total number of aberrations per 100 cells, since a cell may have more than one aberration.
b = Slight precipitate was observed during treatment.
ns = Not scored.
* $P \leq 0.05$, ** $P \leq 0.01$ compared to the relevant control group using a one-sided Fisher's Exact Test. Since several comparisons with a common control were made, an adjustment procedure of Dunnett was used to assess the overall significance of each comparison for doses of test compound.

Reviewer's Comment -Study Design and Data Presentation – ICH guidelines [Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals} states that "[I]f no cytotoxicity is observed, then the lowest precipitating concentration should be used as the top concentrations but not exceeding...10 mM for mammalian cell tests." The Sponsor scored the maximum concentration evaluated that did not result in precipitation. However, these studies were conducted prior to the issuance of this guidance in April 1996.

Sponsor's Conclusions (numbered) and Reviewer's Comments

1. "L-748,731 was negative in the *in vitro* assay for chromosomal aberrations in CHO cells at all concentrations tested with and without S-9. This result confirms the conclusion of the previous *in vitro* chromosomal aberration assay, TT #94-8627." **Reviewer's Comment** - The Reviewer concurs.

The previous Pharmacology/Toxicology Reviewer, Dr. Will Coulter, reviewed the following studies. Additional comments by the current Reviewer are in italics.

5.1.2.iii. *In Vitro* alkaline Elution/Rat Hepatocyte Assay: *[Vol. 1.32; p. D-10]*
Study Nos.: TT #94-8209 (range finding)
TT #94-8220 and TT #94-8221 (alkaline elution)
Compound: L-748,731-000R009
Formulation: Solution in DMSO
Cell Type: Primary rat hepatocytes
Crl:CD®(SD)BR, Sprague-Dawley, males
Duration of Exposure: 3 hr
Negative Control: DMSO 1%
Positive Control: Aflatoxin B_1 in DMSO; 1 µM final concentration
Radiation Positive Control: Following harvest, untreated cell suspensions were irradiated with 3 Gy of gamma radiation
Study Site: Merck, West Point, PA
Date: August 16, 1994
GLP/QAU Statements: Both present and signed.

These studies were conducted to determined whether L-748,731 induces DNA strand breaks without concomitant induction of cytotoxicity in primary rat hepatocytes dosed *in vitro*.

In the alkaline elution assay (#94-8220), the hepatocyte viability was 85% at cell isolation, the concentrations tested were 10, 30, 70, 100, 125, and 150 µM, and the plated cells (2.2×10^6) were incubated 3 hr at 37° C in an atmosphere of 5% CO_2 in air. Cytotoxicity was determined o each sample by trypan blue exclusion and by cellular ATP content. The criteria for a positive result were: a) the induced elution slope (treatment slope (-) negative control slope) ≥0.034, which is a minimum biologically significant increase and b) slopes ≥ 0.034 should not be associated with significant cytotoxicity. Slopes are measured from 3 to 9 hours. Cytotoxicity was defined as significant when cell viability, measured by trypan blue dye exclusion, is 0% of control values and/or when cellular ATP content is 50% of control levels.

Results and Discussion

The results of the range finding study (TT #94-8209) indicated induced toxicity was limited to concentrations ≥ 150 µM. The drug was insoluble at 200 µM, the highest concentration tested. At that dose cell blebbing occurred. Cell blebbing was also seen at 150 µM, a concentration that was said to be just soluble. Hepatocyte viability at cell isolation was 85%. Range-finding results are indicated in the following table: