

IN VITRO MAMMALIAN CHROMOSOME ABERRATION WITH DIDECYLAMINE HYDROCHLORIDE

Study Title: In Vitro Mammalian Chromosome Aberration Test
Study No: GT-0073-TX-2
Study Type: Cytogenetics assay
Volume #, Page #: Vol. 1.18 (Appendix 5-28)
Conducting Laboratory: _____
Study Initiation Date: March 24, 1999
Study Completion Date: June 21, 1999
GLP Compliance: Yes
QA- Reports: Yes (x) No ()
Drug Lot Number: 154-171
Study Endpoint: *In vitro* clastogenesis

METHODOLOGY

Cell line: Chinese Hamster Ovary cells (CHO cells)
Vehicle control (solvent): Ethanol
Negative Controls: Ethanol
Positive Controls: Mitomycin C (MMC) (non-activated assay); Cyclophosphamide (activated assay)
Preparation of test article: Test article (didecylamine HCL) was received from Sponsor and was dissolved in ethanol.
Doses used in assays: Preliminary toxicity tests to determine cell growth inhibition were performed to select the dose levels to be tested in the aberration assays. Maximum dose in these tests was 4000 ug/ml. Concurrent toxicity tests with the selected dose levels were performed to determine the dose levels to be tested in the definitive chromosome aberrations assay.
Metabolic activation system: Aroclor 1254-induced rat liver S9
Aberration assay method: Duplicate cultures of CHO cells were exposed to test article, positive control or solvent alone by adding 500 ul of dosing solution to 4.5 ml cell medium with/out S-9 mixture.
Cell exposure: Cells in non-activated assay were exposed to extract for 4h or continuously for 20h. Colcemid was added to duplicate flasks (0.3 ug/ml) and flasks returned until cell collection. Cells in S-9 activated study were exposed for 4h, washed, returned to incubator and treated with Colcemid two hours before collection.
Cell collection: Two h after colcemid addition, metaphase cells were harvested by _____ Cells were collected approximately 20h after treatment initiation, fixed, and mounted on slides.

ANALYSIS:

Scoring method: Mitotic index was determined for each group. A minimum of 200 metaphase spreads (100 per duplicate flask) were scored for chromatid-type and chromosome-type aberrations. If a positive result was obtained in the non-activated 4h exposure group, the 20h group was not evaluated for aberrations.
Cytotoxic endpoints: Cell growth inhibition
Genetic toxicity endpoints: Number and types of aberrations/100 cells, % of structurally and numerically damaged cells, mean aberrations per cell. Gaps are not included in the % of cells with aberrations or in the frequency of structural aberrations/cell.

Statistical methods: Fisher's exact test. Test was used to compare pairwise the % aberrant cells between treatment and control groups. When the Fisher test was positive at any dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

Criteria for Positive Results: A positive response is defined as a dose-responsive increase in the % of cells with aberrations, with one or more concentrations being statistically significant.

Criteria for Valid Test: (1) Frequency of cells with structural chromosome aberrations in the extraction blank control in the range of the historical negative control. (2) Percentage of cells with aberrations in the positive control statistically increased ($p \leq 0.05$, Fisher's test) relative to extraction blank control.

RESULTS:

Precipitate/Osmolality

Test article was soluble in treatment medium at all concentrations tested. Osmolality of treatment medium of the highest concentrations tested (12 ug/ml) was 299 mmol/kg. Osmolality of the solvent (ethanol) in treatment medium was 330 mmol/kg. The pH of the highest concentration treatment medium was ca. 7.5.

Preliminary toxicity tests

Based upon the results of these toxicity tests the dose levels selected for testing in the aberration assay were as follows:

Treatment Condition	Treatment Time	Recovery Time	Dose Levels (ug/ml)
-S9	4h	16h	0.25, 0.5, 1, 2, 3, 4
-S9	20h	0h	0.06, 0.13, 0.25, 0.5, 1, 2, 4
+S9	4h	16h	1, 2, 4, 6, 8, 10, 12

Aberration test results

The following Tables (Tables 5, 7, 9) show the results of the three definitive aberration assays:

Table 5: Without metabolic activation, 4h treatment, 16h recovery

Table 7: With metabolic activation, 4h treatment, 16h recovery

Table 9: Without metabolic activation, 20h treatment

Table 10 summarizes the results.

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TABLE 5
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Bifedylamine IN THE
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment ^{1,2}	Flask	Mitotic Index ²	Cells Scored	Cells with Aberrations ³		Number of Structural Aberrations ⁴					Severely Damaged Cells ⁵	Average Structural Aberrations Per Cell ^{1,7}	
				Numerical	Structural	Chromatid-type Gaps	Breaks	Exch	Chromosome-type Breaks	Dic			Ring
Ethanol	A	11.6	100	3	1	0	0	0	1	0	0	0	0.010 0.000
	B	10.4	100	2	0	1	0	0	0	0	0	0	
Bifedylamine 0.5 µg/ml	A	8.0	100	4	1	0	1	0	0	0	0	0	0.010 0.000
	B	6.8	100	3	0	0	0	0	0	0	0	0	
1 µg/ml	A	9.4	100	4	0	3	0	0	0	0	0	0	0.000 0.010
	B	11.8	100	4	1	0	1	0	0	0	0	0	
2 µg/ml	A	6.0	100	5	1	1	0	0	0	1	0	0	0.010 0.020
	B	5.4	100	6	2	2	1	0	0	1	0	0	
MMC, 0.05 µg/ml	A	10.2	100	3	13	0	7	4	0	2	0	0	0.130 0.150
	B	8.8	100	5	12	2	7	6	1	1	0	0	

- ¹ CHO cells were treated for 4 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
 - ² Mitotic index = number mitotic figures x 100/500 cells counted.
 - ³ Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.
 - ⁴ Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
 - ⁵ Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
 - ⁶ Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
 - ⁷ Severely damaged cells and pulverizations were counted as 10 aberrations.
- An additional dose level of 0.25 µg/ml was tested as a safeguard against excessive toxicity at higher dose levels but was not required for microscopic examination. Dose levels 3 and 4 µg/ml were not analyzed due to excessive toxicity.

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TABLE 7
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Didecylamine IN THE
PRESENCE OF EXOGENOUS METABOLIC ACTIVATION
4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment ^{1,2}	Flask	Mitotic Index	Cells Scored	Cells with Aberrations ³ (%)		Number of Structural Aberrations						Severely Damaged Cells ⁴	Average Structural Aberrations Per Cell ⁵
				Numerical	Structural	Chromatid-type Gaps	Chromatid-type Breaks	Exchange	Breaks	Dic	Ring		
Ethanol	A	12.8	100	5	1	0	0	0	0	1	0	0	0.010
	B	12.6	100	4	0	1	0	0	0	0	0	0	0.000
Didecylamine 6 µg/mL	A	12.4	100	7	2	0	1	0	0	0	1	0	0.020
	B	11.2	100	5	1	0	0	0	0	0	1	0	0.010
8 µg/mL	A	9.2	100	5	1	1	0	1	0	0	0	0	0.010
	B	8.4	100	8	2	0	1	0	0	1	0	0	0.020
10 µg/mL	A	7.4	100	6	3	0	1	1	0	0	1	0	0.030
	B	6.0	100	5	2	0	0	0	0	2	0	0	0.020
CP, 10 µg/mL	A	5.2	100	5	15	3	7	10	0	3	1	0	0.210
	B	4.6	100	4	14	0	8	10	1	2	0	0	0.210

- ¹ CHO cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
- ² Mitotic index = number mitotic figures x 100/500 cells counted.
- ³ Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.
- ⁴ Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exchange) include quadriradials, triradials and complex rearrangements.
- ⁵ Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
- ⁶ Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
- ⁷ Severely damaged cells and pulverizations were counted as 10 aberrations.
- ⁸ Additional dose levels of 1, 2, and 4 µg/mL were tested as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination. Dose level 12 µg/mL was not analyzed due to excessive toxicity.

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TABLE 9
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Bidecylamine IN THE
ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR CONTINUOUS TREATMENT

Treatment ^{1,2}	Flask	Mitotic Index ²	Cells Scored	Cells with Aberrations ³		Number of Structural Aberrations ⁴						Severely Damaged Cells ⁵	Average Structural Aberrations Per Cell ⁷
				Numerical		Chromatid-type		Chromosome-type					
						Gaps	Breaks	Exch	Breaks	Dic	Ring		
Ethanol	A	6.6	100	2	1	0	0	0	1	0	0	0	0.010
	B	7.4	100	2	0	0	0	0	0	0	0	0	0.000
Bidecylamine 0.25 µg/mL	A	7.6	100	4	1	1	0	0	2	0	0	0	0.020
	B	7.2	100	4	1	0	0	0	0	0	1	0	0.010
0.5 µg/mL	A	6.8	100	5	2	0	2	0	0	0	0	0	0.020
	B	6.0	100	4	0	0	0	0	0	0	0	0	0.000
1 µg/mL	A	3.2	100	5	3	0	0	2	0	1	0	0	0.030
	B	4.0	100	3	0	1	0	0	0	0	0	0	0.000
BMC, 0.05 µg/mL	A	7.2	100	5	11	0	7	1	3	3	0	0	0.140
	B	6.8	100	3	11	0	5	5	7	0	1	0	0.180

- ¹ CHO cells were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
- ² Mitotic index = number mitotic figures x 100/500 cells counted.
- ³ Numerical: includes polyploid and endoreduplicated cells.; Structural: includes cells with only gaps.
- ⁴ Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
- ⁵ Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
- ⁶ Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
- ⁷ Severely damaged cells and pulverizations were counted as 10 aberrations.
- ⁸ Additional dose levels of 0.06 and 0.13 µg/mL were tested as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination. Dose levels 2 and 4 µg/mL were not analyzed due to excessive toxicity.

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TABLE 10

SUMMARY

Treatment	S ⁹ Activation	Treatment Time ¹ (Hours)	Mitotic Index	Cells Scored	Aberrations Per Cell ² (Mean ± SD)	Cells With Aberrations ³ (%)	
						Numerical	Structural
Ethanol	-	4	11.0	200	0.005 ± 0.071	2.5	0.5
Didcylamine							
0.5 µg/mL	-	4	7.4	200	0.005 ± 0.071	3.5	0.5
1 µg/mL	-	4	10.7	200	0.005 ± 0.071	6.0	0.5
2 µg/mL	-	4	5.7	200	0.015 ± 0.122	5.5	1.5
MNC, 0.08 µg/mL	-	4	9.5	200	0.140 ± 0.402	6.0	12.5**
Ethanol	+	4	12.7	200	0.005 ± 0.071	4.5	0.5
Didcylamine							
6 µg/mL	+	4	11.8	200	0.015 ± 0.122	6.0	1.5
8 µg/mL	+	4	8.8	200	0.015 ± 0.122	6.5	1.5
10 µg/mL	+	4	7.7	200	0.025 ± 0.157	5.5	2.5
CP, 10 µg/mL	+	4	4.9	200	0.210 ± 0.581	4.5	14.5**
Ethanol	-	20	7.0	200	0.005 ± 0.071	2.0	0.5
Didcylamine							
0.25 µg/mL	-	20	7.4	200	0.015 ± 0.138	4.0	1.0
0.5 µg/mL	-	20	6.4	200	0.010 ± 0.100	4.5	1.0
1 µg/mL	-	20	3.6	200	0.015 ± 0.122	4.0	1.5
MNC, 0.08 µg/mL	-	20	7.0	200	0.160 ± 0.515	4.0	11.0**

¹ Cells from all treatment conditions were harvested at 20 hours after the initiation of the treatments.

² Severely damaged cells were counted as 10 aberrations.

³ *, p<0.05; **, p<0.01; Fisher's exact test.

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Study Outcome:

- (4) Assay without metabolic activation, 4h treatment, 16h recovery: Mitotic index at the highest dose level evaluated of 2 ug./ml was 48% reduced as compared to vehicle control. There was no statistically significant elevation in the percentage of cells with structural or numerical chromosome aberrations.
- (5) Assay with metabolic activation, 4h treatment, 16h recovery: Growth inhibition was 63% at 13ug/ml. Mitotic index at the highest dose level of 13ug./ml was 80% reduced as compared to the vehicle control. There was no statistically significant elevation in the percentage of cells with structural or numerical chromosome aberrations.
- (6) Assay without metabolic activation, 20h treatment: Mitotic index at the highest dose level of 10 ug./ml was 39% reduced as compared to vehicle control. There was no statistically significant elevation in the percentage of cells with structural or numerical chromosome aberrations.

Study Validity:

The study was valid:

- (6) The frequencies of cells with structural chromosome aberrations in the vehicle controls were in the range of the historical negative controls.
- (7) All three positive controls (MMC in the two assays without metabolic activation; CP in the assay with metabolic activation) showed a significant elevation in the percentage of cells with structural chromosome aberrations.
- (8) For evaluation of numerical aberrations, this study appears to be inadequate. The positive controls did not induce significant increases in the % of cells with these types of aberrations.

SUMMARY:

Under the conditions of the assay (4h treatment, 16h recovery), the chromosome aberration test with didecylamine HCl in CHO cells without metabolic activation was negative.

Under the conditions of the assay (20h treatment), the chromosome aberration test with decylamine HCl in CHO cells without metabolic activation was negative.

Under the conditions of the assay, the chromosome aberration test with didecylamine HCl in CHO cells with metabolic activation was negative.

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IN VITRO MAMMALIAN CHROMOSOME ABERRATION WITH AMINOHEXYLTRIMETHYL AMMONIUM CHLORIDE HYDROCHLORIDE

Study Title: In Vitro Mammalian Chromosome Aberration Test
Study No: GT-0070-TX-2
Study Type: Cytogenetics assay
Volume #, Page #: Vol. 1.18 (Appendix 5-27)
Conducting Laboratory: _____
Study Initiation Date: February 18, 1999
Study Completion Date: May 27, 1999
GLP Compliance: Yes
QA- Reports: Yes (x) No ()
Drug Lot Number: 0171-251
Study Endpoint: *In vitro* clastogenesis

METHODOLOGY

Cell line: Chinese Hamster Ovary cells (CHO cells)
Vehicle control (solvent): Distilled water
Negative Controls: Water
Positive Controls: Mitomycin C (MMC) (non-activated assay); Cyclophosphamide (activated assay)
Preparation of test article: Test article was received from Sponsor and was dissolved in water.
Doses used in assays: Preliminary toxicity tests to determine cell growth inhibition were performed to select the dose levels to be tested in the aberration assays. Maximum dose in these tests was 5000 ug/ml. There was no inhibition of cell growth under any condition. Concurrent toxicity tests with five selected dose levels were then performed to determine the dose levels to be tested in the definitive chromosome aberrations assays.
Metabolic activation system: Aroclor 1254-induced rat liver S9
Aberration assay method: Duplicate cultures of CHO cells were exposed to test article, positive control or solvent alone by adding 500 ul of dosing solution to 4.5 ml cell medium with/out S-9 mixture.
Cell exposure: Cells in non-activated assay were exposed to extract for 4h or continuously for 20h. Colcemid was added to duplicate flasks (0.4 ug/ml) and flasks returned until cell collection. Cells in S-9 activated study were exposed for 4h, washed, returned to incubator and treated with Colcemid two hours before collection.
Cell collection: Two h after colcemid addition, metaphase cells were harvested by _____ Cells were collected approximately 20h after treatment initiation, fixed, and mounted on slides.

ANALYSIS:

Scoring method: Mitotic index was determined for each group. A minimum of 200 metaphase spreads (100 per duplicate flask) were scored for chromatid-type and chromosome-type aberrations. If a positive result was obtained in the non-activated 4h exposure group, the 20h group was not evaluated for aberrations.
Cytotoxic endpoints: Cell growth inhibition
Genetic toxicity endpoints: Number and types of aberrations/100 cells, % of structurally and numerically damaged cells, mean aberrations per cell. Gaps are not included in the % of cells with aberrations or in the frequency

Statistical methods:

of structural aberrations/cell.

Fisher's exact test. Test was used to compare pairwise the % aberrant cells between treatment and control groups. When the Fisher test was positive at any dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

Criteria for Positive Results:

A positive response is defined as a dose-responsive increase in the % of cells with aberrations, with one or more concentrations being statistically significant.

Criteria for Valid Test:

(1) Frequency of cells with structural chromosome aberrations in the extraction blank control in the range of the historical negative control. (2) Percentage of cells with aberrations in the positive control statistically increased ($p \leq 0.05$, Fisher's test) relative to extraction blank control.

RESULTS:

Precipitate/Osmolality

Test article was soluble in treatment medium at all concentrations tested. Osmolality of treatment medium of the highest concentrations tested (5000 ug/ml) was 344 mmol/kg. Osmolality of the solvent (water) in treatment medium was 297 mmol/kg. The pH of the highest concentration treatment medium was ca. 7.0.

Preliminary toxicity tests

In the preliminary tests no substantial cell growth inhibition relative to solvent control was observed at any dose level in the non-activated and S9-activated exposure groups.

Aberration test results

The following Tables (Tables 5, 7, 9) show the results of the three definitive aberration assays:

Table 5: Without metabolic activation, 4h treatment, 16h recovery

Table 7: With metabolic activation, 4h treatment, 16h recovery

Table 9: Without metabolic activation, 20h treatment

Table 10 summarizes the results.

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TABLE 5

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Aminohexyltrimethyl ammonium chloride hydrochloride
IN THE ABSENCE OF ENDOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment ^{1,2}	Ploidy	Mitotic Index	Cells Scored	Cells with Aberrations ³ (%)		Number of Structural Aberrations						Average Structural Aberrations Per Cell ^{4,5}	
				Numerical	Structural	Chromatid-type Gaps	Chromatid-type Breaks	Chromosome-type Exch	Chromosome-type Breaks	Dic	Ring		Severely Damaged Cells ⁶
Water	A	9.4	100	6	0	0	0	0	0	0	0	0	0.000
	B	10.4	100	5	0	1	0	0	0	0	0	0	0.000
Aminohexyltrimethyl ammonium chloride hydrochloride													
1250 ug/ml	A	8.0	100	3	1	0	2	0	0	0	0	0	0.020
	B	7.4	100	4	1	0	0	0	0	1	0	0	0.010
2500 ug/ml	A	8.2	100	4	0	1	0	0	0	0	0	0	0.000
	B	7.8	100	5	1	0	1	0	0	0	0	0	0.010
5000 ug/ml	A	7.0	100	7	2	1	0	1	0	1	0	0	0.020
	B	6.4	100	8	3	1	2	1	2	0	0	0	0.050
MIC, 0.15 ug/ml	A	5.6	100	7	10	0	10	3	0	0	0	0	0.130
	B	7.4	100	4	13	0	8	5	3	0	0	0	0.160

- ¹ CHO cells were treated for 4 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
- ² Mitotic index = number mitotic figures x 100/500 cells counted.
- ³ Numerical: includes polyploid and underduplicated cells.; Structural: excludes cells with only gaps.
- ⁴ Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
- ⁵ Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
- ⁶ Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
- ⁷ Severely damaged cells and pulverizations were counted as 10 aberrations.
- ⁸ Additional dose levels of 315 and 625 ug/ml were tested as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination.

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TABLE 7

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH Aminohexyltrimethyl ammonium chloride hydrochloride
IN THE PRESENCE OF ENDOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment ^{1,2}	Flask	Mitotic Index ³	Cells Scored	Cells with Aberrations ⁴		Number of Structural Aberrations						Severely Damaged Cells ⁵	Average Structural Aberrations Per Cell ^{6,7}
				Numerical	Structural	Chromatid-type	Chromosome-type	Gaps	Breaks	Exch	Breaks		
Water	A	11.2	100	4	0	1	0	0	0	0	0	0	0.000
	B	9.0	100	7	0	2	0	0	0	0	0	0	0.000
Aminohexyltrimethyl ammonium chloride hydrochloride													
1250 ug/mL	A	10.8	100	6	0	0	0	0	0	0	0	0	0.000
	B	10.6	100	5	1	0	0	1	0	0	0	0	0.010
2500 ug/mL	A	9.6	100	3	2	2	3	0	0	0	0	0	0.030
	B	10.4	100	4	2	0	1	0	1	0	0	0	0.020
5000 ug/mL	A	8.0	100	5	2	0	0	0	0	1	1	0	0.020
	B	8.4	100	5	2	0	0	0	0	1	1	0	0.020
CP, 10 ug/mL	A	4.0	100	4	19	4	7	12	2	3	1	0	0.250
	B	5.6	100	5	14	1	12	12	0	2	2	0	0.200

¹ CHO cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.

² Mitotic index = number mitotic figures x 100/500 cells counted.

³ Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.

⁴ Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

⁵ Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

⁶ Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

⁷ Severely damaged cells and pulverizations were counted as 10 aberrations.

⁸ Additional dose levels of 313 and 625 ug/mL were tested as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination.

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TABLE 9

CYTOTHERETIC ANALYSIS OF CMO CELLS TREATED WITH Aminohexyltrimethyl ammonium chloride hydrochloride
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR CONTINUOUS TREATMENT

Treatment ^{1,2}	Flask	Mitotic Index ²	Cells Scored	Cells with Aberrations ³		Number of Structural Aberrations					Severely Damaged Cells ⁴	Average Structural Aberrations Per Cell ^{5,7}	
				(%)	Numerical	Structural	Chromatid-type Gaps	Breaks	Each	Chromosome-type Breaks			Dic Ring
Water	A	7.2	100	3	0	0	0	0	0	0	0	0	0.000
	B	11.0	100	4	0	1	0	0	0	0	0	0	0.000
Aminohexyltrimethyl ammonium chloride hydrochloride 1250 µg/mL	A	8.0	100	4	1	0	0	0	0	1	0	0	0.010
	B	11.2	100	3	0	2	0	0	0	0	0	0	0.000
2500 µg/mL	A	7.0	100	3	0	0	0	0	0	0	0	0	0.000
	B	7.4	100	3	0	1	0	0	0	0	0	0	0.000
5000 µg/mL	A	6.6	100	5	1	0	0	0	0	1	0	0	0.010
	B	7.4	100	4	2	0	1	0	0	1	0	0	0.020
MMC, 0.05 µg/mL	A	9.8	100	4	8	1	7	3	1	1	0	0	0.120
	B	8.6	100	3	10	1	5	4	0	1	0	0	0.100

- ¹ CMO cells were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
- ² Mitotic index = number mitotic figures x 100/500 cells counted.
- ³ Numerical: includes polyploid and aneuploid cells.; Structural: excludes cells with only gaps.
- ⁴ Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
- ⁵ Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
- ⁶ Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
- ⁷ Severely damaged cells and pulverizations were counted as 10 aberrations.
- ⁸ Additional dose levels of 313 and 625 µg/mL were tested as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination.

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TABLE 10

SUMMARY

Treatment	S9 Activation	Treatment ¹ Time (Hours)	Mitotic Index	Cells Scored	Aberrations Per Cell ² (Mean ± SD)	Cells With Aberrations ³ (%)	
						Numerical	Structural
Water	-	4	9.9	200	0.000 ± 0.000	5.5	0.0
Aminoheyltrimethyl ammonium chloride hydrochloride							
1250 ug/ml	-	4	7.7	200	0.015 ± 0.158	3.5	1.0
2500 ug/ml	-	4	8.0	200	0.005 ± 0.071	4.5	0.5
5000 ug/ml	-	4	6.8	200	0.035 ± 0.232	7.5	2.5*
MPC, 0.15 ug/ml	-	4	6.3	200	0.145 ± 0.442	5.5	11.3**
Water	+	4	10.1	200	0.000 ± 0.000	5.5	0.0
Aminoheyltrimethyl ammonium chloride hydrochloride							
1250 ug/ml	+	4	10.7	200	0.005 ± 0.071	5.5	0.5
2500 ug/ml	+	4	10.0	200	0.025 ± 0.186	3.5	2.0
5000 ug/ml	+	4	8.2	200	0.020 ± 0.140	5.0	2.0
CP, 10 ug/ml	+	4	4.8	200	0.265 ± 0.719	4.5	16.5**
Water	-	20	9.1	200	0.000 ± 0.000	3.5	0.0
Aminoheyltrimethyl ammonium chloride hydrochloride							
1250 ug/ml	-	20	9.6	200	0.005 ± 0.071	3.5	0.5
2500 ug/ml	-	20	7.2	200	0.000 ± 0.000	3.0	0.0
5000 ug/ml	-	20	7.0	200	0.015 ± 0.122	4.5	1.5
MPC, 0.08 ug/ml	-	20	9.2	200	0.110 ± 0.386	3.5	9.0**

¹ Cells from all treatment conditions were harvested at 20 hours after the initiation of the treatments.

² Severely damaged cells were counted as 10 aberrations.

³ *, p<0.05; **, p<0.01; Fisher's exact test.

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Study Outcome:

- (7) Assay without metabolic activation, 4h treatment, 16h recovery: Mitotic index at the highest dose level of 5000 ug./ml was 31% reduced as compared to vehicle control. There was a statistically significant elevation in the percentage of cells with structural chromosome aberrations at 5000 ug/ml ($p \leq 0.05$). The dose-response test for the % of cells with structural aberrations was also statistically significant ($p \leq 0.05$). There was no increase in the percentage of cells with numerical chromosome aberrations.

Reviewers Comment:

The Sponsor concluded that, since the % of structurally aberrant cells found at the 5000 ug/ml dose level (2.5%) in this study was within the range of structurally aberrant cells observed with the historical solvent control (0-6%, see APPENDIX), the statistically significant increase in the % of aberrant cells at this dose level was not considered biologically relevant. This is not in agreement with the Sponsor's own criteria for a positive result, and this Reviewer does not agree with their conclusion.

- (8) Assay with metabolic activation, 4h treatment, 16h recovery: Mitotic index at the highest dose level of 5000ug./ml was 19% reduced as compared to the vehicle control. There was no statistically significant elevation in the percentage of cells with structural or numerical chromosome aberrations.
- (9) Assay without metabolic activation, 20h treatment: Mitotic index at the highest dose level of 5000 ug./ml was 23% reduced as compared to vehicle control. There was a slight elevation in the percentage of cells with structural chromosome aberrations, but the effect was not statistically significant. There was no increase in the percentage of cells with numerical chromosome aberrations.

Study Validity:

The study was valid:

- (9) The frequencies of cells with structural chromosome aberrations in the vehicle controls were in the range of the historical negative controls.
- (10) All three positive controls (MMC in the two assays without metabolic activation; CP in the assay with metabolic activation) showed a significant elevation in the percentage of cells with structural chromosome aberrations.
- (11) For evaluation of numerical aberrations, this study appears to be inadequate. The positive controls did not induce significant increases in the % of cells with these types of aberrations.

SUMMARY:

Under the conditions of the assay (4h treatment, 16h recovery), the chromosome aberration test with aminohexyltrimethyl ammonium chloride hydrochloride in CHO cells without metabolic activation was positive.

Under the conditions of the assay (20h treatment), the chromosome aberration test with aminohexyltrimethyl ammonium chloride hydrochloride in CHO cells without metabolic activation was negative.

Under the conditions of the assay, the chromosome aberration test with aminohexyltrimethyl ammonium chloride hydrochloride in CHO cells with metabolic activation was negative.

APPENDIX

**IN VITRO MAMMALIAN CYTOGENETIC TEST USING
CHINESE HAMSTER OVARY (CHO) CELLS**

**HISTORICAL CONTROL VALUES
STRUCTURAL ABERRATIONS
1995-1997**

NON-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control ¹	Positive Control ²
Mean	1.1%	1.2%	27.5%
Standard Deviation	1.0%	1.3%	19.1%
Range	0.0% to 4.5%	0.0% to 6.0%	7.0% to 100.0%

S9-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control ¹	Positive Control ³
Mean	1.3%	1.5%	39.0%
Standard Deviation	1.2%	1.4%	22.8%
Range	0.0% to 5.5%	0.0% to 6.5%	6.5% to 100.0%

¹Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, non-standard solvents and Sponsor-supplied vehicles.

²Positive control for non-activated studies, triethylenemelamine (TEM, 0.25-0.5 µg/ml), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 µg/ml), and Mitomycin C (MMC, 0.08-0.15 µg/ml).

³Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 µg/ml), and benzo(α)pyrene, (B[α]P, 30 µg/ml).

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IN VITRO MAMMALIAN CHROMOSOME ABERRATION WITH 6-DECYLAMINO-HEXYLTRIMETHYL AMMONIUM CHLORIDE HYDROCHLORIDE

Study Title: In Vitro Mammalian Chromosome Aberration Test
Study No: GT-0071-TX-2
Study Type: Cytogenetics assay
Volume #, Page #: Vol. 1.18 (Appendix 5-29)
Conducting Laboratory: _____
Study Initiation Date: March 24, 1999
Study Completion Date: June 21, 1999
GLP Compliance: Yes
QA- Reports: Yes (x) No ()
Drug Lot Number: — 198-129
Study Endpoint: *In vitro* clastogenesis

METHODOLOGY

Cell line: Chinese Hamster Ovary cells (CHO cells)
Vehicle control (solvent): Distilled water
Negative Controls: Water
Positive Controls: Mitomycin C (MMC) (non-activated assay); Cyclophosphamide (activated assay)
Preparation of test article: Test article was received from Sponsor and was dissolved in water.
Doses used in assays: Preliminary toxicity tests to determine cell growth inhibition were performed to select the dose levels to be tested in the aberration assays. Maximum dose in these tests was 5000 ug/ml. Concurrent toxicity tests with seven selected dose levels were then performed to determine the dose levels to be tested in the definitive chromosome aberrations assays.
Metabolic activation system: Aroclor 1254-induced rat liver S9
Aberration assay method: Duplicate cultures of CHO cells were exposed to test article, positive control or solvent alone by adding 500 ul of dosing solution to 4.5 ml cell medium with/out S-9 mixture.
Cell exposure: Cells in non-activated assay were exposed to extract for 4h or continuously for 20h. Colcemid was added to duplicate flasks (0.5 ug/ml) and flasks returned until cell collection. Cells in S-9 activated study were exposed for 4h, washed, returned to incubator and treated with Colcemid two hours before collection.
Cell collection: Two h after colcemid addition, metaphase cells were harvested by _____ Cells were collected approximately 20h after treatment initiation, fixed, and mounted on slides.

ANALYSIS:

Scoring method: Mitotic index was determined for each group. A minimum of 200 metaphase spreads (100 per duplicate flask) were scored for chromatid-type and chromosome-type aberrations. If a positive result was obtained in the non-activated 4h exposure group, the 20h group was not evaluated for aberrations.

Cytotoxic endpoints:

Genetic toxicity endpoints: Cell growth inhibition
Number and types of aberrations/100 cells, % of structurally and numerically damaged cells, mean aberrations per cell. Gaps are not included in the % of cells with aberrations or in the frequency of structural aberrations/cell.

Statistical methods: Fisher's exact test. Test was used to compare pairwise the % aberrant cells between treatment and control groups. When the Fisher test was positive at any dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

Criteria for Positive Results: A positive response is defined as a dose-responsive increase in the % of cells with aberrations, with one or more concentrations being statistically significant.

Criteria for Valid Test: (1) Frequency of cells with structural chromosome aberrations in the extraction blank control in the range of the historical negative control. (2) Percentage of cells with aberrations in the positive control statistically increased ($p \leq 0.05$, Fisher's test) relative to extraction blank control.

RESULTS:

Precipitate/Osmolality

Test article was soluble in treatment medium at all concentrations tested. Visible precipitate was observed in treatment medium at all concentrations tested in the S9-activated group. Osmolality of treatment medium of the highest concentrations tested (4500 ug/ml) was 297 mmol/kg. Osmolality of the solvent (water) in treatment medium was 258 mmol/kg. The pH of the highest concentration treatment medium was ca. 7.0.

Preliminary toxicity tests

Based upon the results of these toxicity tests the dose levels selected for testing in the aberration assay were as follows:

Treatment Condition	Treatment Time	Recovery Time	Dose Levels (ug/ml)
-S9	4h	16h	125, 250, 500, 1000, 1500, 2000, 3000
-S9	20h	0h	1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500
+S9	4h	16h	25, 50, 100, 150, 200, 250, 300, 400

Aberration test results

The following Tables (Tables 5, 7, 9) show the results of the three definitive aberration assays:

Table 5: Without metabolic activation, 4h treatment, 16h recovery

Table 7: With metabolic activation, 4h treatment, 16h recovery

Table 9: Without metabolic activation, 20h treatment

Table 10 summarizes the results.

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TABLE 5

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH 6-decylaminoheptyltrimethyl ammonium chloride hydrochloride IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

4 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment ^{1,2}	Flock	Mitotic Index ³	Cells Scored	Cells With Aberrations ⁴		Number of Structural Aberrations					Severely Damaged Cells ⁵	Average Structural Aberrations Per Cell ^{6,7}	
				Numerical Structural		Chromatid-type	Chromosome-type		Chromatid-type	Chromosome-type			Chromosome-type
						Gaps	Breaks	Exch	Breaks	Dic	Ring		
Water	A	10.8	100	0	4	2	4	0	0	0	0	0	0.040
	B	9.8	100	0	3	0	3	0	0	0	0	0	0.030
6-decylaminoheptyltrimethyl ammonium chloride hydrochloride													
1000 µg/mL	A	7.2	100	3	3	1	3	0	0	0	0	0	0.030
	B	8.0	100	1	6	2	6	0	0	0	0	0	0.060
1500 µg/mL	A	5.6	100	3	5	1	5	0	0	0	0	0	0.050
	B	6.0	100	3	5	1	4	0	0	1	0	0	0.050
2000 µg/mL	A	5.0	100	4	6	1	7	0	0	0	0	0	0.070
	B	5.6	100	3	6	1	6	0	0	0	0	0	0.060
NAC, 0.15 µg/mL	A	9.6	180	2	11	1	9	3	0	0	0	0	0.120
	B	8.4	180	1	11	2	11	0	0	0	2	0	0.130

¹ CHO cells were treated for 4 hours at 37±1°C in the absence of an exogenous source of metabolic activation.

² Mitotic index = number mitotic figures x 100/500 cells counted.

³ Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.

⁴ Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

⁵ Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

⁶ Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

⁷ Severely damaged cells and pulverizations were counted as 10 aberrations.

⁸ Additional dose levels of 125, 250, and 500 µg/mL were tested as a safeguard against excessive toxicity at higher dose levels but were not required for microscopic examination. Dose level 3000 µg/mL was not analyzed due to excessive toxicity.

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TABLE 7

CYTGENETIC ANALYSIS OF CHO CELLS TREATED WITH 6-decylaminohexyltrimethyl ammonium chloride hydrochloride IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

6 HOUR TREATMENT, 16 HOUR RECOVERY PERIOD

Treatment ^{1,2}	Flask	Mitotic Index ²	Cells Scored	Cells with Aberrations ³		Number of Structural Aberrations					Severely Damaged Cells ⁴	Average Structural Aberrations Per Cell ^{5,6}	
				Numerical	Structural	Chromatid-type Gaps	Chromatid-type Breaks	Chromosome-type Exch	Chromosome-type Breaks	Dic			Ring
Water	A	5.8	100	0	2	1	2	0	0	0	0	0	0.020
	B	4.8	100	2	3	0	3	0	0	0	0	0	0.030
6-decylaminohexyltrimethyl ammonium chloride hydrochloride													
1000 µg/mL	A	4.0	100	3	3	2	3	0	0	0	0	0	0.030
	B	4.8	100	4	4	2	3	0	0	0	1	0	0.040
1500 µg/mL	A	5.8	100	2	6	0	6	8	0	0	0	0	0.060
	B	3.8	100	1	4	1	4	8	0	0	0	0	0.040
2000 µg/mL	A	3.8	100	2	6	4	5	1	0	0	0	0	0.060
	B	4.2	100	2	5	3	4	0	0	0	1	0	0.050
CP, 10 µg/mL	A	5.6	100	2	25	2	31	2	0	0	0	0	0.330
	B	5.0	100	2	25	2	26	5	0	0	0	0	0.310

¹ CHO cells were treated for 6 hours at 37±1°C in the presence of an exogenous source of metabolic activation.

² Mitotic index = number mitotic figures x 100/500 cells counted.

³ Numerical: includes polyploid and endoreduplicated cells.; Structural: excludes cells with only gaps.

⁴ Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.

⁵ Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.

⁶ Severely damaged cells include cells with one or more pulverized chromosomes and cells with 10 or more aberrations.

⁷ Severely damaged cells and pulverizations were counted as 10 aberrations.

⁸ Dose levels 2500, 3000, 3500, 4000, and 4500 µg/mL were not analyzed due to excessive toxicity.

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TABLE 9

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH 6-decylaminohexyltrimethyl ammonium chloride hydrochloride IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

20 HOUR CONTINUOUS TREATMENT

Treatment ^{1,2}	Flask	Mitotic Index ³	Cells Scored	Cells with Aberrations ⁴		Number of Structural Aberrations					Severely Damaged Cells ⁵	Average Structural Aberrations Per Cell ⁶	
				Numerical Structural		Chromatid-type		Chromosome-type					
						Gaps	Breaks	Exch	Breaks	Dic	Ring		
Water	A	8.4	100	1	3	0	3	0	0	0	0	0	0.030
	B	8.8	100	0	3	1	3	0	0	0	0	0	0.030
6-decylaminohexyltrimethyl ammonium chloride hydrochloride													
50 µg/ml	A	5.2	100	1	4	0	4	0	0	0	0	0	0.040
	B	5.8	100	1	5	1	5	0	0	0	0	0	0.050
100 µg/ml	A	7.8	100	2	4	1	5	0	0	0	0	0	0.050
	B	6.0	100	0	5	3	5	0	0	0	0	0	0.050
150 µg/ml	A	3.8	100	0	5	0	6	0	0	0	0	0	0.060
	B	5.6	100	1	5	0	3	1	0	1	0	0	0.050
MNC, 0.08 µg/ml	A	6.8	100	0	15	2	14	2	0	0	0	0	0.160
	B	8.0	100	0	14	3	14	2	0	0	0	0	0.160

- ¹ CHO cells were treated for 20 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
- ² Mitotic index = number mitotic figures x 100/500 cells counted.
- ³ Numerical: includes polyploid and endoreduplicated cells.; structural: excludes cells with only gaps.
- ⁴ Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures (Exch) include quadriradials, triradials and complex rearrangements.
- ⁵ Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
- ⁶ Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
- ⁷ Severely damaged cells and pulverizations were counted as 10 aberrations.
- ⁸ An additional dose level of 25 µg/ml was tested as a safeguard against excessive toxicity at higher dose levels but was not required for microscopic examination. Dose levels 200, 250, 300, and 400 µg/ml were not analyzed due to excessive toxicity.

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TABLE 10

SUMMARY

Treatment	99 Activation	Treatment ¹ Time (Hours)	Mitotic Index	Cells Scored	Aberrations Per Cell ² (Mean ± SD)	Cells With Aberrations ² (%)	
						Numerical	Structural
Water	-	4	10.3	200	0.035 ± 0.184	0.0	3.5
6-decylaminohexyltrimethyl ammonium chloride hydrochloride							
1000 µg/mL	-	4	7.6	200	0.045 ± 0.208	2.0	4.5
1500 µg/mL	-	4	5.8	200	0.050 ± 0.218	3.0*	5.0
2000 µg/mL	-	4	5.3	200	0.065 ± 0.267	3.5**	6.0
MNC, 0.15 µg/mL	-	4	9.0	200	0.125 ± 0.374	1.5	11.0**
Water	+	4	5.3	200	0.025 ± 0.157	1.0	2.5
6-decylaminohexyltrimethyl ammonium chloride hydrochloride							
1000 µg/mL	+	4	4.4	200	0.035 ± 0.184	3.5	3.5
1500 µg/mL	+	4	4.8	200	0.050 ± 0.218	1.5	5.0
2000 µg/mL	+	4	4.0	200	0.055 ± 0.229	2.0	5.5
CP, 10 µg/mL	+	4	5.3	200	0.320 ± 0.599	2.0	25.0**
Water	-	20	8.6	200	0.030 ± 0.171	0.5	3.0
6-decylaminohexyltrimethyl ammonium chloride hydrochloride							
50 µg/mL	-	20	5.5	200	0.055 ± 0.269	1.0	4.5
100 µg/mL	-	20	6.9	200	0.050 ± 0.240	1.0	4.5
150 µg/mL	-	20	4.7	200	0.055 ± 0.250	0.5	5.0
MNC, 0.08 µg/mL	-	20	7.4	200	0.160 ± 0.406	0.0	14.5**

¹ Cells from all treatment conditions were harvested at 20 hours after the initiation of the treatments.

² Severely damaged cells were counted as 10 aberrations.

³ *, p<0.05; **, p<0.01; Fisher's exact test.

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Study Outcome:

1. Assay without metabolic activation, 4h treatment, 16h recovery: Growth inhibition was 56% at 2000 ug/ml. Mitotic index at the highest dose level evaluated of 2000 ug./ml was 49% reduced as compared to vehicle control. There was no statistically significant elevation in the percentage of cells with structural chromosome aberrations ($p > 0.05$). There was a statistically significant elevation in the percentage of cells with numerical chromosome aberrations at 1500 and 2000 ug/ml ($p \leq 0.05$ and 0.01). The dose-response test for the % of cells with numerical aberrations was also positive ($p \leq 0.05$).

Reviewers Comment:

The Sponsor concluded that, since the % of numerically aberrant cells found at the 1500 and 2000 ug/ml dose levels (3 and 3.5%) were within the range of numerically aberrant cells observed with the historical solvent control (0-6.5%, see APPENDIX), the statistically significant increase in the % of aberrant cells at this dose level was not considered biologically relevant. In the opinion of this Reviewer, the significance of the increase in numerical aberrations in this test is unclear.

2. Assay with metabolic activation, 4h treatment, 16h recovery: Growth inhibition was 51% at 2000ug/ml. Mitotic index at the highest dose level of 2000g./ml was 25% reduced as compared to the vehicle control. There was no statistically significant elevation in the percentage of cells with structural or numerical chromosome aberrations.
3. Assay without metabolic activation, 20h treatment: Growth inhibition was 50% at 150 ug/ml. Mitotic index at the highest dose level of 150 ug./ml was 45% reduced as compared to vehicle control. There was no statistically significant elevation in the percentage of cells with structural or numerical chromosome aberrations.

Study Validity:

The study was valid:

1. The frequencies of cells with structural chromosome aberrations in the vehicle controls were in the range of the historical negative controls.
2. All three positive controls (MMC in the two assays without metabolic activation; CP in the assay with metabolic activation) showed a significant elevation in the percentage of cells with structural chromosome aberrations.
3. For evaluation of numerical aberrations, this study appears to be inadequate. The positive controls did not induce significant increases in the % of cells with these types of aberrations.

SUMMARY:

Under the conditions of the assay, the chromosome aberration test with 6-decylamino-hexyltrimethyl ammonium chloride hydrochloride in CHO cells without metabolic activation was negative.

Under the conditions of the assay, the chromosome aberration test with 6-decylamino-hexyltrimethyl ammonium chloride hydrochloride in CHO cells with metabolic activation was negative.

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APPENDIX

**IN VITRO MAMMALIAN CYTOGENETIC TEST USING
CHINESE HAMSTER OVARY (CHO) CELLS**

**HISTORICAL CONTROL VALUES
TOTAL NUMERICAL ABERRATIONS
1995-1997**

NON-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control ¹	Positive Control ²
Mean	1.6%	1.8%	2.6%
Standard Deviation	1.5%	1.4%	2.3%
Range	0.0% to 7.0%	0.0% to 6.5%	0.0% to 15.0%

S9-ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells		
	Untreated Control	Solvent Control ¹	Positive Control ³
Mean	1.6%	1.9%	3.2%
Standard Deviation	1.5%	1.4%	2.7%
Range	0.0% to 6.5%	0.0% to 6.5%	0.0% to 11.5%

¹Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, and other non-standard solvents and Sponsor-supplied vehicles.

²Positive control for non-activated studies, triethylenemelamine (TEM, 0.25-0.5 µg/ml), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 0.75-2 µg/ml), and Mitomycin C (MMC, 0.08-0.15 µg/ml).

³Positive control for S9-activated studies, cyclophosphamide (CP, 10-50 µg/ml), and benzo(α)pyrene (B[α]P, 30 µg/ml).

MICRONUCLEUS CYTOGENETIC ASSAY IN MICE WITH COLESEVELAM HYDROCHLORIDE

Study Title: Micronucleus cytogenetic assay in mice
Study No: GT-02-TX-26
Study Type: Cytogenetics assay
Volume #, Page #: Vol. 1.17 (Appendix 5-19)
Conducting Laboratory: _____
Study Initiation: November 25, 1996
Study Completion: March 17, 1997
GLP Compliance: Yes
QA- Reports: Yes (x) No ()
Drug Lot Number: CHOG9601-1391
Study Endpoint: *In vivo* damage to chromosomes or mitotic apparatus

METHODOLOGY

Species: ICR Mice, male and female
Vehicle control: Corn oil
Negative Controls: Corn oil, 20 ml/kg
Positive Controls: Cyclophosphamide (60 mg/kg/day)
Basis of dose selection: Pilot assay results
Doses used in pilot assay Male mice: 1, 10, 100, 1000, 5000 mg/kg/day
Female mice: 5000 mg/kg/day
Doses used in definitive assay: Male and female mice: 1250, 2500, 2500 mg/kg/day
Dose administration: Oral gavage (20 ml/kg)
Animals/groups: Pilot assay: 2 males/group for the four lower doses and 5/sex/group for the high dose.
Main assay: 10/sex/group, with an additional 5/sex/group for the high dose
Dosing schedule: Animals were dosed with vehicle or test article by oral gavage (20 ml/kg) with two administrations separated by about 24 hours
Incubation and sampling times: Main assay: Bone marrow cells were collected 24h and 48h after the second dose

ANALYSIS:

Slide preparation: 2 to 4 slides of femoral bone marrow cells were prepared for each mouse
Scoring method: In an acceptable area, 1000 PCE were scored for the presence of micronuclei. Also, the proportion of PCE to total NCE (normochromatic erythrocytes) per 1000 erythrocytes was recorded as an index of organ toxicity.
Cytotoxic endpoints: PCE/NCE ratio
Genetic toxicity endpoints: # Micronucleated PCE/1000 PCE. Micronuclei are small particles consisting of (fragments of) chromosomes that lag behind at the anaphase of cell division
Statistical methods: Kastenbaum-Bowman tables
Criteria for Positive Results: A positive response is defined as a treatment-related increase in micronucleated PCE (MNPCE), with one or more doses showing statistically significantly increase(s) relative to vehicle control at any sampling time.
Criteria for Valid Test: Vehicle control MNPCE/PCE ratio < 0.5%, and positive control significantly increased relative to vehicle control.

RESULTS:

Pilot assay:

Clinical signs were noted within two hours or later after the second dose administration, and included diarrhea, irregular breathing and distended abdomen in males at 5000 mg/kg/day and lethargy in males and females at 5000 mg/kg/day. No mortality occurred. Therefore, the high dose for the main assay was set at 5000 mg/kg/day.

Study outcome:

Mortality and signs

One male and one female HD animal were found dead after dosing and were replaced with animals from the replacement groups for bone marrow collection. One male not intended for marrow collection also died after dosing. Clinical signs were lethargy, distended abdomens at 5000 mg/kg/day.

Table 2
Clinical Signs Following Dose Administration of CholestaGel[®] (GT31-104NB)
Microinfectious Assay

Treatment	Clinical Observation	Number of Mice Affected/Total Number of Mice Dosed		Number of Mice Died/Total Number of Mice Dosed	
		Males	Females	Males	Females
Case oil, 20 ml/kg/day	Normal	10/10	10/10	0/10	0/10
CholestaGel [®] (GT31-104NB), 1250 mg/kg/day	Normal	10/10	10/10	0/10	0/10
CholestaGel [®] (GT31-104NB), 2500 mg/kg/day	Normal	10/10	10/10	0/10	0/10
CholestaGel [®] (GT31-104NB), 5000 mg/kg/day	Lethargy Distended abdomen	15/15 2/15	15/15 1/15	2/15	1/15
CP, 60 mg/kg/day	Normal	5/5	5/5	0/5	0/5

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Genetic toxicity

There was no statistically significant increase in the ratio of MNPCE/1000 PCE in any dose group at any collection time.

Reductions of 2-10% in the ratio of PCE/total erythrocytes were observed in some test article treated groups compared to vehicle controls, indicating slight bone marrow toxicity.

Table 3
Summary of Bone Marrow Micronucleus Study Using CholestaGel® (GT31-10488)

Treatment	Sex	Time (hr)	Number of Mice	PCE/Total Erythrocytes (Mean +/- sd)	Change From Control (%)	Micronucleated Polychromatic Erythrocytes Number per 1000 PCEs (Mean +/- sd)	Number per PCEs Scored
Corn oil 20 mL/kg/day	M	24	5	0.52 ± 0.04	---	1.0 ± 1.22	5 / 5000
	F	24	5	0.52 ± 0.03	---	0.8 ± 0.45	4 / 5000
CholestaGel® (GT31-10488) 1250 mg/kg/day	M	24	5	0.51 ± 0.03	-2	0.6 ± 0.89	3 / 5000
	F	24	5	0.56 ± 0.06	8	1.0 ± 1.22	5 / 5000
2500 mg/kg/day	M	24	5	0.49 ± 0.03	-6	0.6 ± 0.89	3 / 5000
	F	24	5	0.54 ± 0.05	4	1.2 ± 0.84	6 / 5000
5000 mg/kg/day	M	24	5	0.52 ± 0.05	0	1.2 ± 0.45	6 / 5000
	F	24	5	0.53 ± 0.02	2	1.4 ± 1.14	7 / 5000
CP ^a 60 mg/kg/day	M	24	5	0.25 ± 0.03	-52	31.2 ± 14.50	*156 / 5000
	F	24	5	0.29 ± 0.06	-44	24.4 ± 4.16	*122 / 5000
Corn oil 20 mL/kg/day	M	48	5	0.53 ± 0.08	---	0.6 ± 0.89	3 / 5000
	F	48	5	0.58 ± 0.03	---	0.4 ± 0.55	2 / 5000
CholestaGel® (GT31-10488) 1250 mg/kg/day	M	48	5	0.56 ± 0.03	2	0.2 ± 0.45	1 / 5000
	F	48	5	0.55 ± 0.05	-5	0.6 ± 0.55	3 / 5000
2500 mg/kg/day	M	48	5	0.54 ± 0.03	-2	0.6 ± 0.55	3 / 5000
	F	48	5	0.54 ± 0.07	-7	1.2 ± 0.45	6 / 5000
5000 mg/kg/day	M	48	5	0.53 ± 0.07	-4	0.6 ± 0.89	3 / 5000
	F	48	5	0.52 ± 0.05	-10	0.4 ± 0.55	2 / 5000

^a p<0.01 (Kosterbaum-Bowman Tables)

Study Validity:

The study was valid. The positive control (CP) showed a significant elevation in MNPCE in both male and female mice. The MNPCE/PCE ratio in the vehicle controls at either 24 or 48 h was < 0.5%.

SUMMARY:

Under the conditions of the assay, colesevelam hydrochloride was negative in the in vivo micronucleus assay using male and female mice.

GENETIC TOXICOLOGY

SUMMARY

Three genotoxicity assays were carried out with colesevelam hydrochloride. Since the test compound is a polymer, two *in vitro* assays were done with an extract of the compound (Ames test, CHO cell chromosome aberration test), and one *in vivo* assay was done with the polymer itself (mouse micronucleus assay).

1. In the Ames bacterial reverse mutation assay an extract of the test article was negative with and without metabolic activation
2. In the CHO cell chromosome aberration assay an extract of the test article was positive in the presence of metabolic activation. The extract was negative in the absence of metabolic activation.
3. In the mouse micronucleus assay the test compound was negative.

Both Ames bacterial reverse mutation tests and CHO cell clastogenicity tests were also carried out with four degradants that are found in the drug substance. The four degradants (decylamine HCl, aminohexyltrimethyl ammonium chloride HCl, didecylamine HCl, and 6-decylamino-hexyltrimethyl ammonium chloride HCl) appear at levels approaching — % in the drug substance and drug product during long-term stability studies.

1. In the Ames bacterial mutation assay all four degradants of the test compound were negative with and without metabolic activation.
2. In the CHO cell chromosome aberration assay, two degradants, e.g., decylamine HCl and aminohexyltrimethyl ammonium chloride HCl, were positive under the test condition of 4h treatment and 16h recovery in the absence of metabolic activation. However, under the test condition of 20h treatment, both compounds were negative in the absence of metabolic activation. Under all other conditions, all four degradants were negative.

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REPROTOXICITY

The reproductive toxicity of orally administered colesevelam hydrochloride was assessed in a Segment I study in rats, a Segment II study in rats and rabbits, and a Segment III study in rats.

Table 5.3-19: Reproductive Toxicity Studies

SECTION NUMBER	STUDY TITLE	SPECIES	TREATMENT DURATION	DOSE g/kg	GLP
5.3.4.1	Oral (Diet) Fertility and General Reproduction Toxicity Study of Cholestagel (GT31-104HB) in Rats (Study No. GT-02-TX-12)	Rat	28 days male -15 to Day 7 of gestation	0.2, 1.0, 2.0	Yes
5.3.4.2	Oral (Diet) Developmental Toxicity Study of Cholestagel (GT31-104HB) in Rats (Study No. GT-02-TX-13)	Rat	Days 7 to 17 of gestation	0.3, 1.0, 3.0	Yes
5.3.4.3	Multigenerational Reproductive Toxicity Study in Rats (Segment III) (Study No. GT-02-TX-30)	Rat	Days 6 of gestation to Days 20, 21, or 22 <i>post partum</i>	0.1, 0.3, 1.0	Yes
5.3.4.4	Oral (Stomach Tube) Developmental Toxicity Study of Cholestagel (GT31-104HB) in Rabbits (Study No. GT-02-TX-14)	Rabbit	Days 6 to 18 of gestation	0.1, 0.5, 1.0	Yes

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**ORAL (DIET) FERTILITY AND GENERAL REPRODUCTION TOXICITY STUDY OF
CHOLESTAGEL (GT31-104HB) IN RATS**

Study No: GT-02-TX-12
 Site and testing facility: _____
 Date of study initiation: December 1996
 Date of study completion: February 1997
 GLP compliance: Yes
 QA- Reports: Yes (X) No ()
 Lot Number: CHOG9603-1459

METHODOLOGY

Species/strain: Crl:CD®BR VAF/Plus® (Sprague-Dawley) rats
 Doses employed: 0 ("carrier"), 0 (control article, Sigmacell(D) at 2 g/kg/day), 0.2, 1, 2 g/kg/day

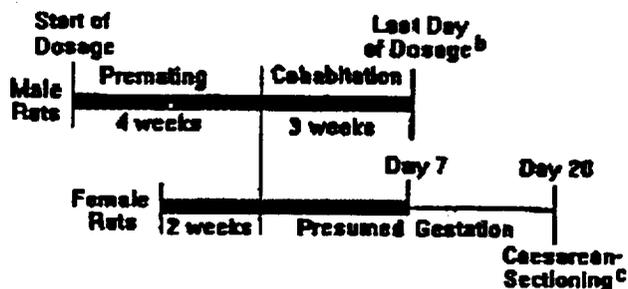
Group	1	2	3	4	5
Dose (g/kg/day)	0 (Carrier)	0 (Control article)*	0.2	1	2
N/sex	25	25	25	25	25

*Concentration of control article in the diet was 2g/kg/day

Route of Administration: Diet
 Study Design: Males were dosed in the diet for 28 days before cohabitation continuing through sacrifice. Females were dosed for 15 days before cohabitation and then through GD7. Males were necropsied on study Day 56-59. Dams were cesarean-sectioned on GD (Gestation Day) 20, or SD (Study Day) 37-38. Gross necropsy was performed on males and females.

STUDY SCHEMATIC

FERTILITY AND GENERAL REPRODUCTION STUDY^a



==== = Dosage Period

- a = For additional details see "Tests, Analyses and Measurements" section of the protocol
- b = Male rats sacrificed, and sperm evaluations performed
- c = Fetal evaluation (external only)

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Number of animals/sex/group: 25
 Parameters evaluated: Clinical signs of toxicity, mortality, body weight, food consumption, estrous cycling, mating performance, litter averages for corpora lutea, implantation sites, litter sizes, live and dead fetuses, resorptions, #dams with resorptions, #dams with viable fetuses, fetal sex ratio, fetal body weights.
 Statistical evaluations: Clinical observations: Variance test
 Continuous data: Variance Analysis or Kruskal-Wallis test, Dunnett's Test, Fisher's exact Test
 Count data: Kruskal-Wallis Test
 Sperm motility: Parametric methods

RESULTS

MALES

Consumed dosages: Very close to or slightly larger than targeted doses
Clinical signs: No treatment-related changes
Mortality: None
Body weight:

Male rat body weight and body weight changes (grams)

Group	1	2	3	4	5
BW Day 1	360	365	362	364	363
Terminal BW (Day 56-59)	512	504	498	497	491
BW gain (Day 1-8)	+37.9	+31.6*	+31.9	+30.8	+31.6
BW gain (Day 1-56)	+147	+133	+131	+125*	+120**
BW gain (Day 1-termination) (Day 56-59)	+153	+139	+137	+133	+129

*p<0.05, **p<0.01, significant difference from control group 1

Comment: There was a reduction in body weight and weight gain in all dose groups. The reduction occurred also in the control article group (Group 2), but over the whole exposure period to a lesser extent than in the group treated with the same dose of test article (Group 5).

Food consumption:

Male rat food consumption

Group	1	2	3	4	5
(Day 1-56) G/day	27.9	28.6	27.8	29.4*	29.8**
(Day 1-56) G/kg/day	62.4	64.1	63.3	67.1**	68.6**

*p<0.05, **p<0.01, significant difference from control group 1

Comment: There was an increase in absolute and relative food consumption in the mid and high dose groups. The increase also occurred in the control article group (Group 2) but to a lesser extent than in the group treated with the high dose of test article (Group 5). Thus, the effects of test article on food consumption and body weight in the males partially appeared to be unspecific effects due to the presence of a polymer in the diet.

Mating and fertility in males: In-life observations

All males mated. There were no differences in mating and fertility parameters evaluated: number of days in cohobitation, number of rats that mated (25 in all groups), time to mating, and fertility index (#pregnancies/#rats that mated, 21/25, 22/25, 21/25, 23/25, 21/25).

Terminal and Necropsy Observations

No necropsy observations related to test article

Organ weights:

Right epididymis absolute weight was decreased in Groups 2 ——— and in Groups 3, 4 and 5 as compared to Group 1 (carrier diet). The effect was significant in groups 2, 4, and 5. For almost all other organs a similar effect was observed but it was not statistically significant. Ratios of organ weight to terminal body weight were not significantly affected by control or test article for any organ. This indicates that the epididymal and other organ absolute weight effects were related to the decrease in body weights in Groups 2, 3, 4, and 5.

Group		1	2	3	4	5
Terminal body weight		512	504	499	498	492
Epididymes right	Absolute wt	0.77	0.72*	0.75	0.71**	0.71**
	Relative wt	0.150	0.144	0.150	0.144	0.144
Testis right	Absolute wt	1.76	1.73	0.75	0.71	0.71**
	Relative wt	0.35	0.34	0.35	0.35	0.34
Seminal vesicles	Absolute wt	1.24	1.17	1.35	1.33	1.26
	Relative wt	0.244	0.234	0.273	0.271	0.257
Prostate	Absolute wt	1.11	0.997	1.05	1.05	0.92
	Relative wt	0.217	0.197	0.210	0.211	0.185

*p<0.05, **p<0.01

Sperm motility:

Caudal epididymal sperm motility (total motile sperm, total nonmotile sperm, total % of motile sperm) was not affected by control or test article, i.e., was comparable among all groups. Caudal epididymal sperm count and concentration were slightly decreased in Groups 2, 4 and 5 (mostly in Groups 2 and 5) as compared to Group 1. However, the effect was not statistically significant.

FEMALES

Consumed dosages:

SD1-SD15: appr. 25% below targeted dose

GD0-GD8: appr. 10% below targeted dose

Clinical signs:

Small mass on forelimb in one HD (Group 5) rat on GD20.

Mortality:

None

Body weight:

Female rat body weight and body weight changes (grams)

Group	1	2	3	4	5
N tested	25	25	25	25	25
N pregnant	21	21b	21	22c	20b

BW Females Day 1	253	251	250	248	251
BW gain (Day 1-8)	+8.8	+9.8	+11.5	+13.2*	+13.9**
BW gain (Day 8-14)	+5.1	+7.4	+9.6	+7.2	+6.5
BW gain (Gest Day 0-8)	+36	+32	+40	+38.5	+37
BW gain (Gest Day 8-14)	+29	+27	+26	+22.7*	+21.6*
BW gain (Gest Day 14-20)	+77	+77	+77	+73	+80

*p<0.05, **p<0.01, significant difference from control group 1

b=restricted to rats with confirmed mating data

c=excluded dam 11790 which delivered on GD14 (unclear mating date)

Food consumption:

Female rat food consumption (g/day)

Group	1	2	3	4	5
N tested	25	25	25	25	25
N pregnant	21	21 ^b	21	22 ^c	20 ^b
(Day 1-8)	19.9	19.6	20.7	19.9	20.8
(Day 8-15)	20.0	20.3	21.5*	20.8	21.7*
Gest Day (0-8)	26	25	27	27	27.5
Gest Day (8-14)	27	26	27	26	26
Gest Day (14-20)	28	27	28	28	27

*p<0.05, **p<0.01, significant difference from control group 1

Comment: The increased food consumption and body weight gain during the pre-mating period (Day 1-15) appeared to be a test-article specific effect. The decrease in body weight gain during GD8-14 was not paralleled by an effect on food consumption and was also test-article specific.

Mating and fertility in females: In-life observations

All females mated. There were no differences in mating and fertility parameters evaluated: estrous stages per 14 days, number of rats with ≥6 days of diestrus or estrus, number of days in cohabitation, number of rats that mated (25 in all groups), time to mating, and fertility index (#pregnancies/#rats that mated, 21/25, 22/25, 21/25, 23/25, 21/25).

Fertility Index

Group	1	2	3	4	5
Rats in cohabitation	25	25	25	25	25
Fertility Index	21/25	22/25	21/25	23/25*	21/25

* Included dam that delivered on presumed GD14

Necropsy Observations:

No significant test article-related effects

Caesarian-sectioning observations:

There were 21-22-21-22-21 litters with live fetuses in the five different Caesarian-sectioned treatment groups. One dam in Group 4 delivered on presumed GD14.

The following parameters (litter averages) did not differ significantly from either carrier or control article group values (Groups 1 and 2) and did not exceed the testing facilities' historical control values:

Corpora lutea, implantations, litter sizes, live fetuses, dead fetuses (there were none), early resorptions, late resorptions, number of dams with any resorptions, number of dams with viable fetuses, number of live fetuses, % live male fetuses, fetal body weights, % resorbed conceptuses per litter. One placenta in the control article group (Group 2) was abnormal (tan mass).

Fetal Gross Alterations:

:No test-article related effects (eg body edema, kinked tail, short limbs, depressed eye bulge)

CONCLUSIONS

Paternal and maternal NOAEL of colesevelam HCl is < 0.2 g/kg/day.

At higher dosages (0.2, 1, 2 g/kg/day) the following effects were observed:

- increase in male food consumption
- reduction in male body weight and body weight gain
- increase in female food consumption values during the premating exposure period
- increase in female body weight and body weight gain during the premating exposure period
- decrease in female body weight and body weight gain during the gestation period immediately after dose discontinuation (GD 8-14)

There were no adverse reproductive effects. The reproductive NOAEL is > 2 g/kg/day.

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ORAL (DIET) DEVELOPMENTAL TOXICITY STUDY OF CHOLESTAGEL (GT31-104HB) IN RATS

Study No: GT-02-TX-13
 Site and testing facility: _____
 Date of study initiation: November 1996
 Date of study completion: December 1996
 GLP compliance: Yes
 QA- Reports: Yes (X) No ()
 Lot Number: CHOG9603-1459

METHODOLOGY

Species/strain: Cri:CD®BR VAF/Plus® (Sprague-Dawley) rats
 Doses employed: 0 ("carrier"), 0 (control article, Sigmacell(D) at 3 g/kg/day), 0.3, 1, 3 g/kg/day

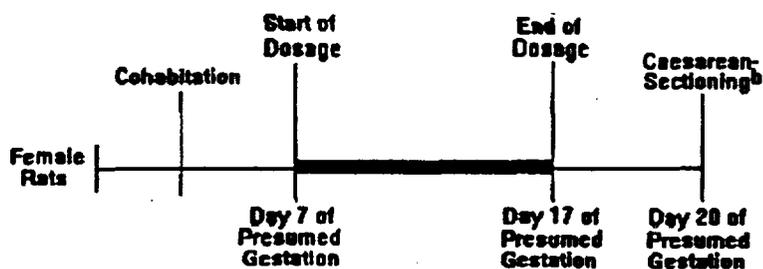
Group	1	2	3	4	5
Dose (g/kg/day)	0 (Carrier)	0 (Control article)*	0.3	1	3
N	25	25	25	25	25

*Concentration of control article in the diet was 3g/kg/day (not specified)

Route of Administration: Diet
 Study Design: Female rats were mated with untreated males and then dosed daily from GD7 through GD17. Dams were sacrificed on GD20, and subjected to gross necropsy.

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STUDY SCHEMATIC
 DEVELOPMENTAL TOXICITY STUDY^a



- = Dosage Period
- ^a = For additional details see "Tests, Analyses and Measurements" section of the protocol
- ^b = Fetal evaluations (all - external, 1/2 per litter - soft tissue or skeletal)

Number of animals/group: 25
 Parameters evaluated: Dams: Clinical signs of toxicity, mortality, body weight, food

consumption, litter averages for corpora lutea, implantation sites, litter sizes, live and dead fetuses, resorptions, #dams with resorptions, #dams with viable fetuses, fetal sex ratio, fetal body weights.

All fetuses: weight, sex and gross alterations; half of fetuses: soft tissue alterations; remaining fetuses: skeletal alterations.

Statistical evaluations:

Clinical observations: Variance test

Continuous data: Variance Analysis or Kruskal-Wallis test, Dunnett's Test, Fisher's Exact Test

Count data: Kruskal-Wallis Test

RESULTS

FEMALES

Consumed dosages:

LD (0.3 g/k/d): 5%-10% above targeted dosage (GD7-GD18)

MD (1 g/kg/d): appr. 10% above targeted dosage (GD7-GD18)

HD (3 g/kg/d): targeted dosage (GD7-20), or 15%-19% above targeted dosage (GD10-GD18)

Clinical signs:

None

Mortality:

None

Necropsy observations:

None treatment-related

Body weight:

BW gain in Group 2 (control article group) slightly reduced in periods GD0-7 (predosing), GD7-10, and GD12-15 as compared to Group 1. BW in this group slightly reduced around GD 14.

BW gain slightly reduced in Group 5 as compared to Group 1 in period GD0-7 (predosing)

Female rat body weight and body weight changes (grams)

Group	1	2	3	4	5
N tested	25	25	25	25	25
N pregnant	24	24	25	25	23
BW GD0	239	239	239	238	239
BW GD7	275	270	273	274	271
BW GD14	324	314**	325	325	322
BW GD20	409	400	414	408	406
BW gain (GD0-GD7)	+36.1	+31.7	+33.8	+35.4	+31.6
BW gain (GD7-GD10)	+16.4	+13.8	+19.3	+18.4	+16.9
BW gain (GD10-GD12)	+17.3	+18.3	+16.8	+16.9	+19.5
BW gain (GD12-GD15)	+22.6	+19.0	+25.7	+22.8	+23.2
BW gain (GD15-GD18)	+36.4	+36.6	+33.8	+36.1	+33.8
BW gain (GD18-GD20)	+41.7	+42.1	+45.3	+39.9	+41.7
BW gain (GD7-GD18)	+92.8	+87.8	+95.6	+94.2	+93.4

**p<0.01 different from Group 1

Food consumption:

Female rat food consumption (g/day)

Group	1	2	3	4	5
N tested	25	25	25	25	25
N pregnant	24	24	25	25	23
GD0-GD7 (g/d)	22.7	21**	22.2	22.0	22.7
GD7-GD10 (g/d)	25.5	23.9**	25.3	25.2	23.4**
GD7-GD18 (g/d)	27.8	26.5*	27.5	28.0	29.0
GD18-GD20 (g/d)	29.6	28.1	30.1	30.3	32.3**

GD0-GD7 (g/kg/d)	88.6	82.6**	86.9	86.0	88.8
GD7-GD10 (g/kg/d)	89.7	85.9*	89	88.4	83.7**
GD7-GD18 (g/kg/d)	88.3	86.5	87.3	89.0	93.2**
GD18-GD20 (g/kg/d)	76.5	74.3	77.1	78.3	84.1**

*p<0.05, **p<0.01 different from Group 1

Comment: Data suggest that in control Group 2 food consumption and body weight were decreased during the dosing period. Test article effects on these parameters were a slight overall increase in food consumption over the whole dosing period with no effect on body weight.

Caesarian-sectioning observations:

There were 24-24-25-25-23 litters with live fetuses in the five groups.

There was an increase in the litter averages for early and total resorptions in the control article group (Group 2) and in the test article-treated groups. However, the increases in the test article-treated groups were not statistically significantly different from Group 2. None of the values in any group was outside the historical control value range.

Effects on resorptions

Group	1	2	3	4	5
N tested	25	25	25	25	25
N pregnant	24	24	25	25	23
Resorptions (litter avg)	0.4	1.0**	0.6	0.7	0.9
Early resorptions (N)	9	23	15	16	21
(litter avg)	0.4	1.0**	0.6	0.7	0.9
Late resorptions (N)	0	0	0	1	0
(litter avg)	0	0	0	0	0
Dams with any resorptions	4	15**	13**	9*	12**
%Resorbed conceptuses/litter	2.5	6.4*	3.8	4.5	6.1

*p<0.05, **p<0.01 (P-values of differences from Group 1)

The following parameters (litter averages) did not differ significantly from either carrier or control article group values (Groups 1 and 2) and did not exceed the testing facilities' historical control values:

Corpora lutea, implantations, litter sizes, live fetuses, dead fetuses (there were none), late resorptions, number of dams with viable fetuses, placental appearance, number of live fetuses, % live male fetuses, live fetal body weights.

Fetal Alterations (External, soft tissue, or skeletal):

Summary findings

Group	1	2	3	4	5
Litters evaluated	24	24	25	25	23
Fetuses evaluated	339	341	373	338	311
Litters with any alterations observed (N)	8	14	10	8	16**
Fetuses with any alterations observed (N)	15	31**	22	12	30**
%Fetuses with any alteration/litter	3.9	8.7	5.9	3.6	9.6

** p<0.01 significantly different from Group 1 value

There were no (control or) test article-related effects on gross external alterations.

According to Sponsor, the significant increase in incidence of fetal alterations in Group 2 was due to reversible delays in ossification (significant effects: thoracic vertebrae, bifid centrum and incompletely ossified 2nd sternal center). However, there were other non-statistically significant soft tissue and skeletal alterations that had increased incidences in this control group and probably contributed to the overall significant increase (see Table below).

According to Sponsor the significant increase in incidence of fetal alterations in Group 5 was also due to reversible delays in ossification, none of them statistically significant. One of these findings increased in Group 5 was thoracic vertebrae centrum, unilateral ossification. However, in the opinion of this Reviewer there were also other soft tissue and skeletal alterations that had increased incidences in this group, that lead to the overall significant increase in fetal alterations in this HD group (see Table below). Most of the litter incidences of these alterations were below 5% and most fetal incidences below 1% (i.e., 1 fetus in 1 litter affected), and none were statistically significant.

There were also several non-statistically significant soft tissue and skeletal alterations that had increased incidences in LD and MD groups. However, the increased incidences of these events were not statistically significant and did not lead to a significant increase in the overall fetal alteration incidence.

All values in all groups were within the historical range of the testing facility.

The following Table summarizes some of the findings that, according to this Reviewer, probably contributed to the significantly increased incidences of fetal alterations in Groups 2 and 5.

Significant and non-significant findings in fetal soft tissue and skeletal alterations

Group	1	2	3	4	5
SOFT TISSUE ALTERATIONS					
Litters evaluated	24	24	25	25	23
Fetuses evaluated	177	178	191	177	161
Cleft palate					
Litter incidence	0	0	0	0	1
Fetal incidence	0	0	0	0	1
Situs inversus					
Litter incidence	0	1	0	0	0
Fetal incidence	0	1	0	0	0
Vessels, innominate absent					
Litter incidence	0	0	0	0	1
Fetal incidence	0	0	0	0	1
SKELETAL ALTERATIONS					
Skull: mandibles fused					
Litter incidence	0	0	0	2	1
Fetal incidence	0	0	0	2	2

Skull: nasals, short					
Litter incidence	0	1	0	0	0
Fetal incidence	0	1	0	0	0
Thoracic vertebrae, bifid centrum					
Litter incidence	0	4**	2	5	3
Fetal incidence	0	6**	3	6	3
Thoracic vertebrae, centrum, unilateral ossification					
Litter incidence	0	1	1	1	2
Fetal incidence	0	1	1	1	2
Sternal centra, 2 nd , incompletely ossified					
Litter incidence	0	2	3	1	0
Fetal incidence	0	5**	4	1	0
Ribs, incompletely ossified					
Litter incidence	0	2	0	0	0
Fetal incidence	0	2	0	0	0
Ribs, wavy					
Litter incidence	0	1	0	0	0
Fetal incidence	0	1	0	0	0
Pelvis, pubis, incompletely ossified					
Litter incidence	6	6	8	3	9
Fetal incidence	9	16	12	6	15
Pelvis, ischium, incompletely ossified					
Litter incidence	2	2	3	2	3
Fetal incidence	3	6	5	4	7

Comment: The statistically non-significant alterations in the test-article treated groups that were not seen in control article Group 2 were cleft palate (Group 5), innominate vessels absent (Group 5), and mandibles fused (Group 4 and 5). All other alterations with increased incidences in test-article treated groups also had increased incidences in control Group 2.

CONCLUSIONS

- Maternal NOAEL is 1 g/kg/day. The next higher dosage of 3 g/kg/day tended to increase absolute and relative food consumption values without an effect on body weight.
- Developmental NOAEL is > 3 g/kg/day. Although there were significant increases in the numbers of litters and fetuses with any alterations in Groups 2 and 5, there were no significant, specifically test article-related effects on fetal external, soft tissue or skeletal morphology.

AN ORAL PRE AND POSTNATAL STUDY OF CHOLESTAGEL® (GT31-104HB) IN THE RAT

Study No: GT-02-TX-30
Site and testing facility: _____
Date of study initiation: September 28, 1998
Date of study completion: February 16, 1999
GLP compliance: Yes
QA- Reports: Yes (X) No ()
Lot Number: TMAC015-1868

METHODOLOGY

Species/strain: Crl:CD®(SD)BR IGS (Sprague-Dawley) rats
Doses employed: 0 (deionized water), 0.1, 0.3, 1 g/kg/day
Route of Administration: Oral (gavage), 10 ml/kg/dose, in two divided doses 6h apart
Study Design: Females (F0 generation) were mated with untreated males, and dosed from Day 6 of Gestation to Day 20, 21 or 22 post partum. F0 females were euthanized on PP Day 21, 22 or 23 and examined by gross pathology. One male and 1 female were selected from each litter to form the adult F1 generation on Day 21 post partum (at weaning). Those not selected were euthanized and examined by gross pathology. The F1 generation was examined for physical development, reflexological/sensory development, behavior and reproductive performance. At approximately 85 days of age, F1 females were mated with F1 males from the same dosage group. F2 pups were euthanized on PP Day 6. F1 males were killed and examined by gross pathology approximately 3 weeks after end of mating period. F1 females were euthanized and examined by gross pathology on PP Day 4, 5, or 6. F1 or F2 pups dying or euthanized as malformed were examined externally and internally.

Number of animals/group: 24
Parameters evaluated: F0 generation: Clinical signs of toxicity, mortality, physical examination, body weight, food consumption, observations at parturition, maternal performance and gross pathology
F1 generation (pups): Condition, sex, body weight, physical development, reflexological/sensory development, gross pathology
F1 generation (adults): Clinical signs, mortality, physical, body weight, physical development, visual function, behavioral performance, parturition, parental and maternal performance and gross pathology.
F2 generation: Condition, sex, body weight.

Statistical evaluations: Continuous data: Variance Analysis with Dunnett's Test, Indices/rates/numbers: X-square test, Fisher's Exact Test, Kruskal-Wallis Test, Mann-Whitney "U" test

Parameters: *Parental:*
Pregnancy rate (%) = (No. of pregnant f/No. of mated f) x100
Mating index (%) = (No. of m mating/No. of m placed for mating) x100 (%)
Fertility index (%) = (No of m producing a pregnancy/No. of m placed for mating) x100
Conception rate (%) = (No. of pregnant f/No.of mated f) x100
Maternal:
Live birth index (%) = (No. of live pups at birth/No. of implantation site scars) x100
Gestation Index (%) = (No. of f rat with live litters/No. of pregnant rats) x100
Litter data:
Viability index (%) = (No. of live pups on PPD4/No.of live pups on PPD0)x100

Survival index (%) = (No. of live pups on PPD7 and 14/No. of live pups on PPD4)x100
 Viability index (%) = (No. of live pups on PPD21/No. of live pups on PPD4)x100

RESULTS

F0 generation

Mortality: One control animal on GD18, with various clinical signs

Clinical signs: None treatment-related

Body weight: No treatment effects

Food consumption: No treatment effects

Gross pathology: None treatment related (number of animals examined: 24-23-23-24; two non-pregnant animals in LD and MD groups were not necropsied due to technical oversight)

Maternal performance (range of group average values in parentheses):

	Group 1	Group 2	Group 3	Group 4
No. of mated females	24	24	24	24
No. of pregnant females	23	23	23	24

No effects on: pregnancy rate (96-100%), live birth index (89-96%), gestation index (96-100%), length of gestation (21.6-21.8 days), duration of parturition (2.7-3.4h), number of implantation scars (14.9-15.5), numbers of live (13.6-14.3), dead (0.13-0.32) and malformed (0-0.05) pups, and pup sex ratio (46.1-50.2%).

F1 generation pups

Viability data (range of group average values in parentheses):

No effects on viability index (99-100%), Day 7 and Day 14 survival index (all 100%), Day 21 lactation index (all 100%). No effects on male, female, or total litter size on Day 0 (13.6-14.3), Day 4 pre-culling (13.5-14.3), Day 4 post-culling (7.9-8.0), and Day 7, 14 and 21 (all 7.9-8.0).

Clinical signs:
None treatment-related

Pup body weight:

Pup body weight (g)	Group 1	Group 2	Group 3	Group 4
Day 0	6.1	6.0	6.3	6.0
Day 7	14.9	14.8	15.8*	14.6
Day 21	50.4	50.7	52.3	49.8

*significantly different from control (group 1) value, p<0.05

Physical and reflexological development:

No significant treatment-related effects on development of pinnae detachment (2.1-2.5 days), righting reflex (2.1-2.3 days), negative geotaxis (8.7-9 days), tooth eruption (9.8-10.2 days), auricular startle (12.1-12.3 days), eye opening (13.5-14.0).

Gross pathology of F1 pups:

Post partum days 0-7 (pups that died or were euthanized malformed):

Group	1	2	3	4		

Males (N)	3	1	2	3		No external/internal findings
Females (N)	4	2	5	3		Various incidental findings, usually in 1 pup of each dose group. No findings in pups of HD group 4.

Post partum days 8-23:

All F1 pups alive on post partum Day 7 survived and were euthanized on Day 21, 22, or 23. No treatment-related gross pathological findings.

F1 generation adults

Mortality:

All survived
(However, data available are from 22 (not 23)-23-23-24 per sex)

Clinical signs:

None treatment-related

Body weight:

No significant effects for males and females during pre-mating and mating, or for females during gestation and lactation

Visual function:

All animals had normal visual placing and pupillary closing (100% in all groups)

Physical development:

No effect on mean day of development of vaginal opening (31.0-31.8 days), and preputial separation (42.2-43.3 days)

Behavioral assessment:

Motor activity:

No significant effects on either Day 35 or Day 60 of assessment

Auditory startle:

No significant effects on auditory startle habituation data (Day 55)

Water maze:

No treatment-related effects on time to complete the maze (7 trials) for males or females, or in the number of errors incurred for males or females.

Gross pathology:

Findings with increased incidence in dosed groups

	Group 1	Group 2	Group 3	Group 4
MALES				
Thymus: Area dark	0	0	1	2
FEMALES				
Adrenal discoloration	0	0	0	4

Parental performance (range of group average values in parentheses):

	Group 1	Group 2	Group 3	Group 4
No. placed for mating (m,f)	22	23	23	24
No. mating	20	20	20	22
No. females pregnant	20	20	20	22

No effects on: mean day of mating (2.7-3.4), mating index (87-91.7%), fertility index (87-91.7%), conception rate (100% all).

Maternal performance (range of group average values in parentheses):

No effects on pregnancy rate (100% all), live birth index (85-93%), gestation index (100% all), length of gestation (21.6-21.9 days), duration of parturition (2.6-3.3h), number of implantation scars (15.5-15.9), numbers of live (13.3-14.6), dead (0.2-0.35) and malformed (0-0.20) pups, and pup sex ratio (49.7-52.8%).

F2 generation pups

Viability data (range of group average values in parentheses):

No effects on viability index on Day 4 (97-99%). No effects on male, female, or total litter size on Day 0 (13.3-14.6) or Day 4 (13.0-14.2)

Clinical signs: None treatment-related

Pup body weight: No treatment effects

Gross Pathology: External/internal findings in pups found dead or euthanized by postpartum day 4, 5, or 6 appeared incidental and not treatment-related. Externally normal pups were discarded without gross pathology examination.

CONCLUSIONS

- Maternal NOAEL > 1 g/kg/day. There was no evidence of maternal toxicity at any dose level.
- Reproductive NOAEL > 1 g/kg/day. There was no effect on reproductive performance during gestation, parturition or lactation, no effect on the survival, physical development, behavior and reproductive performance of the F1 generation, and no effect on the survival and physical condition of the F2 generation pups.

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ORAL (STOMACH TUBE) DEVELOPMENTAL TOXICITY STUDY OF CHOLESTAGEL (GT31-104HB) IN RABBITS

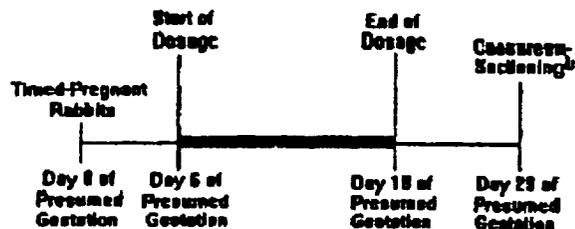
Study No: GT-02-TX-14
Site and testing facility: _____
Date of study initiation: November 1996
Date of study completion: December 1996
GLP compliance: Yes
QA- Reports: Yes (X) No ()
Lot Number: CHOG9603-1459

METHODOLOGY

Species/strain: New Zealand White rabbits [Hra:(NZW)SPF]
Doses employed: 0 (vehicle, deionized water), 0.1, 0.5, 1 g/kg/day
Route of Administration: Oral (stomach tube), 20 ml/kg, same time each day
Study Design: Female rabbits, age 6 months, were mated with untreated males and then dosed daily from GD6 through GD18 . Dams were sacrificed on GD29, and subjected to gross necropsy.

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STUDY SCHEMATIC
RABBIT DEVELOPMENTAL TOXICITY STUDY^a



- = Dosage Period
- ^a = For additional details see "Tests, Analyses and Measurements" section of the protocol
- ^b = Fetal evaluations (external, soft tissue and skeletal)

Number of animals/group: 20
Parameters evaluated: Dams: Clinical signs of toxicity, mortality, body weight, food consumption, litter averages for corpora lutea, implantation sites, litter sizes, live and dead fetuses, resorptions, #does with resorptions, % resorbed conceptuses, #does with viable fetuses, fetal sex ratio, fetal body weights.
All fetuses: weight, gross external alterations, sex, visceral alterations, skeletal alterations.
Statistical evaluations: Clinical observations: Variance test
Continuous data: Variance Analysis or Kruskal-Wallis test, Dunnett's Test, Fisher's Exact Test
Count data: Kruskal-Wallis Test

RESULTS

FEMALES

Clinical signs:

None treatment-related

Mortality:

None

Necropsy observations:

None treatment-related

Maternal body weight:

Body weight: No effects

BW gain reduced significantly in periods GD6-9 and GD12-15, and thus in GD6-19. BW gain not reduced in periods GD0-6, GD9-12, GD15-19, GD19-24, GD24-29

Maternal body weight changes (kg)

Group	1	2	3	4
N tested	20	20	20	20
N pregnant	19	19	20	20
N included in analysis	18A	19	19B	20
BW gain (GD0-GD6)	+0.04	+0.00	+0.01	+0.04
BW gain (GD6-GD9)	+0.08	+0.10	+0.08	+0.05
BW gain (GD12-GD15)	+0.13	+0.11	+0.11	+0.05**
BW gain (GD6-GD19)	+0.32	+0.31	+0.31	+0.23**
BW gain (GD0-GD29)	+0.57	+0.52	+0.52	+0.49

A excluded values for one doe with litter of 3 resorptions, B excluded values for one doe with litter of 3 resorptions
**p<0.01 significantly different from control Group 1

Food consumption:

Reduced non-significantly in periods GD12-15, GD15-19

Maternal food consumption (g/day)

Group	1	2	3	4
N tested	20	20	20	20
N pregnant	19	19	20	20
N included in analysis	18A	19	19B	20
GD12-G15 (g/d)	181	179	180	162
GD15-GD19 (g/d)	181	180	182	171
GD6-GD19 (g/d)	180	179	181	173
GD12-G15 (g/kg/d)	46.3	45.9	45.9	41.3
GD15-GD19 (g/kg/d)	45.2	45	45.5	42.9
GD6-GD19 (g/kg/d)	46.2	46.1	46.6	44.2

Caesarian-sectioning observations:

There were 18-19-19-20 litters with live fetuses in the five groups.

There were no effects on litter averages for corpora lutea, implantation sites, litter sizes, live fetuses (there were no dead fetuses), early and late resorptions, #does with any resorptions, % resorbed conceptuses, #does with viable fetuses, placental appearance, fetal sex ratio, and fetal body weights.

All values in all groups were within the historical control value range.

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Fetal Alterations (External, Soft tissue, or Skeletal):

Fetal alterations - Summary findings

Group	1	2	3	4
Litters evaluated	18	19	19	20
Fetuses evaluated	170	190	164	185
Litters with any alterations observed (N) (%)	7 (39%)	9 (47%)	8 (42%)	8 (40%)
Fetuses with any alterations observed (N) (%)	10 (5.9%)	18 (9.5%)	14 (8.5%)	11 (5.9%)
%Fetuses with any alteration/litter	6.6	9.8	8.0	6.3

There were some gross external alterations with incidences in one or more dosage groups (including the control group). However, they all occurred in only 1 fetus in the relevant dosage group(s) and therefore were neither statistically or biologically significant.

There were several non-statistically significant soft tissue or skeletal alterations that had increased incidences in one or more dosage groups (including the control group). However, the litter incidences of the alterations were either not dose-dependent and therefore not treatment-related, or they occurred in only 1 fetus in 1 MD and/or 1 HD litter (i.e., eyes circumcomeal hemorrhage, and ribs fused) and therefore appeared biologically not significant.

One fetus in the HD group (fetus 3686-2) had multiple alterations, including gross external, soft tissue and skeletal alterations. One other fetus in the HD group from another litter (fetus 3684-1) also had multiple alterations, including gross external and skeletal alterations (malformed digits on right hindpaw, absence of phalanges of 1st and 2nd digits and only 1 phalange in 4th digit of right hindpaw, and presence of only the 4th metatarsal bone in right hindpaw)

3684	1 (11.3)	1 / 9	FETUS 1 FOUR AMP/OC KIDNEYS: KIDNEYS MALFORMED, right hindpaw - fourth; FOUR AMP/OC KIDNEYS: KIDNEYS ABSENT, right hindpaw - first and second	0 / 9		1 / 9	FETUS 1 METATARSALS: 1 FUSED/WT, RIGHT 4TH 8 CYPHOID SPINES: ABSENT, right 1st and 2nd - 2 present, 3rd and 4th 0 METATARSALS: ABSENT, right 1st and 2nd proximal - distal, right 4th medial and distal (0021)a
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SPECIMENS WITH ANY ALTERATIONS		GROSS EXTERNAL EXAMINATION		SOFT TISSUE EXAMINATION		SKELETAL EXAMINATION	
ANIMAL NUMBER	N (%)	S/N	DESCRIPTION	S/N	DESCRIPTION	S/N	DESCRIPTION
3686	1 (11.3)	1 / 9	FETUS 2 BODY: GASTROECHEMIA, portions of liver and intestines protrude through opening in the abdominal wall HEAD: ENOPHTHALMIA; FOUR AMP/OC KIDNEYS: SPYRIDES SPYRIDES; bilobed, fenestrated; FOUR AMP/OC SPYRIDES: FLEXED, bilateral, fenestrated downward at elbow and shoulder SHOULDER: PROTRUSION EYES: SKULL, not covered by skin, orbit contained dark red fluid EARS: PINK SKULL, right; PINK ABSENT, left	1 / 9	FETUS 2 EYES: ENOPHTHALMIA, bilateral TONGUE: PROTRUSION LUNGS: ENOPHTHALMIA NONE ABSENT	1 / 9	SKULL: NOT OBLIQUE, occipital, frontal, parietal, interparietal, supraoccipital, tympanic rings; EYE SOCKET, SKULL, bilateral NOSE: ALA, UNFUSED, bilateral CLAVICULAR: BENT, right RIBS: FLAT, bilateral 1st - 12th SPRINGS: 1ST, INCOMPLETELY OBLIQUE

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All values (% incidence of alteration) were within the historical range of the testing facility.

CONCLUSIONS

- Maternal NOAEL is 0.5 g/kg/day. The 1 g/kg/day dosage reduced body weight gains and absolute food consumption values.
- Developmental NOAEL is > 1 g/kg/day. There were no significant test article-related effects on fetal viability, sex ratio, body weight, or external, soft tissue or skeletal morphology.

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REPRODUCTIVE TOXICOLOGY

SUMMARY

Four reproductive toxicity studies were carried out with colesevelam hydrochloride. Three tests were done in rats (Segment I, II, III) and one in rabbits (Segment II).

RAT STUDIES

Segment I

Reproductive and fertility parameters were not affected by Cholestagel at oral dietary doses as high as 2 g/kg/day in rats.

Segment II

Colesevelam hydrochloride had no adverse effects when administered to presumed-pregnant female rats on fetal development at oral dietary doses up to 3 g/kg/day. There were no significant effects on viability, sex ratios, fetal body weights, or external soft tissue or skeletal morphology of the fetuses in this study.

Segment III

Colesevelam hydrochloride did not cause maternal toxicity and had no adverse effects on reproductive performance during gestation, parturition, or lactation, and no effect on the survival, physical development, behavior, and reproductive performance of the F1 generation at oral gavage doses up to 1 g/kg/day. Also, there were no effects on the survival and physical condition of the F2 generation pups.

RABBIT STUDY

Segment II

Colesevelam hydrochloride had no adverse effects on viability, sex ratio, fetal body weight, or external soft tissue or skeletal morphology of the fetuses of presumed-pregnant female rabbits at oral gavage doses up to 1g/kg/day.

Taken together, these data suggest that colesevelam hydrochloride has no adverse effects on reproduction, fertility, and fetal development in rats and on fetal development in rabbits.

Doses used in reprotoxicity studies expressed as multiples of maximum human dose (4.5 g/day, or 0.075g/kg/day)

	Rat			Rabbit
	Segment I	Segment II	Segment III	Segment II
Doses (g/kg/day)	0.2, 1.0, 2.0	0.3, 1.0, 3.0	0.1, 0.3, 1.0	0.1, 0.5, 1.0
Human dose multiples	2.7x, 13x, 27x	4x, 13x, 53x	1.3x, 4x, 13x	1.3x, 6.7x, 13x

Multiples are calculated on the basis of kg body weight (test compound is non absorbed)

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SPECIAL TOXICITY

Four (4) degradants of colesevelam hydrochloride were identified in stability studies on the drug substance: decylamine HCl, didecylamine HCl, decylamino-6-hexyltrimethyl ammonium chloride hydrochloride, and aminoethyltrimethylammonium chloride hydrochloride. These degradants were present at levels below — % in the batches used in the toxicology studies.

A series of studies was conducted on these degradants to allow their presence in the final product at levels up to — %. These studies included 28-day rat oral toxicity study studies. Each of the degradants was administered via oral gavage with the exception of didecylamine which, for solubility reasons, was administered via the diet. The degradants were tested at doses up to 15 mg/kg. The maximum human dose is 4.5 g/day (60 mg/kg, if body weight is 75 kg). Assuming — % degradant is present in the drug, the maximum human dose of degradant would be —. Thus, the high dose of degradant of 15 mg/kg represents a —fold multiple of the maximum human dose.

A 28-Day Oral Toxicity Study of Decylamine Hydrochloride and Aminoethyltrimethylammonium Chloride Hydrochloride in Sprague-Dawley Rats (Study No. GT-0069/0070-TX-3)

OBJECTIVE

The objective of this study was to determine the potential toxicity of decylamine hydrochloride and aminoethyltrimethylammonium chloride hydrochloride (aminoquat) following at least 28 days of daily oral administration to Sprague-Dawley rats.

METHODS

The animals were randomly assigned to groups by a weight-ordered distribution. Rats (10/sex/group) were treated orally, by gavage, with 0 (water), 1.5, 5.0, or 15 mg/kg (10 ml/kg).

Table 5.3-33: Design for Study No. GT-0069/0070-TX-3

GROUP NUMBER	DOSAGE LEVEL	CONCENTRATION (mg/mL)	NUMBER OF ANIMALS	
			MALES	FEMALES
1	Control (deionized water)	0	10	10
2	1.5 mg/kg decylamine	0.15	10	10
3	5.0 mg/kg decylamine	0.5	10	10
4	15.0 mg/kg decylamine	1.5	10	10
5	1.5 mg/kg aminoquat	0.15	10	10
6	5.0 mg/kg aminoquat	0.5	10	10
7	15.0 mg/kg aminoquat	1.5	10	10

RESULTS

All animals survived to the scheduled sacrifice on Study Days 29 and 30. One female in the 15.0 mg/kg decylamine dosage group (Group 4) had brown material around the nose, an ungroomed coat, and urine-stained abdominal fur. One abnormal ocular observation in a female in the 15.0 mg/kg aminoquat dosage group (Group 7) at the end of the study.

Body weight: No effect in males. Reduced body weight in LD aminoquat females (Group 5) throughout the study. Significant reduction in body weight change in LD aminoquat females (Group 5) during Week 2.

Food consumption: No effects in males. Decrease in food consumption in Group 5 (low-dose aminoquat) females.

Hematology: No hematological effects in males at study termination. Dose-related increase in activated partial thromboplastin time in females treated with aminoquat.

Clinical Chemistry: No effects in males. Dose-related decrease in globulin levels in females treated with aminoquat. Decrease in globulin levels in mid-dose rats treated with decylamine.

Albumin to globulin (A/G) ratios increased in all aminoquat-treated females (Groups 5, 6, and 7).

Urinalysis: No treatment effects.

Gross pathology: No treatment-related effects.

Organ weights: No effects in males. Absolute kidney weight change (??) in low-dose aminoquat females (Group 5). No relative organ weight effects in males or females.

Histopathology: No treatment-related microscopic changes in any tissue in HD males and females (Groups 4 and 7). No significant test material-related effects in selected tissues from LD and MD groups for both test materials.

CONCLUSION

In males, daily oral administration of decylamine or aminoquat for 28 days by oral gavage at dosages up to 15 mg/kg did not cause any significant toxicity. Thus, the NOAEL for either test compound in males is 15.0 mg/kg/day.

In females, treatment-related effects were seen in body weight, food consumption, APTT and serum globulin levels in aminoquat-treated LD, MD and HD groups. Effects on globulin levels were seen in the decylamine-treated MD group. There were clinical observations for a single female treated with 15.0 mg/kg decylamine, and a single female treated with 15.0 mg/kg aminoquat.

A 28-Day Oral (Diet) Toxicity Study in Rats (Study No. GT-0073-TX-3)

OBJECTIVE

The purpose of this study was to evaluate the toxicity of didecylamine when administered in the diet for 28 consecutive days.

METHODS

Sprague-Dawley rats (10/sex/group) were orally dosed with didecylamine for 28 days at doses of 0, 1.5, 5.0, and 15 mg/kg in the feed. Evaluations were made for mortality, clinical observations, body weight, food consumption, clinical pathology parameters, organ weights, and gross and microscopic pathology.

RESULTS

Mortality: No treatment-related effects

Clinical signs: No effects

Body weight, body weight change, and food consumption: No effects.

Ophthalmology: No treatment-related lesions upon terminal evaluation.

Hematology, Coagulation, Clinical chemistry: No treatment-related effects for any dose group.

Gross pathology: No treatment-related findings.

Organ weights: No treatment-related effects on absolute organ weight, relative organ to body weight ratios, or relative organ to brain weight ratios for any dose group.

Histopathology: No treatment-related lesions in any dose group.

CONCLUSION

Didecylamine, when administered orally by gavage for 28 consecutive days to Sprague-Dawley rats, did not cause systemic toxicity. The NOAEL is 15 mg/kg for both male and female animals.

A 28-Day Oral Toxicity Study in Rats (Study No. GT-0071-TX-3)

OBJECTIVE

The purpose of this study was to evaluate the toxicity of decylamino-6-hexytrimethyl ammonium chloride hydrochloride when administered orally by gavage for 28 consecutive days.

METHODS

Sprague-Dawley rats (10/sex/group) were orally dosed with decylamino-6-hexytrimethyl ammonium chloride hydrochloride for 28 days at doses of 0 (water), 1.5, 5.0, and 15 mg/kg (5 ml/kg). Evaluations were made for mortality, clinical observations, body weight, food consumption, clinical pathology parameters, organ weights, and gross and microscopic pathology.

RESULTS

No treatment-related mortality. One animal from the 15 mg/kg dose group was found dead during the course of the study. Gross necropsy revealed a tear in the esophagus due to an accident in gavaging.

Clinical signs: No effects

Body weight, Body weight change, Food consumption: No effects.

Ophthalmology: No treatment-related lesions upon terminal evaluation.

Hematology, Coagulation: Slight reduction in hematocrit and neutrophils in HD males.

Clinical chemistry: No treatment-related effects for any dose group.

Gross pathology: No treatment-related findings.

Organ weights: No treatment-related effects on absolute organ weight, relative organ to body weight ratios, or relative organ to brain weight ratios for any dose group.

Histopathology: No treatment-related lesions in any dose group.

CONCLUSION

Decylamino-6-hexytrimethyl ammonium chloride hydrochloride when administered orally by gavage for 28 consecutive days to Sprague-Dawley rats did not cause significant systemic toxicity. The NOAEL is 15 mg/kg for both male and female animals.

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SUMMARY AND EVALUATION

The current applications (NDA 21,141 capsules; NDA 21,176 tablets) are for the use of the bile acid sequestrant, colestevlam hydrochloride, for the indication of primary hypercholesterolemia. The test compound is an allylamine polymer, to be taken orally, at doses up to 4.5 g/day. Pharmacology and toxicology studies submitted and reviewed for this application included nonclinical efficacy pharmacology studies, studies on the absorption, distribution and excretion of the compound in rats and dogs and a study on drug interaction in the dog, repeat-dose toxicology studies in rats and dogs, dietary carcinogenicity studies on mice and rats, genotoxicity studies of parent compound and four degradants, reproductive toxicity studies in rats and rabbits, and special toxicity studies on four degradants in rats.

Pharmacology

Data on *in vitro* bile acid binding and *in vivo* bile acid excretion in rats and hamsters, and data on the effect of colestevlam hydrochloride on plasma cholesterol in dogs support the mechanism of action of colestevlam hydrochloride as a bile acid sequestering polymer that can reduce plasma cholesterol levels.

ADME

Studies in rats showed that following oral administration of radiolabeled test compound, the radiolabel is almost completely eliminated within 48h by fecal excretion. The stomach tissue retained a minor amount of radiolabel (ca. 0.01% of dose, or 0.1µg eq/g tissue). In dogs small quantities of radiolabeled dose were retained in liver, bile, spleen, mesenteric lymph nodes, caecum, rectum and small intestine, ranging from approximately 0.01 to 0.035 µg eq/g tissue. There was no effect of pretreatment with test compound for one month on tissue retention. Fecal excretion was the major route of elimination. The animal data indicate that small amounts of either polymer itself, or polymer degraded in the intestine, or small soluble low molecular weight residues or degradants present in the dose preparation can be absorbed and distributed systemically. Thus, the data from two animal species studies contradict the statement that the drug is not absorbed.

In the dog, colestevlam hydrochloride did not affect the bioavailability of seven drugs tested.

General toxicology

Rats

Oral dietary toxicity studies in rats were carried out of 90-day and 6-month duration.

Study duration	Doses (g/kg/day)	Route	Human dose multiple*
90 days	0, 0.3, 1.5, 3	Oral (diet)	0x, 5x, 25x, 50x
6-months	0, 0.2, 1.2, 2.4	Oral (diet)	0x, 3.3x, 20x, 40x

*Based on the maximum human dose of 4.5 g/day (60 mg/kg/day), and compared on the basis of body weight (negligible absorption)

NOAEL values were 0.3 g/kg/day (90-day study) and 0.2 g/kg/day (6-month study), which is equivalent to 5x and 3.3x the maximum human dose on body weight basis. Main effects were decreases in serum levels of fat-soluble vitamins (A,D,E,K) in mid- and high-dose males and females in both studies. The decrease in Vitamin K presumably caused hemorrhagic tissue changes, and hemorrhagic syndrome and death in high-dose males. In addition, there were decreases in red cell indices in high-dose males in the 90-day study. Serum transaminases (ALT, AST) were elevated in mid- and/or high-dose males and females in both studies.

Dogs

Oral capsule toxicity studies in dogs were carried out of 13-week and 1-year duration.

Study duration	Doses (g/kg/day)	Route	Human dose multiple*
13 weeks	0, 0.2, 0.67, 2.0	Oral (capsule)	0x, 3.3x, 11x, 33x
1 year	0, 0.2, 0.6, 2.0	Oral (capsule)	0x, 3.3x, 10x, 33x

*Based on the maximum human dose of 4.5 g/day (60 mg/kg/day), and compared on the basis of body weight (negligible absorption)

NOAEL values were < 0.2 g/kg/day (13-week study), and 200 mg/kg/day (1-year study) which is equivalent to < 3.3x and 3.3x the maximum human dose on body weight basis.

There was 1 death of unclear cause in the HD group in the 13-week study. There were abnormal or few feces in MD and HD groups, body weight and food consumption decreases in HD groups, decreases in red cell indices in MD and HD males and females, and decreases in Vitamins A, E, D in LD, MD and HD males and females. Decreased serum cholesterol was seen in LD, MD and HD males and females in the 13-week study, and decreases in phospholipid and cholesterol levels were seen mainly in HD groups in the 1-year study. In the 13-week study, serum alkaline phosphatase was increased in all male dose groups, and serum phosphorus was increased in HD males and females. Increased serum chloride was seen in MD and/or HD of both sexes in both studies, presumably due to high amounts of _____ in the polymer. In the 13-week study, there were no drug-related macroscopic or microscopic effects. In the 1-year study, there was a dose-related decrease in absolute and relative lung weight in males, there were increases in relative heart and kidney weights in HD females, and possibly test-article-related intestinal lesions in both sexes. Clinical pathology changes in the 1-year study were reversible in 4 weeks.

Carcinogenicity

Rat study

A 104-week carcinogenicity study was carried out in the rat with a high dose of 2.4 g/kg/day.

Study duration	Doses (g/kg/day)	Route	Human dose multiple*
104 weeks	0, 0, 0.4, 1.2, 2.4	Oral (diet)	0x, 0x, 6.6x, 20x, 40x

*Based on the maximum human dose of 4.5 g/day (60 mg/kg/day), and compared on the basis of body weight (negligible absorption)

There was a statistically significant dose-tumor positive linear trend in incidence of benign pancreatic acinar cell adenoma in male rats (n = 0-0-0-2-3) (p=0.002). There was also an increased incidence of pancreatic acinar cell hyperplasia in mid and high dose males. This suggests a significant effect of colesivelam hydrochloride, or any of its degradants, on pancreatic tumorigenesis in the rat. The clinical significance of this finding is unclear.

There was a statistically significant dose-tumor positive linear trend in incidence of benign thyroid C-cell adenoma in female rats, when analysis was done using only the data from concurrent control group 2 (vitamin-supplemented group) (p=0.003). The effect was not significant when control group 1 (basal diet) was included since the incidence in this group was much higher and similar to the incidence in the high dose females (n = 20-11-4-5-19). Except for the thyroid C-cell adenoma in females, there were a number of other tumors whose incidence appeared to be decreased in control group 2 as compared to control group 1 (fibrosarcoma, s.c. tissue, in males, adrenal cortical adenoma in females, lymph node lymphosarcoma in females, uterine endometrial stromal polyp in females). This might be taken to believe that vitamin supplementation has the potential to suppress tumor formation. Concomitant administration of colesivelam hydrochloride might then cause vitamin levels to decrease with an obliteration of this effect. The data obtained in this study on vitamin D and E levels in females indeed show a slight increase in vitamin D and E levels in control group 2 versus control group 1. However, in females there were no significant differences in vitamin D or E levels between control group 2 and the three test article treated groups, suggesting vitamin depletion is not underlying the thyroid tumorigenicity. There was also an increased incidence of thyroid C-cell adenoma (n = 8-7-7-5-13) and thyroid C-cell hyperplasia

in males. However, the increased thyroid C-cell adenoma incidence in males was not statistically significant. Taken together, this Reviewer feels that these findings suggest an effect of colesevelam hydrochloride, or any of its degradants, on thyroid C-cell tumorigenesis in the rat. The clinical significance of this finding is unclear.

There was an increase in the incidence of pancreatic islet cell carcinoma in the mid dose female rats (n = 0-0-0-3-1), which was statistically significant according to a pairwise test versus the combined control groups (p=0.035). Since the finding was in the mid dose group its significance is unclear.

The mechanism of induction of tumors by colesevelam hydrochloride as suggested by the findings in the rat study is unclear. One explanation is that the tumors result from a secondary effect of the drug related to its primary pharmacodynamic action, e.g., decreases in serum vitamin or lipid changes. Another possible explanation is that, since some of the compound or its degradants are probably absorbed systemically, the compound is directly causing the tumorigenicity, possible via a genotoxic mechanism. In this regard, it is worthwhile noting that a polymer extract and two of the four degradants tested in a chromosomal aberration assay were found to be positive under particular conditions of the assay (see Genetic Toxicology).

Mouse Study

A 104-week carcinogenicity study was carried out in the mouse with a high dose of 3.0 g/kg/day.

Study duration	Doses (g/kg/day)	Route	Human dose multiple*
104 weeks	0, 0, 0.3, 1.0, 3.0	Oral (diet)	0x, 0x, 5x, 17x, 50x

*Based on the maximum human dose of 4.5 g/day (60 mg/kg/day), and compared on the basis of body weight (negligible absorption)

There were no significant tumor findings. Note, however, that toxicity in the mouse carcinogenicity study dose groups was minimal, and that the doses used were therefore not optimal.

Genetic Toxicology

Colesevelam Hydrochloride

Three genotoxicity assays were carried out with colesevelam hydrochloride. Since the test compound is a polymer, two *in vitro* assays were done with an extract of the compound (Ames test, Chinese Hamster Ovary (CHO) cell chromosome aberration test) and one *in vivo* assay was done with the polymeric drug substance itself (mouse micronucleus assay).

An extract of the test article was negative in the Ames bacterial reverse mutation assay with and without metabolic activation.

An extract of the test article was positive in the CHO cell chromosome aberration assay with metabolic activation. The positive response occurred at the highest extract level evaluated of 100 ul/ml where cell growth inhibition as compared to solvent control was 52%. Although at this dose level as well as at all the other dose levels tested (7-100 ug/ml) precipitate was observed in the treatment medium containing the test article extract this Reviewer feels that this positive finding can not be dismissed and should be mentioned in the label. The extract was negative in the absence of metabolic activation.

The test compound was negative in the mouse micronucleus assay. However, the validity of an *in vivo* mouse micronucleus assay is disputable since only a very small amount of the test compound and/or of small molecular weight species present in the drug substance is absorbed, and the bone marrow in the test species is most likely not exposed to sufficiently high doses of the drug or any of its fragments.

Degradants

In addition to testing an extract of the parent compound, both Ames bacterial reverse mutation tests and CHO cell clastogenicity tests were carried out with four degradants of the test

compound. The four degradants have been identified at levels approaching — % in the drug substance and drug product during long-term stability studies. The degradants are decylamine HCl, aminoethyl-trimethyl ammonium chloride HCl (aminoquat), didecylamine HCl, and 6-decylamino-hexyltrimethyl ammonium chloride HCl (decylaminoquat).

All four degradants were negative in the Ames bacterial mutation assay with and without metabolic activation.

In the CHO cell chromosome aberration assay, two degradants, e.g., decylamine HCl and aminoethyltrimethyl ammonium chloride HCl, were positive under the test condition of 4h treatment and 16h recovery in the absence of metabolic activation. The positive responses occurred at the highest dose levels evaluated of 15 ug/ml and 5000 ug/ml, where cell growth inhibition was 52% and 5%, respectively. However, the two degradants were negative under the test condition of 20h treatment in the absence of metabolic activation. The two degradants were also negative in the presence of metabolic activation. The two other degradants (didecylamine HCl and 6-decylamino-hexyltrimethyl ammonium chloride HCl) were negative under all conditions.

The positive effects of both a polymer extract and of two of the four degradants in the CHO cell chromosome aberration assay may be a cause for concern, in particular since positive carcinogenicity findings were also obtained in the rat, and since animal studies have shown that there is a small amount of absorption of the test compound or its soluble components. The positive outcome of these tests was in all cases due to an elevation in the percentage of cells with structural chromosome aberrations at the highest dose level tested. However, the positive outcomes at the highest dose levels were not accompanied by largely increased cytotoxicity as compared to the next lower dose levels tested. Nevertheless, testing in a 20h treatment regimen of the two degradants that were found to be positive in the 4h treatment assays did not reproduce the positive results. Another consideration with regard to the significance of the positive outcome of the clastogenicity tests is the fact that there are no structural alerts obvious from the structure of the degradants.

Taken together, this Reviewer feels that the study results obtained with the two degradants that tested positive under one assay condition (4h treatment) but not the other (20h treatment) were equivocal, and recommends mention of these equivocal findings in the product label.

Reproductive Toxicity

Four reproductive toxicity studies were carried out with colesevelam hydrochloride. Three tests were done in rats (Segment I, II, III) and one in rabbits (Segment II).

Doses used in reprotoxicity studies expressed as multiples of maximum human dose (4.5 g/day, or 0.075g/kg/day)

	Rat			Rabbit
	Segment I	Segment II	Segment III	Segment II
Doses (g/kg/day)	0.2, 1.0, 2.0	0.3, 1.0, 3.0	0.1, 0.3, 1.0	0.1, 0.5, 1.0
Human dose multiples	2.7x, 13x, 27x	4x, 13x, 53x	1.3x, 4x, 13x	1.3x, 6.7x, 13x

Multiples are calculated on the basis of kg body weight (test compound is non absorbed)

Segment I (rat)

Reproductive and fertility parameters were not affected by colesevelam hydrochloride at oral dietary doses as high as 2 g/kg/day in rats.

Segment II (rat)

Colesevelam hydrochloride had no adverse effects when administered to presumed-pregnant female rats on fetal development at oral dietary doses up to 3 g/kg/day. There were no significant effects on viability, sex ratios, fetal body weights, or external soft tissue or skeletal morphology of the fetuses in this study.

Segment III (rat)

Colesevelam hydrochloride did not cause maternal toxicity and had no adverse effects on reproductive performance during gestation, parturition, or lactation, and no effect on the survival,

physical development, behavior, and reproductive performance of the F1 generation at oral gavage doses up to 1 g/kg/day. Also, there were no effects on the survival and physical condition of the F2 generation pups.

Segment II (rabbit)

Colesevelam hydrochloride had no adverse effects on viability, sex ratio, fetal body weight, or external soft tissue or skeletal morphology of the fetuses of presumed-pregnant female rabbits at oral gavage doses up to 1g/kg/day.

These data suggest that colesevelam hydrochloride has no adverse effects on reproduction, fertility, and fetal development in rats and on fetal development in rabbits.

Special Toxicity

Special toxicity studies of 28-day duration with the four degradants identified in the drug substance were carried out in rats. NOAEL for didecylamine and 6-decylamino-hexyltrimethyl ammonium chloride HCl (decylaminoquat) were 15 mg/kg, which is equivalent to — the maximum human dose. NOAEL for males for decylamine and aminohexyl-trimethyl ammonium chloride HCl (aminoquat) were also 15 mg/kg, i.e., — the maximum human dose. For females, however, the NOAEL for the latter two compounds was <1.5 mg/kg, or less than — the maximum human dose. The effects seen at the LOAEL low dose of 1.5 mg/kg were decreased body weight and food consumption, elevated APTT and decreased serum globulin levels.

APPEARS THIS WAY
ON ORIGINAL

RECOMMENDATION

Pending agreement with the Sponsor on the relevant sections of the package insert, Pharmacology/Toxicology recommends approval of colesevelam hydrochloride for the indication of adjunctive therapy to diet and exercise for the reduction of elevated LDL cholesterol in patients with primary hypercholesterolemia.

Reviewer signature



/S/

Gemma Kuijpers

4/19/00

Team leader signature Concurrence/Non-concurrence

/S/

Ronald Steigerwalt, Ph.D.

See team leader memo to file

4/19/00

**APPEARS THIS WAY
ON ORIGINAL**

Cc: NDA Arch
HFD-510
HFD-510/Steigerwalt/Kuijpers/Koch

LABELING REVIEW

Carcinogenesis, Mutagenesis, Impairment of Fertility

A 104-week carcinogenicity study with colesevelam _____ (Welchol™) was conducted in CD-1 mice, at oral dietary doses up to 3 g/kg/day. This dose was approximately 50 times the maximum recommended human dose of 4.5 g/day, based on body weight, mg/kg.

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Pregnancy

Pregnancy Category B.

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Pediatric Use

The safety and efficacy of colesevelam hydrochloride (Welchol™) have not been established in pediatric patients.

Geriatric Use

There is no evidence for special considerations when colesevelam hydrochloride (Welchol™) is administered to elderly patients.