PHM3

Date of study initiation: Oct 27, 1998
Date of study completion: Dec 11, 1998
GLP Compliance: Yes
Drug Lot # 7231:040
Study end point: Bacterial reverse mutation in vitro (Ames test)
Strains: Salmonella typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and E.coli strain WP2uvrA.
Dose selection criteria: Highest dose was selected on the basis of the toxicity and precipitates of the test article. The condition of the background lawn was evaluated for the evidence of toxicity using a light microscope. Following criteria were used for the toxicity and degree of precipitation of the test material.

Description	Characteristics
Normal	Distinguished by a healthy microcolony lawn
Slightly reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
Moderately reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
Severely reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
Absent	Distinguished by a complete lack of any microcolony lawn over >90% of the plate.
Obscured by precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.
Non-interfering precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than 10% of the revertant colony count.
Interfering precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count.

In the preliminary toxicity assay maximum dose tested was 5000-µg per plate for TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA in the absence and presence of S -9 fractions of the liver homogenates. No precipitate or toxicity was observed at 5000µg dose. The highest dose employed in the assay was 5000µg/plate. The solubility limit of the test substance is 500mg/ml. The stock solution used for achieving the maximum amount per plate was 100 mg/ml. Generally the highest dose chosen for the Ames assay is about 5000µg-1000µg as the limit dose, if no toxicity is detected in the preliminary assay. Using that approach, the sponsor chosen a limit dose as the maximum dose for the mutagenicity assay.

Test agent stability consideration: Not mentioned in the report. However, a certificate of analysis of the test article is provided in the report. The test article dilutions were prepared immediately before use.

Metabolic activation system: Aroclor- treated male Sprague-Dawley rat liver homogenates were used as the source of metabolic activation system. Each assay plate contained 0.5 ml of S-9 or sham mixtures. The compositions of the S-9 mixtures were liver homogenates (10% protein), 5mM glucose-6-phosphate, 4

mM NADP, 8 mM MgCl and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The sham S-9 mixtures contained 100-mM phosphate buffer.

Controls:

Vehicle used in the assay was sterile distilled water.

Appropriate positive controls were used. The positive controls are shown in the following table.

Strain	S-9 activation	Positive control	Concentration/plate (µg/plate)
All S. typhimurium, E.coli	+	2-aminoanthracene	S. typhimurium (1.0) E. coli (10)
TA 98	-	2-nitrofluorene	1.0
TA 100, TA 1535	-	Sodium azide	1.0
TA1537	-	9-aminoacridine	75
WP2 uvrA	-	Methyl methanesufonate	1000

Exposure conditions:

Agar media supplemented by L-histidine, D-biotin and L-tryptophan to a final concentration of 50 µL were used. 0.5 ml 0f S-9 mixtures or phosphate buffer, 100 µL of the tester strain ($0.3X10^9$ cells/ml), 50 µL of vehicle or test article or the positive control were added to 2 ml molten selective agar at 45C. After mixing, the medium was plated on the surface of the agar plate. The plate was incubated for 48-72 hours at 37 and counted thereafter for number of revertant colony.

Doses used in the definite study were 100, 333, 1000, 3333 and 5000µg.

Analysis:

Three plates for each concentration were used in the assay. Number of colonies was counted by automatic colony counter or by hand unless the assay was the preliminary toxicity assay or the plate showed toxicity.

Mean and standard deviation of the mean for the number of revertant colony was determined.

Criteria for a positive results:

The test article must cause a dose-related increase in the revertants per plate in at least one tester strain with a minimum of two increasing concentrations of the test article. Criteria for a positive response for TA 1535 and TA 1537 were an increase in the revertant colonies of equal or greater than 3 times mean vehicle control. Criteria for TA 98, TA 100 and WP2 uvrA were an increase in number of revertants of equal or greater than two times the mean vehicle control.

The mean of each positive control should exhibit at least three-fold increase in the number of revertants of the vehicle control.

At any concentration, a reduction of more than 50% in the reverent colony compared to the vehicle control was considered to be a toxic response.

Results:

Preliminary toxicity study at maximum 5000µg per plate showed no precipitate and toxicity to tester strains TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA. The sponsor selected 5000 µg as the highest amount in the mutagenicity assay.

The data for the mutagenicity assay in the absence of S-9 mixtures are shown in the following table.

Dose (µg)	TA 98	TA 100	TA 1535	TA 1537	WP2 uvrA
0	10	138	8	5	13
100	10	153	9	3	10
333	11	151	8	2	9
1000	11	147	9	4	9
3333	8	155	11	4	4
5000	11	151	6	5	4
Positive control	409	534	412	515	178

The data in the presence of S-9 mixtures are shown below.

Dose (µg)	TA 98	TA 100	TA 1535	TA 1537	WP2 uvrA
0	14	180	7	3	16
100	11	158	7	6	10
333	17	178	9	5	9
1000	15	153	9	5	7
3333	15	176	5	4	5
5000	16	167	7	5	4
Positive control	531	848	72	129	273

Above data suggest that levobetaxolol is not mutagenic in Ames assay.

Second Study:

The sponsor attached another report for Ames assay Protocol # N-86-46 submitted on page 5-03179 vol 9. The assay was conducted at ( ) according to the GLP. The study was initiated on Sept 5, 1986 and completed on Dec 2, 1986. Since the procedures of the assay were similar to that described above. The study was conducted according to the GLP.

Method:

The batch # for S-(-) betaxolol used was ERM 1740:080 was dissolved in sterile deionized water. The experiment was conducted using TA 1535, TA 1537, TA 1538, TA 98 and TA 100 strains of S. typhimurium. The positive controls were sodium azide (water was used as the solvent), 2-nitrofluorene and quinacrine mustard (DMSO was used as the solvent) in the absence of the activating system. In the presence of S-9 activation, 2-anthramine was used as a positive control and DMSO was used to dissolve it.

Preliminary assay was conducted using levobetaxolol ranging from 1.22µg per plate to 10000 µg per plate using TA 100. Results of the preliminary assay suggest that 10,000 µg was toxic to the plate as evident from the reduction of the number of revertant colonies and appearance of thin microcolonies. Mutagenicity test was conducted at 8 doses ranging from 1-10,000µg/plate. The assay was conducted using three plates per dose level.

The positive controls are shown in the following table.

Positive control	Strain	Concentration, µg/plate
Sodium azide	TA 1535, TA 100	10
2-Nitrofluorene	TA 1538, TA 98	10
Quinacrine mustard	TA 1537	5.0
2-Anthramine (in the presence of S-9 mixtures)	All strains	2.5

The incubation mixtures consisted of 2 ml of agar media, 0.05-0.1 ml of the solution of test chemical, 0.2 ml of the organism (number/ ml not given) and 0.2 ml of phosphate buffer for the non-activation system. The mixtures were plated on the selective medium in a plate-contained histidine and biotin. The plates were incubated at 37C for two days. Number of colonies was counted using an automated counter. For the plates containing activation system, 0.5 ml of S-9 mixtures were added instead of the phosphate buffer.

Data sets were evaluated as positives for stains TA 1535, TA 1537 and TA 1538 if a dose response was observed over a minimum of 3 test concentrations and the increase in revertants were equal to or greater than three times the solvent.

For strain TA 98 and TA 100, the results were considered to be positive if the dose response was observed over three test concentrations and the increase in revertant colonies were double or higher than the solvent control.

The mutation assays were conducted using three plates per dose level.

Results:

The results of the experiment without S-9 mixtures are shown in the following table.

Test System	TA 1535	TA 1537	TA 1538	TA 100
Solvent	14.7	9.3	10.0	130
Positive control	669	202	837	560
1 µg	19.3	10.3	12.0	149
10	17.7	12.3	13.7	133.3
100	23.3	13.0	11.3	145.3
500	22.7	11.3	15	149.7
1000	25	9	13.0	143.3
2500	22.3	12.3	13.0	170.7
5000	28.7	10.0	25.0	162.3
10000	18.7	8.0	27.3	137.3

APPEARS THIS WAY
ON ORIGINAL

APPEARS THIS WAY
ON ORIGINAL

The results in the presence of the activation system are shown in the following table.

Test system	TA 1535	TA 1537	TA 1538	TA 100
Solvent control	11.0	10.0	19.3	142.3
Positive control	287.7	105.7	961.7	832.0
1.0µg	13.7	16.0	21.7	146
10µg	7.7	15.0	20.3	152.7
100µg	15.0	17.7	17.3	154.0
500µg	9	13.7	15.3	149.0
1000µg	11.3	11.7	23.7	154.3
2500µg	11.7	11.0	24.0	164.7
5000µg	12.0	13.3	29.0	169.3
10000µg	10	13.0	42.3	173.7

The solvent system was sterile deionized water.

Results showed that S (-) betaxolol had an increase in revertants at 5000 and 10000µg in strain TA 1538. Also the sponsor stated that due to contamination, the test was not conducted in the first experiment using TA 98. Therefore, the assay was repeated using TA 1538 and TA 98 strains. The result of the repeat assay in the absence of S-9 mixtures is shown below.

Concentration/ system	TA 1538	TA 98
Solvent control	14.3	23.3
Positive control	1645.3	1157.7
1.0 µg	13.3	27.7
10.0µg	14.3	23.0
100µg	15.3	23.7
500µg	16.0	21.7
1000µg	15.3	22.7
2500µg	13.3	20.3
5000µg	21.7	22.7
10000µg	11.3	16.7

The data with the activation system are shown below.

Concentration/system	TA 1535	TA 98
Solvent	29.7	29.7
Positive control	1550	1752
1.0µg	27.3	36.7
10µg	31.3	43.7
100µg	28.3	45.3
500µg	42.3	39.0
1000µg	38.3	37.3
2500µg	8.5	8.9
5000µg	55.7	52.7
10000µg	56.7	51.3

Above data suggest that in the presence of S-9 mixtures, TA 1535 and TA 98 strains increased number of revertant colonies at 5000 and 10000µg although the increase was not similar to that is qualified under the criteria for a positive response. Therefore the experiment was repeated at 2500, 5000 and 10000µg doses in the presence of S-9 mixtures. Results are shown below.

Concentration/system	TA 1538	TA 98
Solvent	23	29
Positive control	1751	1941
2500µg	39.3	40.3
5000µg	43.7	59.3
10,000µg	25.7	34.7

Above data suggest that the increase in revertant colonies at 2500µg and higher doses was not reproducible.

Reviewer's conclusion: It was concluded that S (-) betaxolol was negative in the Ames assay. The positive controls showed increase in the revertants colonies.

Sponsor's summary and conclusion:

Under the conditions of this study, test article levobetaxolol hydrochloride was concluded to be negative in the bacterial reverse mutation assay.

Cytogenetic study:

In vitro cytogenetic assay measuring chromosome aberration frequencies in Chinese hamster ovary cells (CHO) using S (-) betaxolol hydrochloride.

Page 5-03213, vol 9, Protocol: N-86-50

Study dates:

Initiated: July 29, 1986

Completed: Sept 12, 1986

GLP compliance: Yes

Drug Lot #: ERM 1740:080

Study end point: In vitro clastogenesis.

Cell Line: Chinese hamster ovary cells (CHO) in culture.

Dose selection criteria:

The test material is soluble at 500 mg/ml McCoy's culture medium. Toxicity and cell cycle kinetics data were used for the selection of doses. Preliminary study showed that the test substance inhibited cell mitosis at 500µg/ml to 5 mg/ml. Cell cycle kinetics data showed that there was a delay in the cell cycle at 167µg/ml. Therefore, 10-hour harvest time and a dose range between 37.5 µg/ml through 500µg/ml was chosen in the absence of S-9 mixtures. In the presence of S-9 mixtures, dose range of 37.5 µg/ml to 500 µg/ml was chosen for the assay at 10-hour harvest time.

The sponsor stated that analysis of the data on the cell cycle kinetics from the repeat experiments conducted for sister chromatid exchange assay using the same cell line suggests that the increase of the

harvest time was possible. Accordingly mitotic cells were harvested after 20-hours. The concentrations chosen for the experiment with 20-hour harvest time without S-9 mixtures were between 45 and 600 μg/ml. The concentrations in the presence of S-9 mixtures were between 70 μg/ml to 700 μg/ml.

Metabolic activation system:

Following mixtures were used for metabolic generating system:

1. S-9 liver homogenates, 15μL/ml.

2. NADP, 1.5 mg/ml.

3. Isocitric acid, 2.7 mg/ml.

The liver homogenates were prepared from male S-D rats previously treated with Aroclor 1254 for the induction of hepatic enzymes.

Controls: McCoy's 5a medium at 10 μL/ml of the reaction mixture was used as the solvent and negative control.

Positive controls:

Mitomycin C was used as a positive control in the absence of S-9 mixtures. The concentrations used for the 10-hour harvest time were 500 ng/ml and 1μg/ml The concentrations used for 20-hour harvest time were 40 and 80 ng/ml.

Cyclophosphamide was used as a positive control in the presence of S-9 mixtures. The concentrations used for 10-hour harvest time were 25 and 50 μg/ml. The concentrations used for 20-hour harvest time were 12.5 and 17.5 μg/ml. These positive controls are appropriate for the test system.

The sponsor stated that only one dose was actually analyzed in each of the aberration assay.

Exposure conditions:

The culture was initiated by seeding 1.2 to 1.5 million cells per flask. The culture was treated with the test article one day after the initiation of the culture.

Cells were exposed to the test article continuously until about 2.5 hours before the harvest time for the non-activation system, for example 7.25 or 17.25 hours for 10 and 20-hour harvest time, respectively. Following the treatment, the test substance was washed from the cells and Colcemid (0.1μg/ml) was added for arresting cells at metaphase. The metaphase cells were harvested after 2 and half-hours after Colcemid addition.

Cells were treated with test substance for two hours at 37C in the presence of S-9 mixtures for the metabolic activation assay. At the end of the treatment period with the test substance, cells were washed with the fresh medium and reincubated with the culture medium for the remaining period (7.25 or 17.8 hours). Colcemid was added 2.5 hours before the harvest time for the arrest of the cells in the metaphase. Cells at metaphase were treated with hypotonic KCl solution (0.075M), fixed with methanol: glacial acetic acid (3:1) mixtures before preparing slides for the determination of the chromosomal aberrations.

The harvest time mentioned above was approximate. The harvest time referred in the result for the 20-hour assay was 20.1 hours.

The slides were stained with 5% Giemsa solution at pH 6.8.

Doses of S (-) betaxolol used in the definitive study:

In the absence of S-9 mixtures: Concentrations used were 175, 250, 375 and 500 µg/ml for 10-hour harvest time. Concentrations used for 20-hour harvest time were 150, 300 and 450 µg/ml.

In the presence of S-9 mixtures: The concentrations used for 10 hour harvest time were 175, 250, 375 and 500 µg/ml. Concentrations used for the 20 hour harvest time were 70, 175 and 350 µg/ml. At 525 and 700 µg/ml complete toxicity was noted. Therefore, results up to 350 µg/ml have been reported.

Analysis:

100 cells at metaphase for each duplicate culture were scored for each dose of the test substance. Single culture (100 cells) was used for the negative or solvent cultures. About 25-50 cells were scored for the positive controls. For the experiment without S-9 mixtures at 20-hour harvest time, 150 and 50 cells were scored at 450 µg/ml. Cultures were analyzed for the chromosomal aberration. All slides except the positive controls were blinded for the analysis of the chromosomes. Following changes were recorded.

1. Overall chromosomal aberration frequencies.
2. The % of cells with any aberrations.
3. % Of cells with more than one aberration.
4. Evidence of dose dependent damage.
5. Number of breaks.

Chromatid and isochromatid gaps were noted but not considered for chromosomal aberrations.

Cytotoxic endpoints:

Visual observations of toxicity was made by the assessment of the % confluence of the cell monolayer and presence of dead cells floating in the medium Prior to the harvest of the cultures.

Statistical methods:

Fisher's exact test with an adjustment for multiple comparisons was conducted. Data for the treated groups were compared with the results of pooled solvent control for % of cells with aberrations. The statistical significance was established at $p>0.05$.

Criteria for positive results: The sponsor did not mention the criteria for a positive response in the report. However, analysis of statistical significance has been given in the report for determining statistically significant response.

Results:

Number of aberrations per cell at 10 hours harvest time in the absence of S-9 mixture is shown in the following table.

Treatment	Cells scored	# of aberrations/cell	% cells with aberration	% cells with >1 aberration
Negative and solvent control	200	0.06	5.5	0.5
Mitomycin C 1.0μg/ml	25	0.48	28.0*	8.0*
S (-) betaxolol, 175 μg/ml	200	0.04	3.0	0.5
250 μg/ml	200	<0.06	4.5	2.0
375 μg/ml	200	0.03	2.5	0.5
500 μg/ml	200	<0.03	2.5	0.5

* Significantly greater than pooled negative and solvent controls, $p<0.01$

Cytotoxicity e.g. a reduction in monolayer confluence and visually observable mitotic cells was present at 375 and 500 μg/ml. However no significant increase in the aberrations was observed at 10-hour harvest time in the absence of S-9 mixtures.

Data for 20-hour harvest time in the absence of S-9 mixtures are shown below.

Treatment	Cells scored	# of aberrations/cell	% cells with aberrations	% cell with more than 1 aberrations
Negative and solvent control	200	0.01	0.5	0
Mitomycin C, 80 ng/ml	25	0.44	28.0*	12.0*
S (-) betaxolol, 150 μg/ml	200	0	0	0
300 μg/ml	200	0.01	0.5	0
450 μg/ml	200	0.02	1.5	0

* Statistically significant than pooled negative and solvent controls, $p<0.01$

The data suggest that there was no drug-induced aberration after 20 hours harvest of cells. The sponsor stated that cytotoxicity was noted at 450- 500μg/ml.

Results of the chromosomal aberration assay in the presence of S-9 mixtures at 10-hour harvest time are shown below.

Treatment	Cells scored	# of aberrations per cell	% cells with aberrations	% cells with >1 aberrations
Negative and solvent controls	200	<0.05	5.0	0.5
Cyclophosphamide, 50 μg/ml	50	0.14	14.0*	0
S (-) betaxolol, 175 μg/ml	200	0.02	1.5	0
250 μg/ml	200	<0.05	4.5	0.5
375 μg/ml	200	0.04	3.5	0.5
500 μg/ml	200	<0.03	2.5	1.0

*Statistically significant compared to pooled negative and solvent control data, $p<0.01$

Above data suggest that there was no treatment-related aberration in the presence of S-9 mixtures at 10-hour harvest time.

Data for the 20-hour harvest time are shown in the following table.

Treatment	Cells scored	# of aberrations	% cells with aberrations	% cells with >1 aberrations
Negative and solvent control	200	0.01	1.0	0
Cyclophosphamide 17.5 µg/ml	25	0.60	40*	12*
S (-) betaxolol 70 µg/ml	200	0.02	1.0	0.5
175 µg/ml	200	0.02	1.5	0
350 µg/ml	200	0.02	1.5	0

*Statistically significant than pooled data from the negative and solvent control, $p<0.01$

Complete toxicity was noted at 525 and 700 µg/ml. The result did not show treatment-related aberrations.

Reviewer's conclusion: It was concluded that S (-) betaxolol did not induce clastogenic property in vitro in CHO cells in the presence or absence of S-9 mixtures.

Sponsor's conclusion:

The test article, S (-) betaxolol hydrochloride, is considered negative for inducing chromosomal aberrations in Chinese hamster ovary cells under both the metabolic activation and nonactivation conditions of this assay.

Sister chromatid exchange:

Title: Mutagenicity evaluation of S (-) betaxolol hydrochloride in an in vitro cytogenetic assay measuring sister chromatid exchange in Chinese Hamster Ovary (CHO).

Page 5-03253, Vol: 9.

Date of study initiation: July 29, 1986

Date of study completion: September 3, 1986
GLP compliance: Yes

Drug Lot # ERM 1740:080

Study end point: Chromosomal mutation characterized by exchange of chromosomal materials between sister chromatids. CHO cells in culture were exposed to the test article for two approximately two-cell cycles. Sister chromatid exchange frequencies were determined from metaphase cells (M_2).

Methods:

Cell Line: CHO cell line in culture.

Dose selection criteria:

Cellular toxicity due to S (-) betaxolol was determined in the preliminary assay. The concentrations that showed two cycles of mitosis in the presence of the test compound were used for the SEC assay. The highest dose was chosen to avoid cytotoxicity. The cytotoxicity was examined on the basis of percent confluence of the cell monolayer in the culture flask and on the basis of the presence of dead cells. Doses used in the preliminary assay were between 167µg/ml and 5 mg/ml.

Test agent stability: The test article was dissolved in McCoy's 5a culture medium to obtain a stock solution at 500-mg/ml concentration. The sponsor has not provided any stability data of the test substance under the experimental conditions.

Metabolic activation system:

The composition of the metabolic activation system is shown below:

1. 15 µl/ml of S-9 fraction of the liver homogenates prepared from Aroclor treated male S-D rats.
2. NADP 1.5 mg/ml.
3. Isocitric acid 2.7 mg/ml.

Controls:

Vehicle: McCoy's 5a culture medium.

Negative controls: McCoy's 5a culture medium.

Positive controls:

Mitomycin C was used in the absence of S-9 mixtures as the positive control at 5 ng/ml and 10 ng/ml.

Cyclophosphamide was used as a positive control in the presence of S-9 mixtures at 1.5 µg/ml and 2.0 µg/ml.

However, the analysis was performed on one dose only.

Concentrations used in definitive study in the absence of S-9 mixtures: 5, 16.7, 50 and 167 µg/ml in the first trial. Doses used in the second trial were 150, 200 and 300 µg/ml.

Concentrations used in the definitive study in the presence of S-9 mixtures:

16.7, 50, 167.0 and 500 µg/ml in the first trial. Concentrations analyzed in the second trial were 400, 500, 600 and 700 µg/ml. Due to increase cytotoxicity at 700 µg/ml, the SCE was analyzed up to 600µg/ml concentration.

Exposure conditions:

Non activation system:

The CHO cells were grown for 24 hours before the treatment by seeding about $1.0X10^4$ cells per 75-cm^2 flask in 10 ml of complete McCoy's 5a medium. Mutagenic effect was examined by incubating the cell

with the test substance or positive control. 5-Bromo-2'-deoxyuridine (BrdUrd) was added at 10μM final concentration approximately 2 hours after adding the test article or positive control to the cell. The cells were reincubated for another 23 hours after adding BrdUrd. The cells were washed with phosphate buffered saline. Cells were further incubated with the medium containing BrdUrd (10μM) and Colcemid (0.1 μg/ml) for another 2.5 hours. The total period of the incubation was about 27.5 hours.

The sponsor stated that there was a delay in the cell cycle kinetics at 300 μg/ml of S (-) betaxolol. Therefore, an additional harvest was performed after incubating the cells for additional 9 hours.

Metabolic activation assay:

Cells were exposed to the test substance and S-9 mixtures for two hours at 37C for the metabolic activation. The test material and the activation system were washed from the cell with buffered saline at the end of two hours. Cells were recultured in the media containing 10 μM BrdUrd for 23 hours. At the end of the incubation period, Colcemid (0.1 μg/ml) was added. Cells were harvested about 2.5 hours after addition of Colcemid. Due to delay in the cell cycle kinetics, an additional harvest was carried out at 600-μg/ml dose of the test substance after a further incubation for 9 hours.

Analysis:

Metaphase cells (M_2) were collected fixed and were [redacted] Slides were air dried and examined under [redacted]

Cultures that showed cell cycle delay were further incubated so that enough cells at metaphase (M_2) could be scored.

Fifty cells at metaphase (M_2) per concentration were analyzed for SCE from each of the top four concentrations. Fifty cells were read from each of the solvent and negative controls. At least 20 cells were read from one dose level of the positive control. All slides except positive control were coded for analysis.

Slides were also examined for the presence of delay of cell cycle. One hundred metaphase cells were examined if they were in M_1 or M_1+ M_2 cycle for estimating cell cycle inhibition.

Cells were scored for total SCE, SCE per chromosome and SCE per cell.

Cytotoxic endpoints:

Toxicity to the test compound was determined by assessing the percent of confluence of the cell monolayer in the flask and on the basis of the dead cells in the culture. In the preliminary, concentrations of S (-) betaxolol above 500 μg/ml showed cytotoxicity in the absence of S-9 mixtures. Cytotoxicity was observed at 1.67 mg/ml concentration of S (-) betaxolol in the presence of S-9 mixtures. Therefore, results were analyzed at lower than the toxic doses. However, cytotoxicity was monitored for individual test.

Statistical analysis:

Statistical significance was determined using Student t-test for comparing SCE frequencies with the negative and solvent control.

Criteria for a positive response:

If an increase in SCE is observed, one of the following criteria must be met for a positive response.

1. Two-fold increase in SCE frequency at one dose or more compared to the solvent and negative control.

2. A positive assessment is made in the absence of doubling the SCE if there was statistically significant increase at a minimum of three concentrations and if there was an evidence of positive dose response.
3. In some cases, statistically significant increase with neither doubling nor a presence of dose (concentration) response observed. These results were assessed on the basis of the reproducibility, magnitude of the response and from the proportion of the concentration level.

Results:

In the absence of S-9 mixtures:

Data for the sister chromatid exchange (SCE) in the absence of S-9 mixtures are shown in the following table.

Treatment	Cells scored	# of chromosomes	# of SCE	SCE/Chromosome	SCEs/Cell	Time in BrdU (hr)	% increase over solvent control
Negative control McCoy's 5a	50	1038	426	0.41	8.52	25.5	12
Solvent, McCoy's 5a, 10µl/ml	50	1044	382	0.37	7.64	25.5	
Mitomycin C (5.0 ng/ml)	50	1033	1381	1.34	27.6	25.5	262
S (-) betaxolol, 5.0µg/ml	50	1037	397	0.38	7.94	25.5	4
S (-) betaxolol, 16.7µg/ml	50	1047	353	0.34	7.0	25.5	
S (-) betaxolol, 50µg/ml	50	1036	379	0.37	7.58	25.5	
S (-) betaxolol 167µg/ml	50	1037	468	0.45	9.36	25.5	23

The percent of metaphase cells (M_2) were 95, 94, 95, 92, 94, 90 and 38 for negative control, solvent control, positive control, S (-) betaxolol 5, 16.7, 50 and 167 µg/ml, respectively. Data suggest that there was a delay in the cell cycle at 167 µg/ml concentration of S (-) betaxolol. There was an increase in the SCE at 167 above the solvent control. However, it was not statistically significant.

The sponsor decided to conduct another experiment at doses from 50 to 400µg/ml of S (-) betaxolol in the absence of S-9 mixtures because there was an increase in the SCEs /cell at 167 µg/ml. The sponsor analyzed SCE at 150, 200 and 300 µg/ml. No mitotic cell was observed at 400µg/ml due to cytotoxicity. There was 25% decrease in the monolayer confluence at 300µg/ml. The data for the repeat experiment are shown below.

Treatment	Total cells scored	# of chromosomes	# of SCE	SCEs/chromosome	SCEs/cell	Time in BrdU, hr	% increase over solvent control
Control, negative McCoy's 5a	50	1039	374	0.36	7.48	25.4	
Solvent, McCoy's 5a, 10μl/ml	50	1041	406	0.39	8.12	25.4	
Mitomycin C, 5.0 ng/ml	50	1048	1349	1.29	26.98	25.4	232
S (-) betaxolol, 150μg/ml	50	1044	423	0.41	8.46	25.4	4
S (-) betaxolol, 200μg/ml	50	1045	466	0.45	9.32	25.4	15
S (-) betaxolol, 300μg/ml	50	1045	593	0.57	11.86	34.4	46

The percent of metaphase cells at M_2 were 86, 82, 84, 42, 12 and 19 for negative control, solvent control, mitomycin C, S (-) betaxolol at 150, 200 and 300μg/ml, respectively. S (-) betaxolol treated cultures showed cell cycle delay at concentrations shown in the above table. Increase in the SCE was statistically significant at 300 μg/ml only. However, the P value was not indicated in the table. The harvest time at 300 μg/ml was increased to 34.4 hours due to non-availability of enough M_2 cells at the end of 25 hour.

The results suggest that S (-) betaxolol was not mutagenic in the assay due to lack of a statistically significant mutation at 150 and 200μg/ml. Also the response at 300 μg/ml was not considered to be mutagenic due to cytotoxicity.

APPEARS THIS WAY
ON ORIGINAL

In the presence of S -9 mixtures:

The experiment was conducted at doses between 16.7µg/ml and 5.0 mg/ml. Cytotoxicity was observed at 500µg/ml and above concentrations of S (-) betaxolol. Results were analyzed at 16.7 to 500 µg/ml and data are presented in the following table.

Treatment	Total cells scored	# of chromosomes	# of SCE	SCEs/chromosome	SCEs/cell	Time in BrdU, hr	% increase over solvent
Negative control, McCoy's 5a	50	1048	418	0.40	8.36	25.5	2
Solvent control, McCoy's 5a, 10 µl/ml	50	1046	411	0.39	8.22	25.5	
Cyclophosphamide, 1.5 µg/ml	50	1043	1463	1.40	29.26	25.5	256
S (-) betaxolol, 16.7µg/ml	50	1046	427	0.41	8.54	25.5	4
S (-) betaxolol, 50 µg/ml	50	1045	370	0.35	7.40	25.5	
S (-) betaxolol, 167 µg/ml	50	1047	426	0.41	8.52	25.5	4
S (-) betaxolol, 500 µg/ml	50	1042	703	0.67	14.06	25.5	71

Statistically significant increase in the SCE was noted at 500 µg/ml. The percent of M_2 (mitotic) cells were 94, 92, 91, 95, 97, 84 and 36 at negative control, solvent control, cyclophosphamide 1.5 µg/ml, S (-) betaxolol at 16.7, 50, 167 and 500 µg/ml, respectively. The sponsor stated that cytotoxicity characterized by reduction in monolayer confluency, floating cellular debris and a decrease in the M_2 cells were observed. Since the mutation was observed at a toxic dose, the sponsor repeated the study to examine the reproducibility of effect of the drug for SCE at comparable doses. Accordingly, the test was repeated at doses from 200µg/ml to 700 µg/ml.

Cytotoxicity, for example 90% reduction of monolayer confluency and cell debris were observed at 600 µg/ml concentration of S (-) betaxolol. Results were analyzed at 400, 500 and 600 µg/ml as shown in the following table.

Treatment	Total cells scored	# of chromosomes	# of SCE	SCEs/chromosome	SCEs/cell	Time in BrdU, hr	% increase over solvent
Negative control, McCoy's 5a medium	50	1050	384	0.37	7.68	25.4	
Solvent control, McCoy's 5a, 10µl/ml	50	1050	370	0.35	7.40	25.4	
Cyclophosphamide 1.5 µg/ml	25	526	710	1.35	28.40	25.4	284
S (-) betaxolol, 400 µg/ml	50	1047	441	0.42	8.82	25.4	19
S (-) betaxolol, 500µg/ml	50	1047	730	0.70	14.60	25.4	97
S (-) betaxolol, 600 µg/ml	50	1049	1576	1.50	31.4	34.4	326

The percent of M_2 (mitotic cells) was 84, 92, 84, 72, 55 and 66 for the negative control, solvent control, cyclophosphamide, S (-) betaxolol at 400, 500 and 600 µg/ml, respectively.

The harvest time after adding BrdU at 600 µg/ml was increased to 34.4 hours due to the drug induced delay of the cell replication. The increase in SCE at 600µg/ml was statistically significant.

The response at 500 µg/ml was considered to be positive for mutation. Although cell cycle delay was observed at 500 µg/ml concentration of S (-) betaxolol, the effect of the drug on mutation was statistically significant, almost double in the SCE frequency and reproducible. A positive dose response trend was also observed in the experiment.

The ICH guidelines for cytotoxicity in the mutagenicity assay suggest that the inhibition of the cell proliferation and culture confluency should be higher than 50% for considering cytotoxicity. However, these criteria of cytotoxicity were not met at 500µg/ml concentration of S (-) betaxolol in the repeat assay. Therefore, S (-) betaxolol is considered to be mutagenic in the assay in the presence of S-9 mixtures at a non-cytotoxic concentration in the repeat assay.

Reviewer's conclusion: It is concluded that S (-) betaxolol is mutagenic in the presence of S-9 mixture in SCE assay.

Sponsor's conclusion:

Under the conditions and evaluation criteria for this study, S (-) betaxolol hydrochloride is considered negative for inducing sister chromatid exchange in Chinese hamster ovarian cells under conditions of metabolic nonactivation. However, S (-) betaxolol hydrochloride is considered positive under conditions of metabolic activation.

Mouse lymphoma assay:

Title: Mouse lymphoma forward mutation assay with S (-) betaxolol.

Page 5-03302, vol 9

Date of study initiation: Aug 1, 1986
Date of study completion: Sept 29, 1886

GLP compliance: Yes

Drug lot number: ERM 1740:080

Methods:

Cell line: Mouse lymphoma cells in culture, L5178Y, TK $^{+/-}$ Forward mutation assay.
Dose selection criteria:

Five or six concentrations were selected to cover a range of toxicity to cellular growth. The concentrations for the mutagenicity assay were chosen (5 or 6 concentrations) to cover the cell growth from 80-100% of the control to almost 10-20% survival compared to the control.

Stability and solubility of test agent:

S (-) betaxolol is soluble in water at 100 mg/ml. Stock solution was prepared prior to the assay. The stock solution was diluted in water to the appropriate concentrations. Final dilution of the stock solution was conducted in the culture medium. The test material is soluble in the culture medium up to 5000 µg/ml.

Metabolic activation system:

S-9 fraction of rat liver homogenates was obtained from a commercial source. It was prepared by treating male rats with Aroclor-1254. Liver tissues were homogenized and 9000g supernatants were separated. Each batch of S-9 homogenates was checked for sterility, aryl hydroxylase activity and protein content. NADP and isocitric acid (the concentration is not specified in the report) were added in the S-9 homogenates for the metabolic activation.

Controls:

Solvent control:

Water is used as the solvent control for S (-) betaxolol. The sponsor has not mentioned specific solvent used for 3-Methylcholanthrene. However, the methods stated that if organic solvent is necessary for dissolving compounds used in the assay, either DMSO or ethanol or acetone is used. The sponsor stated that at least two solvent controls were included in each assay.

Negative control:

Water used in the assay is considered to be the negative control.

Positive controls:

Ethylmethane sulfonate (EMS) is used as the positive control in the non-activation assay at 0.25-0.5 µl/ml final concentration. 3-Methylcholanthrene is used in the metabolic activation assay at 1-4 µg/ml as the positive control.

Exposure conditions:

Non-activation assay:

The cells were seeded at $6x10^6$ cells per tube. Cells were grown in RPMI 1640 medium. The dosed tubes were incubated at 37C for 4 hours. The cells were washed twice and reincubated for two days for the expression of TK-/- phenotype cells. Cell counts were adjusted to $3x10^5$/ml by dilution after 24 hours of the treatment with the test substance for maintaining optimal growth of cells. Cells were again counted on day 2. At the end of the expression period, cells ($3x10^6$) from each tube for mutagenicity test was sampled and distributed to three 100-mm dishes so that each dish contains $1X10^6$ cells. Cells were exposed to the selective medium containing 3µg/ml of 5-trifluorothymidine (TFT) for 10-14 days. The mutation frequency was determined.

The cloning efficiency was determined by growing cells in non-selective medium. The samples were distributed to 3 dishes each containing $1x10^6$ cells. The cloning efficiency is determined by diluting the sample and seeding each of three dishes with 200 cells in cloning medium (non-selective medium). All dishes were incubated for 10-14 days at 37C and colonies were counted at the end of the incubation period.

Activation assay:

This assay is similar to that described for the non-activation assay. However, liver S-9 homogenates and cofactors were added during the four-hour pretreatment period with the test or appropriate positive-control.

Doses used in the definitive study:

Non-activation system:

S (-) betaxolol concentrations in the absence of S-9 metabolic mixtures were 75, 100, 150, 200, 250 and 300 µg/ml in the first assay. The concentrations used in the second assay were 100, 150 200 and 250 µg/ml.

Activation assay:

The concentrations used in the activation assay were 75, 150, 200, 250, 300 and 400µg/ml. The experiment was repeated at 150, 200, 250, 300, 350 and 400 µg/ml.

Analysis:

Auto or manual counting: The colonies were counted with an electronic colony counter.

Cytotoxic end points:

Preliminary cytotoxicity due to the test substance was determined in the absence or presence of S-9 metabolic activation mixtures. The test substance was incubated up to 5 mg/ml for four hours with the cells at 37C. The cells were washed and resuspended in the growth medium and incubated for another 24

hours. Cytotoxicity was determined by counting cells. The concentration that shows a reduction in the cell growth compared to the solvent control was determined.

Little or no survival was considered to be cytotoxic.

Percent relative growth:

This parameter provides the effectiveness of the treatment and toxicity to each treatment during the expression time. The percent relative growth was determined by growth of cells over two-day of expression time of the cells multiplied by the average cloning efficiency (%) of the solvent control.

Genetic toxicity endpoints/results:

Non-activation system:

To determine actual number of cells capable of forming colonies, the cloning efficiency was examined by the following equation:

Cloning efficiency = Mean colonies per plate in the absence of selective media /Total cells per plate X 100

Mutant frequency/10^6 cells was calculated by dividing the total number of colonies in each of 3 dishes by total count in the set of three dishes and multiplied by $2x10^{-4}$.

Relative growth (%) of mutants compared to the solvent control was monitored as the indication of cytotoxicity. The highest dose was chosen so as to show cytotoxicity compared to the solvent control (see cytotoxic end points). All assays below 50% cloning efficiency were considered to be unacceptable.

Criteria for positive results:

The sponsor set following criteria for a positive response.

1. Minimal criterion necessary for considering a mutagenic response to be one hundred fifty percent of the concurrent solvent control mutant frequency plus $10x10^{-6}$.
2. A dose related or toxicity-related increase in mutant frequency in at least three doses.
3. If an increase of about two times the minimal criteria or greater is observed for a single dose near the highest testable toxicity, the test material will be considered mutagenic. A smaller increase at a single concentration near highest testable toxicity will require a confirmatory test.
4. An apparent increase in mutagenic activity as a function of decreasing toxicity is not an acceptable evidence of toxicity.
5. Treatments that induce less than ten- percent relative growth is included in the assay but are not used as primary evidence for mutagenicity.

The report does not include any statistical methods.

Results:

Non activation system:

The test substance was examined at 25 to 700 µg/ml for the mutation assay. However, the test substance was lethal at 400µg/ml. Therefore, mutant induction was examined at 6 concentrations ranging from 75 to 300µg/ml. According to the criteria of the assay, mutation frequency of $81X10^{-6}$ and above is considered to be a positive response for the assay. S (-) betaxolol did not show mutagenicity. The highest concentration used to examine mutation showed about 22% growth of the colony compared to the solvent

control. The positive control showed increase mutation at 0.25 and 0.40 µl/ml. The sponsor repeated the study at concentrations that show lower cellular growth, for example about 10-20% of the solvent control.

The data for the first assay is shown in the following table.

Treatment	Suspension growth	Cloning efficiency	Relative growth (%)	Mutant frequency (10^{-6} units)
Solvent control	17.4	87.8	100	50.5
Solvent control	15.1	91.3	100	44.5
Solvent control	12.2	90.2	100	46.9
EMS (0.25µl/ml)	10.8	88.5	71.3	339.0
EMS (0.4µl/ml)	8.6	58.5	37.6	385.7
	Average solvent control=14.9	Average solvent control=89.8		
	Relative to solvent control (%)	Relative to solvent control (%)		
S (-) betaxolol, 75µg/ml	96.8	111.4	107.8	45.0
S (-) betaxolol, 100µg/ml	100.9	98.6	99.5	44.4
S (-) betaxolol, 150µg/ml	101.2	116.6	118.0	39.5
S (-) betaxolol, 200µg/ml	77.6	105.0	81.5	41.0
S (-) betaxolol, 250µg/ml	53.7	100.0	53.7	46.4
S (-) betaxolol, 300µg/ml	25.9	88	22.8	59.1

There was no mutagenicity observed in the first assay as shown in the above table.

The data for the second experiment is shown in the following table.

Treatment	Suspension growth	Cloning efficiency	Relative growth (%)	Mutant frequency (10^{-6} units)
Solvent control	20.4	69.3	100	68.3
Solvent control	16.6	80.7	100	59.1
Solvent control	19.5	74.3	100	56.0
EMS, 0.25µl/ml	9.1	52.2	33.6	422.4
EMS, 0.40µl/ml	9.7	46.2	31.7	584.1
	Average solvent control, 18.8	Average solvent control, 74.8		
	Relative to solvent control (%)	Relative to solvent control (%)		
S(-) betaxolol, 100µg/ml	98.3	115.2	113.2	45.6
S(-) betaxolol, 150µg/ml	69.6	101.6	70.7	52.6
S(-) betaxolol, 200µg/ml	55.7	76.6	42.7	89.5
S(-) betaxolol, 250µg/ml	25	79.1	19.8	76.0

S (-) betaxolol at 400 μg/ml concentration showed lethal cytotoxicity. The mutation was analyzed up to 250μg/ml concentration. Mutation frequency above 101.7x10-6 is considered to be a positive as one of the criteria set for a positive response. Results showed that S (-) betaxolol is not mutagenic in the absence of the S-9 metabolic activation system.

Metabolic activation system:

Concentration of S (-) betaxolol from 75-500 μg/ml were used in the assay. However, mutation frequency was analyzed up to 300 μg/ml because excessive toxicity was observed at 500 μg/ml. The mutation frequency above 81.1×10^{-6} was considered to be one of the criteria for a positive response in the assay. The data are shown in the following table.

Treatment	Suspension growth	Cloning efficiency	Relative growth (%)	Mutant frequency (10^{-6} units)
Solvent control	15.9	81.5	100	45
Solvent control	13.4	96.8	100	49.6
Solvent control	13.7	96.7	100	47.6
MCA, 2.5μg/ml	7.6	41.8	24.2	359.4
MCA, 4.0μg/ml	8.7	58.7	38.8	321.0
	Average solvent control, 14.3	Average solvent control, 91.7		
	Relative to solvent control (%)	Relative to solvent control (%)		
S (-) betaxolol, 75μg/ml	99.9	106.3	106.2	45.8
S (-) betaxolol, 150μg/ml	98.0	93.6	91.7	42.3
S (-) betaxolol, 200μg/ml	81.1	108.7	88.2	40.5
S (-) betaxolol, 250 μg/ml	71.2	92.0	65.5	51.4
S (-) betaxolol, 300 μg/ml	54.5	91.6	49.9	54.0
S (-) betaxolol, 400 μg/ml	29.2	83.1	24.3	63.9

The positive control showed mutation in the assay. There was no increase in the mutation frequency up to 400 μg/ml concentration of S (-) betaxolol. However, the percent relative growth was about 24% at 400μg/ml. The sponsor repeated the assay so as to analyze the mutagenicity of S (-) betaxolol at concentrations that are more cytotoxic to the cell.

The experiment was repeated at 150, 200, 250, 300, 350 and 400μg/ml concentrations. The data for the repeat experiment are shown in the following table.

Treatment	Suspension growth	Cloning efficiency	Relative growth (%)	Mutant frequency (10^{-6} units)
Solvent control	16.2	75.8	100	45.3
Solvent control	15.6	76.7	100	57.4
Solvent control	14.2	69.8	100	61.1
MCA 2.5 µg/ml	9.9	50.2	43.9	332.2
MCA 4.0 µg/ml	6.8	51.3	30.8	327.9
	Average solvent control, 15.3	Average solvent control, 74.1		
	Relative to solvent control (%)	Relative to solvent control (%)		
150 µg/ml	110.7	89.3	98.9	70.5
200µg/ml	59.8	103.5	61.9	56.1
250µg/ml	66.8	82.6	55.2	76.8
300µg/ml	49.9	91.8	45.8	67.6
350µg/ml	34.9	70.4	24.6	85.0
400µg/ml	8.9	69.5	6.2	57.6

Results of the repeat experiment showed that there was no increase in the mutant frequency above $91x10^{-9}$ (a limit set as one of the criteria for a positive response) at a concentration that inhibited cellular growth substantially compared to the solvent control (6.2%). The positive control showed mutation of the cell.

Reviewer's summary for the L5178Y mouse lymphoma assay:

Results of the assay showed cloning efficiency of the solvent control above 50%. The assay was acceptable. It is concluded that S (-) betaxolol is not mutagenic in the forward mutation assay in CHO cells in the absence and presence of S-9 liver homogenates and cofactors. The positive controls showed mutation of the cell.

Sponsor's conclusion:

The test material, S (-) betaxolol hydrochloride was inactive in the mouse lymphoma forward mutation assay with and without metabolic activation.

Cell transformation assay:

Descriptive title: Evaluation of S (-) betaxolol in the in vitro transformation of BALB/C-373 cell assay.

Page 5-03326, vol 9.

Date of study initiation: August 1, 1986

Date of study completion: September 12, 1986

GLP compliance: Yes

Drug Lot Number: ERM 1740:080

Study end point:

The assay evaluated morphological transformation potential of mouse BALB/c-3T3cell line in culture so that a dense piled colonies are formed on top of monolayer of normal cells.
Methods:

Cell line: BALB/c-3T3 mouse cell line.

Dose selection criteria: Cytotoxicity to the cell in vitro.

Cytotoxicity was determined on the basis of the cell survival at fifteen doses between 0.97 to 16000 µg/ml concentrations. On the basis of the cytotoxicity data, doses between 7.5 and 110 µg/ml were chosen for the transformation assay.

Test agent stability and solubility:

S (-) betaxolol is soluble in the culture medium up to 20,000 µg/ml. Stock solution of S (-) betaxolol was prepared at 15.98 mg/ml and diluted serially to a range of 0.977 to 16,000µg/ml.

Controls:

Vehicle: Culture medium was used as the solvent for S (-) betaxolol.

Negative control: Culture media was used as the negative control. When organic solvent was used to dissolve any test material, aliquot of the organic solvent was used as the negative control. The sponsor stated that if any substance used in the assay was insoluble in water, DMSO or acetone or ethanol was used to dissolve it. The maximum amount of the organic solvent was 0.5%. S (-) betaxolol is soluble in the medium. However, the sponsor has not specified which solvent was used to dissolve the positive control.

Positive controls: 3-Methylcholantherene (MCA) was used at 2.5 to 5 µg/ml used as a positive control.

Exposure conditions:

Doses used in the definitive study:

Following doses of S (-) betaxolol were used for the transformation assay.

7.5, 30, 50, 80, 110µg/ml.

Analysis:

Auto or manual counting: Manual counting.

Cytotoxic endpoints:

Cytotoxicity was determined by observing the survival of colonies. The growth and formation of colonies of BALB/c-3T3 cells after 72 hours of treatment were examined. Each dose was applied to the culture vessel seeded with approximately 200 cells per vessel. There was no survival of colonies at 250 µg/ml and above concentrations. Only 1 % survival relative to control culture medium was noted at 125 µg/ml. Cytotoxicity data are shown in the following table.

Treatment	Average colonies/culture	% relative cell survival
Culture medium control	84.7	100
250 µg/ml	0	0
125µg/ml	0.7	0.8
62.5 µg/ml	26.3	31.1
31.3 µg/ml	66.3	78.3
15.6 µg/ml	68.3	80.7
7.81 µg/ml	78.7	92.9
3.91 µg/ml	77.0	90.9
1.95 µg/ml	84.7	100
0.977 µg/ml	83.3	98.4

The best response for transformation assay is obtained if the assay is carried out at concentrations of test substance that provide 10-50% survival. Five concentrations ranging 7.5-110 µg/ml were selected for the assay based on the cytotoxicity data so as to cover non-cytotoxic to cytotoxic concentrations.

Genetic toxicity end points:

Approximately 200 cells per vessel were exposed for 72 hours with the test substance. Cells were washed and allowed to grow for 3-5 days for colony formation. Survival colonies were stained and counted. Relative survival for each dose of S (-) betaxolol or positive control was determined by comparing number of colony counts to that of the negative control. The highest dose is expected to show about 90% reduction in the colony forming ability.

For the transformation assay, culture vessels were seeded with $1x10^4$ to $3x10^4$ BALB/c3T3 cells per vessel. After 24 hours of cellular growth in culture, cultures were treated with appropriate concentrations of S (-) betaxolol or positive control or solvent control. The cultures were incubated for 72 hours. Cultures were washed with the medium and reincubated for four weeks with refeeding twice a week. The assay was terminated by fixing the cell monolayer with 100% methanol and stained with 10% Giemsa stain. The stained culture vessels were examined under the microscope to determine number of foci of transformed cells.

Evaluation of transformed loci:

Transformed cells were identified as dense mass of foci that stained deeply and superimposed on the surrounding monolayer of cells. The foci are variable in size and morphological features as described below:

1. A dense pile of cells that exhibit criss-cross orientation of fibroblastic cells at the periphery of the focus and further invasion into the monolayer.
2. Other foci are rounded cells with necrosis at the center.
3. Foci without necrotic center and presence of large number of cell exhibiting criss-cross pattern of overlapping cells throughout most of the colony.

Foci were scored according to the size as described below:

1. Foci exceed 4 mm in diameter were scored +++
2. Foci 204 mm of diameter were scored ++

In contrast to the transformed cells, the normal phenotype cells showed uniformly stained monolayer of round contact inhibited cells.

Criteria for a valid test:

Following criteria were used for a valid test:

1. Negative control to have macroscopically visible BALB/c-3T3 cell cloning with colony efficiency higher than 15.
2. Higher than 50% cell survival in one of the concentrations of the test material.
3. Presence of a cytotoxic dose response.
4. Presence of continuous monolayer of cell in the negative, positive and solvent controls as well as the assay with S (-) betaxolol.
5. Spontaneous frequencies of transformed cell in the negative control should not exceed 2 foci.

Statistical methods:

The transformed foci data were transformed into its log base 10 value to achieve normal distribution pattern. Significant differences from the solvent control were determined using Bailey's modified Student's t-test.
P values less than 0.01 was considered to be a strong positive response. P value between 0.05 to 0.01 was considered to be a weak positive response.

Criteria for positive results:

1. At least one of the positive control treatments resulted in increase of transformed cell at 99% confidence level ($p<0.01$).
2. At least twelve culture vessels per assay condition available for analysis.
3. At least three treatment levels of the test material should be available for analysis.

APPEARS THIS WAY
ON ORIGINAL

Results:

Results of the transforming activity and relative cell survival are shown in the following table.

Treatment	Relative Survival (%)	Total Cultures	Total Foci	Average Foci/Culture	Transforming Activity	P value
Negative control, Culture medium	100	40	6	0.15	0.09	
Positive control, 3-MC 2.5μg/ml	22.9	20	135	6.75	6.13	≤0.01
S (-) betaxolol, 7.5μg/ml	96.8	20	4	0.20	0.13	<0.7
S (-) betaxolol, 30μg/ml	77.6	20	14	0.70	0.47	<0.05
S (-) betaxolol, 50μg/ml	65.0	20	5	0.25	0.19	<0.4
S (-) betaxolol, 80μg/ml	24.5	20	5	0.25	0.17	<0.5
S (-) betaxolol, 110μg/ml	11.3	20	1	0.05	0.04	<0.5

The data show that the positive control induced an increase in the transformed cells. S (-) betaxolol at 30 μg/ml significantly elevated number of transformed colonies. However, there was no dose response in the transformation activity and there was no increase in the transformed cell colonies at concentrations that showed lower cell survival (cytotoxicity). Therefore, the response at 30μg/ml is considered to be incidental.

Reviewer's conclusion: The assay results are acceptable. It is concluded that S (-) betaxolol is not mutagenic in the cell transformation assay.

Sponsor's Conclusion:

Results of the study showed that under the conditions and evaluation criteria of the study, S (-) betaxolol hydrochloride was evaluated as inactive in the BALB/C 3T3 cell transformation.

Reviewer's summary of the mutagenicity studies:

In vitro genotoxicity studies were conducted in Ames assay, chromosomal aberration, sister chromatid exchange, mouse lymphoma cells and cell transformation assay in the mouse BALB/c-373 cell line. S (-) betaxolol is not mutagenic in above assays except sister chromatid exchange assay in which an increase in mutation was noted at 500 and 600 μg/ml concentrations of (S-) betaxolol in the presence of S-9 liver homogenates.

In the Draft #1 of the review dated Dec 15, 1999, it was mentioned that the sponsor needs to complete the ICH requirements and accordingly completion of in vivo micronucleus test was recommended. However, the medical team leader (HFD-550) suggested in his response to the review that repetition of micronucleus study is not required. The reviewer reconsidered the earlier recommendation and compared the results between betaxolol and levobetaxolol for mutagenicity assays. The findings are summarized as follows.

Test	NDA 19-270 (Betaxolol)	NDA 21-114 (Levobetaxolol)
Ames Test	Up to 2000 µg/plate not mutagenic in activated and non-activated systems	Not mutagenic up to 10000 µg/plate in activated and non-activated systems.
Mouse lymphoma	Not mutagenic in non-activated system up to 500 µg/ml and in activated system up to 1500 µg/ml	No effect up to 400 µg/ml in the presence and absence of S (9) mixtures.
SCE	No effect on non-activated system. Significant increase at 1000 µg/ml in activation system	Positive at 500 µg/ml in the presence of S-(9) mixtures
Chromosomal Aberration in CHO cell line	No effect up to 1000 µg/ml	No effect up to 500 µg/ml
In vitro cell transformation assay	Not mutagenic at 1-20 µg/ml	No effect up to 110 µg/ml
In vivo micronucleus test in mice	Up to 500 mg/kg/day had no effect	Not done with levobetaxolol

From the similarity of the data between racemic and levobetaxolol, it is concluded that the in vivo micronucleus study with levobetaxolol would not provide further insight into the mutagenic potential of the drug. However, it is recommended that the positive findings in the SCE assay in the presence of metabolic activation systems should be included in the package insert.

Sponsor's summary of mutagenicity studies:

Levobetaxolol was evaluated in a series of five in vitro genotoxicity assays. Under the conditions of metabolic activation, levobetaxolol HCl was considered positive in the Sister Chromatid Exchange (SCE) assay in Chinese Hamster Ovary Cells. This finding was not substantiated in additional more sensitive assays including the mouse lymphoma mutation assay, in vitro transformation of BALB/C-3T3 cells, in vitro chromosome aberration in CHO cells, and the Ames Salmonella E. Coli/ microsome reverse mutation assay.

Labeling considerations:

It is concluded that S (-) betaxolol is mutagenic in vitro in the sister chromatid exchange assay in Chinese Hamster Ovarian cells in culture in the presence of metabolic activation system. This information needs to be addressed in the package insert.

Special toxicological studies:

The reviewing chemist for the NDA requested to discuss the toxicity profiles of N-lauroylsarcosine (SNLS) that will be used at 0.03% in the S (-) betaxolol ophthalmic suspensions. N-lauroylsarcosine is a surfactant that has not been used in an approved ophthalmic formulation in the US. However, it is approved in UK as in 0.25% Betoptic suspensions at 0.03%. Considering the drop size of 30µL and one-drop BID dose in each eye, maximum amount of N-lauroylsarcosine that will be used for both eyes will be about 36 µg.

The sponsor has submitted a summary of safety studies conducted for N-lauroylsarcosine in page 5-03360 vol 9 of the NDA. The sponsor concluded that SNLS is used in toothpaste. Therefore, safety of the surfactant when in contact of the oral mucosa is known. The amount of SNLS in the toothpaste is not mentioned in the summary report.

The acute dose of SNLS for 50% mortality in male albino rats is 2175 mg/kg/oral. The sponsor summarized a two-year study in Wistar rats treated with 0.1 and 0.2% NLSA in the diet. Results of the

BEST POSSIBLE COPY

study suggest that at the end of two year of dosing, hyperplasia of the stratified squamous epithelium of cardiac mucosa of the stomach was observed. However, similar change was not present at the end of six months.

SNLS is not a primary irritant to the skin when applied at 20% aqueous solution for two weeks in the rabbit skin. SNLS also did not show allergic reactions to the guinea pig skin.

Albino rabbits at 0.1 ml 5% w/v aqueous solution of SNLS showed minimal conjunctival irritation up to 24 hours without any damage to the cornea.

Reviewer's conclusion: Based on the preclinical data it is unlikely that SNLS would cause safety concern to the eye when used acutely. However, there is no preclinical data on the safety of SNLS in eye after chronic uses. Therefore, the safety of SNLS needs to be examined from the clinical safety data if any.

The review chemist also asked for the opinion of pharmacologist regarding the use of 0.75% polystyrene divinylbenzene sulfonic acid in the S (-) betaxolol formulation. The approved racemic betaxolol ophthalmic formulation contains about 0.25% of polystyrene divinylbenzene sulfonic. Therefore, safety of the inactive ingredient needs to be examined from the safety database for the clinical use.

Reviewer's overall summary and evaluation:

Betaxolol is an approved drug for the treatment of increased intraocular pressure in glaucoma and ocular hypertension. The sponsor submitted data for review of the effect of its levo-isomer (levobetaxolol) for the same indications. Levobetaxolol is a potent cardioselective β_1-adrenoceptor antagonist. It is about 37 times more potent than the R (+) enantiomer. Levobetaxolol is effective at 150-µg single dose for reducing experimentally elevated intraocular pressure in the monkey model.

In vitro experiments suggest that levobetaxolol inhibits calcium influx (by blocking calcium channels) in some of the smooth muscle preparations. The effect is independent of its ß-adrenoceptor activity due to a lack of specificity to stereo isomers of betaxolol. The sponsor suggested that in addition to ß-adrenoceptor blockade, blockade of calcium channels contribute to the mechanism of action in glaucoma. However, a definite conclusion on the contribution of levobetaxolol in glaucoma as a calcium channel inhibitor can not be made due to following reasons:

1. Lack of specificity of its effect in all tissues tested for blockade of calcium channels.
2. Lack of direct evidence to support that calcium channel blockade contributes to the efficacy in glaucoma.

Therefore, the reviewer recommends that only ß-adrenergic activity needs to be mentioned as one of the mechanisms of its pharmacodynamic effect in glaucoma.

Levobetaxolol did not show analgesic and antipyretic effects in rodents up to 10 mg/kg doses. Several in vivo studies are reported in rodents and dogs for evaluation of pharmacological side effects when given systemically. However, levobetaxolol did not show side effects in GI, lung and CNS up to 10 mg/kg SC or IV doses. Levobetaxolol increased the hypertensive effects of epinephrine in anesthetized dogs without showing anticholinergic or antihistaminic effect at 0.1-mg/kg dose. Levobetaxolol did not show changes in the blood pressure, heart rate and ECG up to 1-mg/kg I.V doses. However, left ventricular dp/dt and end diastolic pressure was reduced at 0.3 and 1.0 mg/kg I.V doses in anesthetized beagle dogs. Data suggest that levobetaxolol reduced the force of contractions of the heart at 0.3 mg/kg and above doses.

Levobetaxolol is bioavailable in the systemic circulation when given as ophthalmic drops at 2 drops of a 0.5% ophthalmic preparation two times a day in the right eye in rabbits. It is also distributed to the anterior and posterior chambers of the eye following ophthalmic delivery in rabbits. In addition to its efficacy in the target organ (eye), levobetaxolol may show untoward effects in the heart. Therefore, precaution needs to be taken for recommending levobetaxolol in glaucoma patients who has been

diagnosed for cardiovascular diseases also. Indeed, such warning is already included in the proposed package insert.

Levobetaxolol does not undergo inversion to the relatively less effective R-isomer of betaxolol. Therefore, pharmacological efficacy of the drug will not be compromised by the inversion of active to inactive stereoisomeric form.

Chronic safety study of levobetaxolol is conducted in rabbits following ocular delivery of ophthalmic suspensions up to 2.5%, two drops two times a day for one year. The drug was well tolerated without toxicity to systemic organs and eyes. However, conjunctival discharge and dilatation of the lacrimal gland were noted as side effects at 0.5-2.5%. Animals at 2.5% 4 drops/day dose also showed increased incidences of constipation in the rabbit study although single dose pharmacodynamic study did not show a decrease in the GI motility up to 10 mg/kg SC dose in mice.

Considering 50μl drop size for the rabbit study, daily doses were 0.33, 0.66 and 1.65 mg/kg (3.96, 7.9 and 19.8 mg/m ^{2}) in rabbits. The maximum human dose for 70-kg patient will be 8.6 μg/kg (318 μg/m^2). Rabbit to human dose ratio for 0.5% ophthalmic drops is about 12 when data are normalized to the surface area. The plasma concentration data between rabbits and human at 0.5% ophthalmic suspensions showed a plasma concentration ratio of 14. It is possible that human subjects may show conjunctival discharge similar to that observed in rabbits considering the low margin of safety (12-14) at 0.5% ophthalmic doses between the rabbits and humans. Constipation in rabbits was observed at about 62 fold higher doses than clinical doses in rabbits when doses were compared as mg/m ^{2} basis.

The information on the possibility of conjunctival discharge and constipation is shared with the medical reviewer to examine whether similar changes (conjunctival discharge and constipation) were observed in the patients. The medical reviewer informed the pharmacology reviewer that conjunctival discharge was observed in the clinical studies and the information is included in the package insert. However, constipation is not reported in the adverse reaction profiles in the clinical study.

Levobetaxolol is tolerated up to 1250 mg/kg oral single doses in rats. Tremors, difficulty in breathing, salivation and reduced activity were clinical signs noted within 30 minutes after the dosing.

Oral safety study was conducted in rats at 3, 30 and 100 mg/kg doses of levobetaxolol for three months. A dose of 100 mg/kg of betaxolol was added for comparison. Mortality was noted at 100-mg/kg dose of betaxolol in rats. However, levobetaxolol was tolerated without mortality.

Levobetaxolol up to 30 mg/kg doses did not show treatment related pathological changes in male and female rats. However, at higher doses for example, 100 mg/kg showed increased incidences of cardiac inflammation, alveolar histiocytes in the lung and hyperplasia of transitional epithelium of the kidney in female rats. Male rats showed increased hemorrhage in the mesenteric lymph nodes at 100-mg/kg dose. The differences in the toxicity in the heart and kidney between male and female rats were due to higher plasma levels of levobetaxolol in female rats.

The proposed recommended human dose is two drops of 0.5% levobetaxolol per day in each eye. Considering the drop size of 30 μl, daily dose for both eyes in 70-kg subject will be about 8.6 μg/kg. The no effect dose in the oral toxicity in rats (30 mg/kg) is about 3500 times more than the recommended human dose. The average C_{max} in rats at 30 mg/kg is 1049 ng/ml. The average C_{max} in humans is 0.547 ng/ml. The ratio of C_{max} between rats at 30-mg/kg/oral dose to 0.5% one-drop BID dose per eye is about 2000 folds. Based on the margin of safety extrapolate from the 30 mg/kg oral dose in rats, it is expected that the ophthalmic doses in humans would not cause pathological changes in the heart and kidney.

Segment II reproductive safety study was conducted at oral doses of 4, 12 and 36 mg/kg doses during organogenesis in rabbits. Maternal toxicity was observed at 36-mg/kg dose. Fetal toxicity for example increased incidences of rudimentary 13th rib were observed at 4 mg/kg (48 mg/m ^{2}). Increased resorptions

of the fetuses, postimplantation loss, a decrease in the number of live fetuses and mal-aligned sternebrae were noted at 12 mg/kg (144 mg/m ^{2}). The ratio of rabbit (4 mg/kg) to human (8.6 μg/kg) dose ratio is about 465. The ratio of rabbit (48-mg/ m^2) to human doses (318 μg/m ^{2}) when equal surface area is considered will be about 150. The reviewer recommends pregnancy category C for the label.

In vitro mutagenicity studies were conducted in Ames assay, chromosomal aberration assay in Chinese Hamster Ovarian (CHO) cell line, sister chromatid exchange in CHO cell line, mouse lymphoma assay in L5178Y cell line and cell transformation assay in mouse BALB/C-373 cell line. Levobetaxolol showed mutagenicity in the presence of metabolic activation mixtures in the sister chromatid exchange assay. The information needs to be indicated in the package insert.

Sponsor's summary and conclusions of non-clinical pharmacology and toxicology (Page 3-00034, vol 1):

Betaxolol is a ß-adrenergic blocking drug. Levobetaxolol is the more biologically active enantiomer of betaxolol and is primarily responsible for reducing the intraocular pressure. Alcon Laboratories Inc. has developed levobetaxolol ophthalmic suspension for the topical ocular treatment of glaucoma. Based on the preclinical pharmacology and toxicology studies performed by Alcon Laboratories, following conclusions are made for the NDA.

Levobetaxolol reduces intraocular pressure; it is a neuroprotectant and enhances blood flow in ocular vessels. Levobetaxolol will be effective against chronic glaucoma degenerative processes. Experimental glaucoma models based on increased intraocular pressure are not suitable for experimentally demonstrating neuroprotective activity of betaxolol, because the concomitant reduction of intraocular pressure by betaxolol complicates the interpretation of preservation of ocular function. Therefore, the demonstrated ability of betaxolol to function as a neuroprotectant must be extrapolated from experimental models that typically require substantially higher drug dose levels.

1. Levobetaxolol is a potent and highly selective ß-adrenoceptor ligand with relative selectivity for β_1 receptors and also possesses some affinity for L-type calcium channel.
2. Levobetaxolol will retain the desirable safety profile and relative freedom from respiratory side effects experienced with betaxolol.
3. No effects were noted in general pharmacology screening with levobetaxolol up to 0.3 mg/kg, 30 fold the anticipated daily maximum clinical dose of 0.01 mg/kg (two 25 μl drops of 0.5% twice daily in a 50 kg person.
4. Minimal and transient decrease in blood pressure and cardiac contractility were noted at 1 mg/kg.
5. At 1 mg/kg dose, levobetaxolol modified the cardiovascular responses to isoproterenol, histamine and acetylcholine.
6. No bronchoconstriction was noted with levobetaxolol up to 3 mg/kg in guinea pigs. Two animals died at 10-mg/kg IV infusion.
7. Cardioselectivity of levobetaxolol is preserved in vivo.
8. The degree of ß-adrenergic blockade in S –timolol is stronger than levobetaxolol.
9. Under the condition of metabolic activation, levobetaxolol showed positive mutagenicity response in Sister Chromatid Exchange assay in CHO cell line. This finding is not substantiated in additional In vitro mutagenicity assays.
10. The maximum tolerated oral dose in rats is 1250 mg/kg.
11. A three-month oral gavage study in Sprague-Dawley rats showed no toxic effect at 30 mg/kg/day. An increase in subacute inflammation in the heart was noted at 100-mg/kg dose of levobetaxolol and betaxolol in female rats.
12. One year topical ocular safety study with a three month interim evaluation was conducted with 0.5, 1.0 and 2.5% S (-) betaxolol ophthalmic suspensions (BID) in New Zealand white rabbits. This study demonstrated that 0.5%-2.5% S (-) betaxolol ophthalmic suspensions do not possess a cumulative ocular irritation potential and do not demonstrate any apparent systemic toxicity in rabbits.
13. No evidence of teratogenicity was observed in New Zealand rabbits treated up to 36 mg/kg/day of levobetaxolol HCl. Similarly no teratogenicity was observed in this study with rabbits treated with 36

mg/kg/day of betaxolol HCl. Both levobetaxolol HCl and betaxolol HCl at 36 mg/kg/day induced spontaneous abortion/premature delivery. An increase in the number of early and late resorptions occurred with 12 and 36 mg/kg/day levobetaxolol and 36-mg/kg/day betaxolol HCl.

14. The safety of n-lauroyl sarcosine has been substantiated by its status as a cosmetic ingredient and food additive. In addition, safety of topical ophthalmic product has been established by the sponsor.
15. The toxicology safety profile for levobetaxolol HCl appears to mirror that of racemate. 2.5% levobetaxolol ophthalmic suspension is safe in topical ocular studies in rabbits. The sponsor calculated the safety factor as 125 fold on the basis of the dose (mg/kg) ratio between the dose in rabbits to maximum human dose.

Reviewer's conclusion:

Levobetaxolol up to 2.5% at 4 drops per day is safe for the chronic use in rabbits. The dose is about 1.65 mg/kg or 19.8 mg/m ^{2}. The proposed clinical dose is 0.5% two drops per eye. The human dose is about 8.6 µg/kg (318 µg/m ^{2}) for both eyes. The highest dose used in the rabbits was about 62 times the proposed maximum human dose when compared as mg/m ^{2} basis. The side effects in the rabbit study were increased conjunctival discharge and constipation. On the basis of the preclinical ophthalmic safety study and the oral rat study, human use of 0.5% ophthalmic suspensions of levobetaxolol is recommended to be safe. There were positive findings for mutagenicity in <u>in vitro</u> sister chromatid exchange assay in CHO cell lines. Levobetaxolol should be considered for the pregnancy category C.

Reviewer's recommendations:

Internal:

The pharmacology reviewer informed the medical reviewer regarding conjunctival discharge and constipation as the side effects of the drug for long-term use for reducing intraocular pressure in glaucoma. The medical review suggests that conjunctival discharge is reported in the clinical study and finding will be incorporated in the label. No action is necessary in this regard.

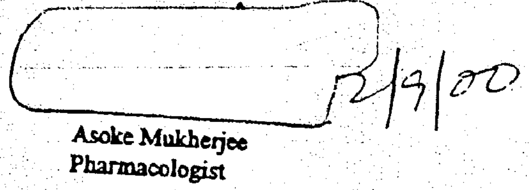
External recommendation to the sponsor: No suggestions made at this time

Labeling review, reviewer's recommendations:

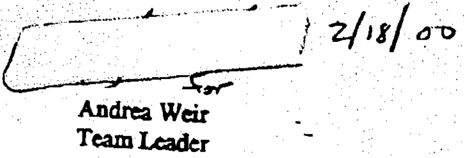
DRAFT LABELING

DRAFT

LABELING



Asoke Mukherjee
Pharmacologist



Andrea Weir
Team Leader

Addendum to Review:

Appendix attachment: Pages 5-02669 and 5-02670 of vol 8.

Draft date: Dec 15, 1999 Draft #1. Feb 9, 2000 Draft #2

Memorandum of non-concurrence:

Disagree that constipation and/or lacrimal gland dilatation are necessarily β-adrenergically related.

Alcon LABORATORIES

OBC Study No. E571-874

Table I: Gross Necropsy Findings
Males

Test Article	S Betaxolol				RS Betaxolol
Dose mg/kg	0	10	30	100	100
# Animals examined	15	15	15	15	15
# Animals with no findings	14	13	13	14	11
Findings/# Animals with findings					
Cavities, thoracic - clear fluid					1
Heart – enlarged	1				
Thymus – small		1			1
Focus(i), red			1		
Spleen – small					1
Lymph node, other, renal - enlarged			1		
Liver – discoloration, pale		1			
discoloration, red					1
Kidneys – pelvis, calculus			1		
pelvis dilation			1		
pelvis, focus, white					1
cortex, focus, white					1
Cyst					1
Bladder – thick			1		
Calculus			1		
Prostate – firm					1
Esophagus – perforation					1
Stomach – focus, red, depressed				1	1
discoloration, red					1
Dilation					1
Adrenals – discoloration, red					1
Duodenum – discoloration, red					1
Jejunum – discoloration, red					1
Ileum – discoloration, red					1
Cecum – discoloration, red					1

APPEARS THIS WAY ON ORIGINAL

BEST POSSIBLE COPY

Alcon LABORATORIES

Table II: Gross Necropsy Findings
Females

Test Article	S Betaxolol				RS Betaxolol
Dose mg/kg	0	10	30	100	100
# Animals examined	15	15	15	15	15
# Animals with no findings	15	14	11	11	13
Findings/# Animals with findings					
Lung – discoloration, red					–
Spleen – capsule, adhesion					
Thymus – discoloration, red			1		
Lymph node mandibular – discoloration, red				1	1
Lymph node, other, iliac - enlarged				2	
- renal enlarged				1	
Liver, discoloration, pale					
Kidneys – pelvis, dilation			1	2	1
unilateral, enlarged		1			
unilateral, small				2	–
				1	
Bladder – diverticulum				1	–
Calculus					
Thick				1	
				2	
Uterus – horns, dilation				1	
Stomach – locus, black			2		
				1	

APPEARS THIS WAY
ON ORIGINAL

BEST POSSIBLE COPY