

MICROBIO

Appendix C



財團法人生物技術開發中心
DEVELOPMENT CENTER FOR BIOTECHNOLOGY

SERIAL NO: DV-TR-CA00022E
PROJECT CODE: DV-TA00199
PAGE 1 OF 19



0302

***IN VITRO* CHROMOSOME ABERRATION ASSAY
PRODUCT CODE MICRSOY-20 (MS-20)**

FINAL REPORT

DEVELOPMENT CENTER FOR BIOTECHNOLOGY
DRUG DEVELOPMENT DIVISION



Signature Page

Study Director:

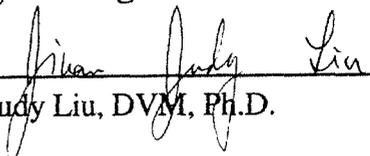
 Jan 130 / 2002
Shwu-Fei Lee-Chen, Ph.D.

Investigators:

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Facility Manager:

 Jan 130 / 2002
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QUALITY ASSURANCE STATEMENT

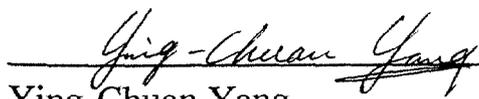
The Quality Assurance Unit (QAU) has inspected the conduct of different phases of the study according to a predetermined testing schedule. To the best of our knowledge, there were no deviations from the protocol, protocol amendment and standard operating procedures that would affect the integrity of this study.

This report has been audited by the QAU in accordance with the appropriate standard operating procedures of Drug Development Division, DCB. The report is considered to describe the methods and procedures used in the study, and the reported results accurately reflect the raw data generated during this study.

The Genetic Toxicology Laboratory of Drug Development Division, DCB has been recognized by the Council of Chinese National Laboratory Accreditation (CNLA) as an accredited laboratory, and chromosome aberration assay is one of the registered testing item within the field of Biological Testing. We now had the CNLA logo attached on the cover page of this report for compliance

Listed below are the phases in this study that were audited by the QAU and the dates the audits were performed and findings reported to management.

<u>Audit Date</u>	<u>Phase Audited</u>	<u>Date Reported to Study Director</u>	<u>Date Reported to Management</u>
Dec. 6, 2001	Protocol	Dec. 10, 2001	Dec. 10, 2001
Jan. 15, 2002	Test article preparation Test article treatment without S9 activation	Jan. 17, 2002	Jan. 18, 2002
Jan. 17, 2002	Test article preparation Test article treatment with S9 activation	Jan. 18, 2002	Jan. 18, 2002
Jan. 25, 2002	Raw data; study records	Jan. 25, 2002	-----
Jan. 28, 2002	Final report	Jan. 30, 2002	Jan. 30, 2002


Ying-Chuan Yang
Quality Assurance Officer

Jan. 30 / 2002
Date



TESTING FACILITY

- A. Name: Genetic Toxicology Laboratory, Drug Development Division, Development Center for Biotechnology
- B. Address: 103, Lane 169, Kang-Ning St., Hsi-Chih City, Taipei County 221, Taiwan, R.O.C.

SPONSOR

- A. Name: MICROBIO Co., Ltd.
- B. Address: No. 81, Gauyang N. Rd., Lung Tan Shiang, Tao Yuan, Taipei, Taiwan, R.O.C.
- C. Representative: William Lu

TEST ARTICLE (The information was supplied by the sponsor prior to study initiation)

- A. Name/Identification: Product code MicrSoy-20 (MS-20)
- B. Receiving Date: Oct. 25, 2001
- C. Batch/Lot Number: 20010209
- D. DCB Code: DV00199-a
- E. Ingredients: MS-20 is a Chinese medicine. The components are very complicated. Until now, its effective components are still unable to determine.
- F. Storage Conditions: Room temperature and desiccation in a dark bottle
- H. Expiration Date: Feb. 09, 2004
- I. Physical Appearance: Dark-brown liquid with prune juice odor

Statements:

1. The test article is a proprietary product of the sponsor; therefore the sponsor will be responsible for the requirements listed under "Test Article" of the GLP regulation.
2. The testing result is effective for submitted sample only, and shall not be excerpted from the contents of this report without the written approval of the testing facility.
3. The testing result and report are generated by DCB for the test article submitted by the sponsor, and are intended for petition to government agency for product registration.

TEST SCHEDULE

- A. First Date of Target Cells Seeding : Jan. 14, 2002
- B. First Date of Test Article Treatment: Jan. 15, 2002
- C. First Date of Chromosome Slides Preparation: Jan. 16, 2002
- D. Chromosome Slides Observation Period: Jan. 21 ~ 23, 2002

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Ovary Cells



IN VITRO CHROMOSOME ABERRATION ASSAY

Product Code MicrSoy-20 (MS-20)

SUMMARY

The test article, Product code MicrSoy-20 (MS-20), supplied by MICROBIO Co., Ltd. was studied with chromosome aberration (CA) assay in Chinese hamster ovary (CHO) cells in the absence and presence of Aroclor 1254-induced rat liver S9. The preliminary assay was used to establish the dose ranges for the chromosome aberration assay. The definite chromosome aberration assay was used to evaluate the clastogenic potential of the test article.

Test article stock solution of Product code MicrSoy-20 (MS-20) was prepared in McCoy's 5A medium and filtered through 0.2 μm membrane after adjusting pH value to the range of 7.0 ~ 7.2. The culture medium was used to further dilute test article solution to desired concentrations for cell treatment. According to the preliminary toxicity test of chromosome aberration assay, the top concentration was set as 100 $\mu\text{l/ml}$ for 3-hour and 20-hour treatment in chromosome aberration assay. Additional four lower concentrations with a dilution factor of 2 were conducted in both the presence and absence of S9 activation for 3-hour treatment, as well as 20-hour treatment without S9 activation.

Chromosome aberration was analyzed with three test schemes: 3-hour exposure both without and with S9 activation (schemes I and II) and 20-hour continuous exposure without S9 (scheme III). All the tests were conducted in duplicate cultures and with solvent control and positive controls concurrently. Cultures exposed for 3 hours with and without S9 were harvested about 20 hours from the beginning of test article treatment. Colcemid at 0.1 $\mu\text{g/ml}$ was added to all the cultures 2 hours before harvesting. A minimum of 100 metaphases for each culture and 200 for each treatment were scored. The results showed that the requirements for a valid test were met. The concurrent cytotoxicity assay indicated that the tested concentrations used in test schemes I and III had reached above 50% of cytotoxicity, whereas the scheme II had tested up to 100 $\mu\text{l/ml}$ and reduction of mitotic cells was noted. The top analyzable concentrations for test schemes I, II and III were 50, 100 and 25 $\mu\text{l/ml}$, respectively.



Therefore, Product code MicrSoy-20 (MS-20) was concluded to be negative in the chromosome aberration assay in Chinese hamster ovary cells under the tested conditions.

INTRODUCTION

The objective of this study was to evaluate the potential of Product code MicrSoy-20 (MS-20) to induce structural chromosome aberration in Chinese hamster ovary cells. The chromosome aberration induced in various stages of cell cycle and different metabolic conditions was assessed via three test schemes.

MATERIALS AND METHODS

A. Test System

Chinese hamster ovary cells (CHO-K1) were obtained from American Type Culture Collection (ATCC, repository number CCL-61), Rockville, MD, USA. The CHO-K1 cell line has an epithelial-like morphology and a modal chromosome number of 20. The population doubling time of test cells is about 12~14 hours. The frozen lot of cells was tested and found to be free of mycoplasma contamination (ELISA method, Boehringer Mannheim). Cells used in the CA assay were within five subpassages from frozen stock in order to assure karyotypic stability. Karyology of CHO-K1 cells were analyzed in the untreated cells for the chromosome aberration assay (see Results and Discussion section).

B. Culture Condition

CHO-K1 cells were stored in liquid nitrogen. After thawing, cells were maintained as monolayers in McCoy's 5A medium (GIBCO, USA) supplemented with 10% heated-inactivated fetal bovine serum, 2.2 g/l NaHCO₃, 2 mM L-glutamine, 1% penicillin-streptomycin solution, pH 7.0 ~ 7.2 in a humidified incubator at 37 ± 1 °C and 5 ± 1% CO₂ in air.

C. Metabolic Activation System

Postmitochondrial fraction (S9) of livers of Sprague-Dawley rats induced with Aroclor 1254 was used for metabolic activation and was prepared by MOLTOX™, Inc.



U.S.A. S9 containing medium was freshly prepared by mixing 0.315 mg/ml NADP, 0.152 mg/ml G-6-P and S9 in serum-free McCoy's 5A medium. The final concentration of S9 was 1%.

D. Concentration-range Finding Test

The optimal concentration levels for chromosome aberration assay were determined by the toxicity of test article. According to the OECD guideline, the top concentration should be the lowest concentration showing greater than 50% cytotoxicity, if possible. The cytotoxicity test of preliminary chromosome aberration assay of test article showed no significant toxicity up to 50 μ l/ml. Therefore, 100 μ l/ml was chosen as the top concentration for 3-hour and 20-hour treatments in chromosome aberration assay.

E. Test Article Preparation and Test Concentrations

Test article stock solution of Product code MicrSoy-20 (MS-20) was prepared in McCoy's 5A medium and filtered through 0.2 μ m membrane after adjusting pH value to the range of 7.0 ~ 7.2. The culture medium was used to further dilute test article solution to desired concentrations for cell treatment. The top concentrations of test article used in different stages were determined as described above. A minimum of three analyzable concentrations has to be obtained for the chromosome aberration assay. Therefore, additional lower concentrations were added for cell treatment. The lower concentrations were separated basically by a factor of 2. Following concentrations were used for cell treatment under different test schemes.

Scheme	Treating Condition	Concentration (μ l/ml)
I	-S9, 3 hours	0, 6.25, 12.5, 25, 50, 100
II	+S9, 3 hours	0, 6.25, 12.5, 25, 50, 100
III	-S9, 20 hours	0, 6.25, 12.5, 25, 50, 100

F. Controls

Negative control was McCoy's 5A medium. In the chromosome aberration test without S9, mitomycin C [CAS no. 50-07-7] (Boehringer Mannheim Art.107409,



Germany) at 1 μ M for 3-h treatment was used as the positive control. Cyclophosphamide [CAS no. 6055-19-2] (Sigma Cat. No. C0768, USA) at 40 μ M for 3-h treatment was the positive control for the test with S9. Positive controls were prepared in dimethyl sulfoxide (Merck Art. 2931, Germany) and added to culture medium with exponentially growing cells.

G. Procedures of Chromosome Aberration Assay

1. Preparation of Target Cells

Exponentially growing CHO-K1 cells were seeded in complete McCoy's 5A medium at approximately 5×10^5 cells/ 60 mm dish. The dishes were incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO_2 in a humidified incubator overnight before treatment.

2. Treatment of Target Cells

Treatment was carried out in duplicate cultures by adding freshly prepared test article or control solutions to desired concentrations. Chromosome aberration assay was performed with 3 schemes. Scheme I, cells were treated with test article for 3 h in the absence of S9. Scheme II, cells were treated for 3 h in the presence of S9. In these two test schemes, cells were kept growing in fresh medium and mitotic cells were harvested at approximately 20 h from the beginning of treatment. On the scheme III, cells were continuously treated for 20 h without S9 activation. Colcemid was added to the culture medium at a final concentration of 0.1 $\mu\text{g}/\text{ml}$ during the last 2 h of incubation before harvest. Concurrent measures of cytotoxicity for all treated and negative control cultures were performed at the time of cell harvest by cell counting.

3. Chromosome Preparation

Mitotic cells were collected by the shake-off technique. Mitotic cells were treated with hypotonic solution and fixed with a mixture of methanol and acetic acid (3:1, v/v). Cells were dropped on cleaned slides and stained with 3% Giemsa.

4. Data Recording

For each treatment, 200 metaphases were examined in blind-coded slides under microscope with 1000X magnification. Mitotic cells with chromosome numbers 18 ~ 21 were observed for the presence of aberrant chromosomes. Aberrant chromosome



morphologies including chromosome gap (G), chromosome break (B), dicentric (D), ring (R), chromatid gap (g), chromatid break (b), exchange (e) and multiple aberrations (MA) were recorded separately on the data sheet for each culture. Percents of aberrant cells from each culture and each treatment were calculated excluding cells with chromosome and chromatid gaps.

H. Criteria for a Valid Test

The frequency of aberrant cells in the concurrent negative control must not exceed 3 percents. The percents of aberrant cells in the concurrent positive controls should be statistically increased ($p < 0.05$) relative to the negative control.

I. Interpretation of Results

All conclusions were based on sound scientific judgement. However, as a guide to interpret the data, both the number of concentrations significant and the trend probability (Galloway et al., 1985) for a test article are taken into account. For a given concentration, percent of aberrant cells is analyzed by one-tail binomial test and compared pair-wisely to the negative control. According to standard normal distribution, set α value 0.01 as the significant level. If there is at least one of the test concentration giving significant results, trend test is proceeding to determine the existence and extent of a CA-inducing concentration-responsiveness. However, a test result is considered negative in the test system if none of test points gives significant increase of frequency of CA than negative control.

J. Data Retention

All raw data, documentation, records, protocols and final reports generated as a result of this study will be inventoried and archived by the Quality Assurance Unit at DCB's archives located in the Drug Safety Building. All slides will be stored in Genetic Toxicology Laboratory at DCB. The retaining duration of those records and slides will be in accordance with the relevant regulations.



RESULTS AND DISCUSSION

A. Karyology Analysis

Karyotypic stability was analyzed in CHO cells of the solvent control for the chromosome aberration assay. The distribution of chromosome numbers of analyzed cells is shown in Table 1.

Table 1. Karyology Analysis of Test Cells

No. of chromosomes	<18	18	19	20	21	22	>22
No. of cells	0	4	22	22	2	0	0

Since all of cells analyzed containing chromosome numbers within 20 ± 2 , this lot of cells met the requirements for chromosome aberration assay.

B. Concurrent Cytotoxicity Test

Measurement of cytotoxicity for all treated and negative control cultures in the chromosome aberration assay was conducted at the time of mitotic cell harvest and recorded in Table 2. This cytotoxicity was measured by counting cell numbers and normalized by the corresponding negative control value. Treatments with Product code MicrSoy-20 (MS-20) to CHO cells at 100 $\mu\text{l/ml}$ in test schemes I and III have produced more than 50% cytotoxicity. Although the top concentration of scheme II produced only 40% cytotoxicity, a slight reduction of mitotic cells was noted. Therefore, all three test schemes have been tested to maximum analyzable concentrations.

C. Chromosome Aberration Assay

Numbers of cells with chromosome aberrations and types of chromosome aberrations given separately for each treated and control cultures are presented in Tables 3, 4 and 5. Table 6 summarized the frequency of chromosome aberration of each concentration from three test schemes of study. The top analyzable concentrations for test schemes I, II and III were 50, 100 and 25 $\mu\text{l/ml}$, respectively. It was noted, the percents of aberrant cells of vehicle controls from three test schemes I, II and III were all 0%. The positive controls induced significant increase in percents of aberrant cells over the



negative control values. There was a minimum of three analyzable concentration levels obtained for each test scheme. Thus, this assay data met the criteria for acceptance.

Chromosome aberrations induced by Product code MicrSoy-20 (MS-20) for three test schemes were analyzed. No significant increase on the percents of aberrant cells was found at all other tested concentrations when compared with the data of vehicle controls (Table 6).

CONCLUSION

The criteria for a valid study were met as described in the protocol. It is concluded that under the tested conditions both with and without metabolic activation, the sample of Product code MicrSoy-20 (MS-20) did not significantly increase frequency of chromosome aberrations in Chinese hamster ovary cells.

COMMENTS AND/OR PROBLEMS

This study was conducted in compliance with (1) Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR 58), FDA, U.S.A., 1987; (2) Good Laboratory Practice for Nonclinical Laboratory Studies, Department of Health, R.O.C., 3rd edition, 2000, with the exceptions of test article identification and related analyses.

To the best of our knowledge, there were no deviations from the study protocol and protocol amendment that would affect the integrity of this study. No problems were encountered that would adversely affect the study results or interpretation.

REFERENCES

1. Galloway, S.M., Aardema, M.J., Motoi Ishidate, Jr., Ivett J.L., Kirkland, D.J., Morita, T., Mosesso, P., and Sofuni, T. (1994) International Workshop on Standardization of Genotoxicity Test Procedures. "Report from working group on *in vitro* tests for chromosomal aberrations". Mutation Research 312:241-261.
2. Galloway, S.M., Bloom, A.D., Resnick, M., Margolin, B.H., Nakamura, F., Archer, P., and Zeiger, E. (1985) Development of a Standard Protocol for *In Vitro* Cytogenetic Testing with Chinese Hamster Ovary Cells. Environ. Mutag. 7: 1-51.



3. Margloin, B.H., Resnick, M.A., Rimpo, J.Y., Archer, P., Galloway, S. M., Bloom, A. D., and Zeiger, E. (1986) Statistical Analyses for *In Vitro* Cytogenetic Assays Using Chinese Hamster Ovary Cells. *Environ. Mutag.* 8: 183-204.
4. OECD Guideline for the Testing of Chemicals #473: *In Vitro* Mammalian Chromosome Aberration Test, 1997.
5. Quality Manual, Drug Development Division, Development Center for Biotechnology, 6th edition, 1999.



Table 2. Concurrent Cytotoxicity Analysis of Product Code MicrSoy-20 (MS-20) in Chinese Hamster Ovary Cells

Conc. ($\mu\text{l/ml}$)	Cell number ($\times 10^5$ cells)	Survival (%)	Cytotoxicity (%)
<u>Scheme I (-S9, 3 h)</u>			
0	34.0	100.0	0.0
6.25	31.2	91.8	8.2
12.5	32.8	96.5	3.5
25	21.2	62.4	37.6
50	16.6	48.8	51.2
100	10.6	31.2	68.8
<u>Scheme II (+S9, 3 h)</u>			
0	28.2	100.0	0.0
6.25	28.0	99.3	0.7
12.5	25.6	90.8	9.2
25	25.0	88.7	11.3
50	21.8	77.3	22.7
100	16.8	59.6	40.4
<u>Scheme III (-S9, 20 h)</u>			
0	36.8	100.0	0.0
6.25	35.4	96.2	3.8
12.5	30.4	82.6	17.4
25	21.6	58.7	41.3
50	5.6	15.2	84.8
100	0.0	0.0	100.0



Table 3. Effects of Product Code MicrSoy-20 (MS-20) on the Induction of Chromosome Aberrations in CHO Cells for 3-h Treatment in the Absence of S9

Treatment Conc. (µl/ml)	Aberrant Cells (%) ^a	Number of Chromosome Aberrations/100 Cells							
		G ^b	B	D	R	g	b	e	MA
Negative control	0	0	0	0	0	0	0	0	0
Negative control	0	0	0	0	0	0	0	0	0
6.25	0	0	0	0	0	0	0	0	0
6.25	0	0	0	0	0	0	0	0	0
12.5	0	0	0	0	0	0	0	0	0
12.5	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
25	1	0	0	0	0	0	0	1	0
50	0	0	0	0	0	0	0	0	0
50	1	0	0	0	0	2	1	0	0
100		Too few metaphases							
100		Too few metaphases							
Positive control ^c	21	1	1	0	1	1	1	20	0
Positive control ^c	25	0	0	0	1	1	2	22	1

^a Aberrant cells were calculated excluding cells with gaps.

^b G: chromosome gap B: chromosome break D: dicentric R: ring
g: chromatid gap b: chromatid break e: exchange MA: multiple aberrations

^c Positive control was 1 µM mitomycin C for 3 h.

Data from duplicate cultures were recorded independently by two observers.



Table 4. Effects of Product Code MicrSoy-20 (MS-20) on the Induction of Chromosome Aberrations in CHO Cells for 3-h Treatment in the Presence of S9

Treatment Conc. (μ l/ml)	Aberrant Cells (%) ^a	Number of Chromosome Aberrations/100 Cells							
		G ^b	B	D	R	g	b	e	MA
Negative control	0	0	0	0	0	0	0	0	0
Negative control	0	0	0	0	0	0	0	0	0
6.25	0	0	0	0	0	0	0	0	0
6.25	0	0	0	0	0	0	0	0	0
12.5	0	0	0	0	0	0	0	0	0
12.5	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0	0
50	2	0	0	0	0	0	0	2	0
100	1	0	0	0	0	1	0	1	0
100	4	0	0	0	0	0	0	4	0
Positive control ^c	20	0	0	0	0	0	9	11	0
Positive control ^c	17	0	0	1	1	0	8	7	0

^a Aberrant cells were calculated excluding cells with gaps.

^b G: chromosome gap B: chromosome break D: dicentric R: ring
g: chromatid gap b: chromatid break e: exchange MA: multiple aberrations

^c Positive control was 40 μ M cyclophosphamide for 3 h.

Data from duplicate cultures were recorded independently by two observers.

Table 5. Effects of Product Code MicrSoy-20 (MS-20) on the Induction of Chromosome Aberrations in CHO Cells for 20-h Treatment in the Absence of S9

Treatment Conc. (µl/ml)	Aberrant Cells (%) ^a	Number of Chromosome Aberrations/100 Cells							
		G ^b	B	D	R	g	b	e	MA
Negative control	0	0	0	0	0	0	0	0	0
Negative control	0	0	0	0	0	0	0	0	0
6.25	0	0	0	0	0	0	0	0	0
6.25	0	0	0	0	0	0	0	0	0
12.5	0	0	0	0	0	1	0	0	0
12.5	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	1	0	0	0
50		Too few metaphases							
50		Too few metaphases							
100		Too few metaphases							
100		Too few metaphases							
Positive control ^c	22	0	1	0	1	1	1	20	0
Positive control ^c	23	0	0	0	0	0	3	18	2

^a Aberrant cells were calculated excluding cells with gaps.

^b G: chromosome gap B: chromosome break D: dicentric R: ring
g: chromatid gap b: chromatid break e: exchange MA: multiple aberrations

^c Positive control was 1 µM mitomycin C for 3 h.

Data from duplicate cultures were recorded independently by two observers.



Table 6. Summary of the Results of Chromosome Aberrations of Product Code MicrSoy-20 (MS-20) in Chinese Hamster Ovary Cells

Treatment	Concentration (μ l/ml)	Treating Hour	S9 (-/+)	Aberrant Cells (%)
<i>Scheme I (-S9, 3 h)</i>				
Vehicle control	0	3	-	0
Test article	6.25	3	-	0
	12.5	3	-	0
	25	3	-	0.5
	50	3	-	0.5
	100	3	-	too few metaphases
Positive control		3	-	23
<i>Scheme II (+S9, 3 h)</i>				
Vehicle control	0	3	+	0
Test article	6.25	3	+	0
	12.5	3	+	0
	25	3	+	0
	50	3	+	1
	100	3	+	2.5
Positive control		3	+	18.5
<i>Scheme III (-S9, 20 h)</i>				
Vehicle control	0	20	-	0
Test article	6.25	20	-	0
	12.5	20	-	0
	25	20	-	0
	50	20	-	too few metaphases
	100	20	-	too few metaphases
Positive control		3	-	22.5

1. All data were scored from 200 metaphase cells of each treatment (duplicate cultures).
2. Positive control used in each test scheme was described in Tables 3 ~ 5.

Appendix A

◆ Test Article Information Sheet



Test Article Information Sheet

DV00199-a

1/1

DV-QA00033E

Sponsor : MICROBIO Co., Ltd.Address : No.81 Gauyang N. Rd., Lung tan Shiang, Tao Yuan, TaiwanTelephone : 886-3-4710888 Fax : 886-3-4710288Delivery Date : 10 / 25 / 2001 (MM / DD / YY)Category : Health Food Herb Medicine Drugs Cleanser Medical Devices
 Cosmetics Pesticides Others : _____1. Sample Name : Product code MicrSoy-20(MS-20)

2. a. Ingredients :

MS-20 is a Chinese medicine. The components are very complicated. Until now, its effective components are still unable to determine.

b. Purity : _____

3. Batch / Lot No. : 20010209

4. Physical Appearance :

a. Powder Liquid Others : _____b. Odor : No Yes : Prune Juicec. Color : Dark-brown5. How Supplied (Amount / Pack) : 180 ml/Bottle6. Amount Supplied : 2 Bottle



Test Article Information Sheet

DV00199-a

Solubility (Approx. _____ g/L)
H₂O Soluble , DMSO _____, Other Solvents _____

8. Storage

a. Storage Temperature : Room Temperature Refrigeration Frozen

b. Other Environment Condition : Desiccation Protect from Light
 Others : _____

c. Expiration Date : 02/ 09/ 2004 (MM / DD / YY)

9. Treatment of Residual Samples

Retrieved by the Sponsor

Managed by DCB with Extra Fees

Disposed by DCB with Waste Disposal Method Provided :

10. Handling Precautions and Others

Directions: To drink 1~5c.c. daily by dilution with 100c.c. water before breakfast.
It's not suggested to drink water in 10 minutes after MS-20. After 10 minutes later, we suggest you to drink water as usual. Before dilution, the product can be stored at room temperature after opening, but please use the product immediately after dilution.

Undiluted product has a high acidity of pH around 3.8

MS-20 has two packages which are 180ml/Bottle and 30ml/Bottle

Product Chemist :

Jaeon Kim

10 / 25 / 2004 (Signature)
(MM / DD / YY)

Sponsor Representative :

[Signature]
William Lee

10 / 25 / 2004 (Signature)
(MM / DD / YY)

Appendix B

◆ Protocol and Protocol Amendment



***IN VITRO* CHROMOSOME ABERRATION ASSAY- PRODUCT CODE MicrSoy-20 (MS-20)**

PROTOCOL

**DEVELOPMENT CENTER FOR BIOTECHNOLOGY
DRUG DEVELOPMENT DIVISION**



Signature Page

Study Director:

Shwu-Fei Lee-Chen Dec. 10 / 2001
Dr. Shwu-Fei Lee-Chen, Ph.D.

Investigators:

Yann-Hur g Shieh, B.S.

Chun-Han, Shih, M.S.

Quality Assurance Officer:

Ying-Chuan Yang Dec. 10 / 2001
Ying-Chuan Yang, M.S.

Facility Manager:

Jiann Judy Liu Dec 10 / 2001
Dr. Jiann Liu, DVM, Ph.D.

Sponsor's Representative:

William Lu Dec 15 / 2001
William Lu



IN VITRO CHROMOSOME ABERRATION ASSAY PRODUCT CODE MICRSOY-20 (MS-20)

I. PURPOSE

The objective of chromosome aberration assay is to assess the potential of test article to induce structural chromosome mutations in Chinese hamster ovary cells. This study should provide a rational basis of risk assessment in man.

II. TESTING FACILITY

- A. Name: Genetic Toxicology Laboratory, Drug Development Division, Development Center for Biotechnology
- B. Address: 103, Ln. 169, Kang-Ning St., Hsi-Chih City, Taipei County 221, Taiwan, R.O.C.

III. SPONSOR

- A. Name: MICROBIO Co., Ltd.
- B. Address: No. 81, Gaiyang N. Rd., Lung Tan Shiang, Tao Yuan, Taipei, Taiwan, R.O.C.
- C. Representative: William Lu

IV. TEST ARTICLE (To be supplied by the sponsor prior to study initiation)

- A. Name/Identification: Product code MicrSoy-20 (MS-20)
- B. Receiving Date: Oct. 05, 2001
- C. Batch/Lot Number: 20010209
- D. DCB Code: DV00199-1
- E. Ingredients: MS-20 is a Chinese medicine. The components are very complicated. Until now, its effective components are still unable to determine.
- F. Storage Conditions: Room temperature and desiccation in a dark bottle
- G. Expiration Date: Feb. 09, 2004
- H. Physical Appearance: Dark-brown liquid with prune juice odor

Statement:

The test article is a proprietary product of the sponsor, therefore the sponsor will be responsible for the requirements listed under "Test Article" of the GLP regulation (21CFR§58.105, FDA).



V. TEST SCHEDULE

- A. Proposed First Date of Target Cells Seeding: Dec. 17, 2001
- B. Proposed First Date of Test Article Treatment: Dec. 18, 2001
- C. Proposed First Date of Chromosome Slides Preparation: Dec. 19, 2001
- D. Proposed Chromosome Slides Observation Period: Dec. 24 ~ 28, 2001

VI. TEST SYSTEM

- A. Cells: Chinese hamster ovary cells (CHO-K1)
- B. Source: American Type Culture Collection (ATCC, repository number CCL-61), Rockville, MD, U.S.A.
- C. Modal Chromosome Number: 20
- D. Population Doubling Time: About 12~14 hours
- E. Mycoplasma: Negative
- F. Media and Culture Condition:

CHO-K1 cells are stored in liquid nitrogen. After thawing, cells will be maintained as monolayers in McCoy's 5A medium (GIBCO, U.S.A.) supplemented with 10% fetal bovine serum, 0.22% sodium bicarbonate, 1% penicillin-streptomycin solution, pH 7.0~7.2 in a humidified incubator at 37 ± 1 °C and 5 ± 1 % CO₂ in air.

- G. Metabolic Activation:

Postmitochondrial fraction (S9) of livers of Sprague-Dawley rats induced with Aroclor 1254 will be used for metabolic activation and was prepared by MOLTOX™, U.S.A. S9 containing medium will be freshly prepared by mixing NADP, G-6-P and S9 in serum-free McCoy's 5A medium (SOP : DCB-DV-TE00217).

VII. EXPERIMENTAL DESIGN

- A. Test Article Preparation and Concentration-range Finding Test

The solution of test article will be prepared according to the Sponsor's information. Test article stock solution of Product code MicrSoy-20 (MS-20) will be prepared in McCoy's 5A medium and filtered through 0.2 µm membrane after adjusting pH value to the range of 7.0 ~ 7.2. Cell culture medium will be used for further dilution of test article solution to desired concentrations.

A preliminary assay with MTT cytotoxicity test was performed to determine the top concentrations for chromosome aberration assay. The results of MTT assay showed that in the absence of S9 the test article did not produce more than 50% cytotoxicity until the concentration reached at 50 $\mu\text{l/ml}$ for 3-h treatment or at 25 $\mu\text{l/ml}$ for 20-h treatment. In addition, by referring colony formation test results of HPRT gene mutation assay, the top concentrations are set as 50 $\mu\text{l/ml}$ and 25 $\mu\text{l/ml}$ for 3-hour and 20-hour treatments, respectively, in the chromosome aberration assay.

B. Exposure Concentrations

According to the regulatory guidelines, the top concentration of chromosome aberration assay should be the lowest concentration to produce greater than 50% cytotoxicity, if possible. The maximum concentration for soluble non-toxic substance is 5 mg/ml.

The top concentrations of chromosome aberration assay of MS-20 are set as described above. Five concentration levels with a dilution factor of 2 will be conducted in both the presence and absence of S9 activation for 3-hour treatment, and 20-hour treatment without S9 activation. The following concentrations will be used for cell treatment in treatment schemes I, II and III.

Treatment Scheme	Treating Condition	Concentration ($\mu\text{l/ml}$)
I	-S9, 3 hours	0, 3.125, 6.25, 12.5, 25, 50
II	+S9, 3 hours	0, 3.125, 6.25, 12.5, 25, 50
III	-S9, 20 hours	0, 1.5625, 3.125, 6.25, 12.5, 25

C. Controls

1. Positive Control

In the chromosome aberration test without S9 activation, mitomycin C [CAS no. 50-07-7] (Boehringer Mannheim Art.107409, Germany) at 1 ~ 2 μM for 3-hour treatment will be used as the positive control. Cyclophosphamide [CAS no. 6055-19-2] (Sigma Cat. No. C0768, U.S.A) at 20 ~ 40 μM for 3-hour treatment will be the positive control for the test with S9 activation. Positive controls will be prepared in dimethyl sulfoxide (Merck Art. 2931, Germany) and added to culture medium.

2. Solvent Control (Negative Control)

McCoy's 5A medium will be used as the solvent control.



D. Procedures of Chromosome Aberration Assay

1. Preparation of Target Cells

Exponentially growing CHO-K1 cells will be seeded in complete McCoy's 5A medium at approximately 5×10^5 cells/ 60 mm dish. The cells will be incubated overnight before treatment. Duplicate cultures will be used at each concentration of test article and for negative and positive control cultures.

2. Treatment of Target Cells

Freshly prepared test article or control solutions will be directly added to the cells and incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO_2 for desired treatment hours. Chromosome aberration assay will be performed with 3 treatment schemes. In scheme I, cells will be treated with test article for 3 hours in the absence of S9. In scheme II, cells will be treated for 3 hours in the presence of S9. In these two test schemes, mitotic cells will be harvested at approximately 20-hour after the beginning of treatment. In scheme III, cells will be continuously treated for 20 hours without S9 activation. Colcemid will be added 2 h before each harvest at a final concentration of $0.1 \mu\text{g/ml}$.

If there is precipitate interfered with cell harvest, the culture of scheme III will be washed after 20-h treatment before adding colcemid. Cells will then be cultured for additional 2-4 hours before harvesting for mitotic cells.

3. Concurrent measures of cytotoxicity by cell counting for all treated and negative control cultures will also be recorded.

4. Chromosome Slides Preparation

Mitotic cells will be harvested by the shake-off technique. Collected cells will be treated with hypotonic solution and fixed with a mixture of methanol and acetic acid (3:1, v/v). Chromosome slides will be prepared by dropping cell suspension on clean slides and stained with 3 % Giemsa.

VIII. OBSERVATION AND EXAMINATION

A. Mitotic cells with chromosome no. 18 ~ 22 will be observed for the presence of aberrant chromosomes. For each treatment, 200 metaphases will be examined in blind-coded slides under microscope with 1000X magnification. Number of metaphases scored may be reduced when significant cytotoxicity or cell cycle delay occurred.

B. Cells without observable aberrant chromosome are recorded as N (Normal). Aberrant chromosome morphologies including chromosome gap (G), chromosome



break (B), dicentric (D), ring (R), chromatid gap (g), chromatid break (b), and exchange (e) will be recorded separately on the data sheet for each culture. Cells with multiple aberrant chromosomes are recorded as MA. Polyploidy and endoreduplication will also be recorded when these events are observed but will not be calculated unless significant numbers are seen. Percents of structurally aberrant cells will be calculated excluding cells with chromosome and chromatid gaps.

- C. Number of cells with chromosome aberrations and type of chromosome aberrations will be recorded separately for each treated and control culture.

IX. STATISTICAL ANALYSIS

A. Acceptance Criteria

Negative control

The percent of aberrant cells in the negative control must not exceed 3.

Positive control

The percent of aberrant cells in the positive control should be statistically increased ($p < 0.05$) relative to the negative control.

- B. For a given concentration, percent of aberrant cells will be analyzed by one-tail binomial test and compared pairwise to the negative control. According to standard normal distribution, set α value 0.01 as the significant level.
- C. If there is at least one of the test concentration giving significant results, trend test is proceeded to determine the existence and extent of a CA-inducing concentration-responsiveness.
- D. Final interpretation of the assay will take into account both the number of concentrations significant and the trend probability (Galloway et al., 1985). However, a test result is considered negative in the test system if none of test points gives significant increase of frequency of CA than negative control.

X. RECORDS RETENTION

All raw data, documentation, records, protocols and final reports generated as a result of this study will be inventoried and archived by the Quality Assurance Unit at DCB's archives located in Drug Safety Building. The slides related to this experiment will be retained in genetic toxicology laboratory. The retention duration of those records and slides will be in accordance with the relevant regulations.

XI. REGULATORY REQUIREMENTS

This study will be performed in compliance with (1) Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR 58), FDA, U.S.A., 1987; (2) Good Laboratory Practice for Nonclinical Laboratory Studies, Department of Health, R.O.C., 3rd edition, 2000; (3) General Requirements for the Competence of Calibration and Testing Laboratories (ISO/IEC Guide 25), ISO/IEC, third edition, 1990; (4) Specific Criteria for Biological Testing, Chinese National Laboratories Accreditation, R.O.C., 2nd edition, 2000, with the exceptions of "test article" requirement.

XII. REFERENCES

1. Galloway, S. M., Aardema, M. J., Motoi Ishidate, Jr., Ivett, J. L., Kirkland, D. J., Morita, T., Mosesso, P., and Sofuni, T. (1994) International Workshop on Standardization of Genotoxicity Test Procedures. "Report from working group on *in vitro* tests for chromosomal aberrations". Mutation Research 312: 241-261.
2. Galloway, S. M., Bloom, A. D., Resnick, M., Margolin, B. H., Nakamura, F., Archer, P., and Zeiger, E. (1985) Development of a Standard Protocol for *In Vitro* Cytogenetic Testing with Chinese Hamster Ovary Cells. Environ. Mutag. 7: 1-51.
3. Margloin, B. H., Resnick, M. A., Rimpo, J. Y., Archer, P., Galloway, S. M., Bloom, A. D., and Zeiger, E. (1986) Statistical Analyses for *In Vitro* Cytogenetic Assays Using Chinese Hamster Ovary Cells. Environ. Mutag. 8: 183-204.
4. OECD Guideline for the Testing of Chemicals #473: *In Vitro* Mammalian Chromosome Aberration Test, 1997.
5. Quality Manual, Drug Development Division, Development Center for Biotechnology, 6th ed., 1999.

Protocol Amendment

Protocol Serial No.: DV-PR-CA00022E

Amendment No.: 1

Project Code: DV-TA00199

Study Title: *In Vitro* Chromosome Aberration Assay – Product code MicrSoy-20 (MS-20)

Date Issued: Jan. 08, 2002

Changed from:

V. TEST SCHEDULE

- A. Proposed First Date of Target Cells Seeding: Dec. 17, 2001
- B. Proposed First Date of Test Article Treatment: Dec. 18, 2001
- C. Proposed First Date of Chromosome Slides Preparation: Dec. 19, 2001
- D. Proposed Chromosome Slides Observation Period: Dec. 24 ~ 28, 2001

VII. A. Test Article Preparation and Concentration-range Finding Test

P.5, Line 6,the top concentrations are set as 50 µl/ml and 25 µl/ml for 3-hour and 20-hour treatments, respectively, in the chromosome aberration assay.

VII. B. Exposure Concentrations

Treatment Scheme	Treating Condition	Concentration (µl/ml)
I	-S9, 3 hours	0, 3.125, 6.25, 12.5, 25, 50
II	+S9, 3 hours	0, 3.125, 6.25, 12.5, 25, 50
III	-S9, 20 hours	0, 1.5625, 3.125, 6.25, 12.5, 25

Changed to:

V. TEST SCHEDULE

- A. Proposed First Date of Target Cells Seeding: Jan. 14, 2002
- B. Proposed First Date of Test Article Treatment: Jan. 15, 2002
- C. Proposed First Date of Chromosome Slides Preparation: Jan. 16, 2002
- D. Proposed Chromosome Slides Observation Period: Jan. 21 ~ 25, 2002



VII. A. Test Article Preparation and Concentration-range Finding Test

P.5. Line 4 ..., and referring the cytotoxicity results of the preliminary CA test, the top concentrations are set as 100 µl/ml for both 3-hour with and without S9 and 20-hour with S9 treatments in the chromosome aberration assay.

VII. B. Exposure Concentrations

Treatment Scheme	Treating Condition	Concentration (µl/ml)
I	-S9, 3 hours	0, 6.25, 12.5, 25, 50, 100
II	+S9, 3 hours	0, 6.25, 12.5, 25, 50, 100
III	-S9, 20 hours	0, 6.25, 12.5, 25, 50, 100

Reasons for Change:

After cells were treated with MS-20 in schemes I, II and III, the cytotoxicity recorded for the top concentrations were 43.7%, 20.6% and 45.2%, respectively. Therefore, the top concentrations of 3 schemes are adjusted to 100 µl/ml. Additional 4 concentrations with a dilution factor of 2 will be used to treat cells. The exposure concentrations are adjusted to ensure covering the concentration that causes more than 50% cytotoxicity and obtaining three analyzable concentrations for chromosome assay.

Approved by:

Study Director: Shu-ji Loh Jan 10/1/2002

Facility Manager: Jian-fu Liu Jan 10/1/2002

Sponsor Representative: William Lu Jan 10/1/2002

Appendix C

- ◆ Historical Control Data of Chromosome Aberrations Assay in Chinese Hamster Ovary Cells

Historical Control Data of Chromosome Aberrations Assay in Chinese Hamster
Ovary Cells

	Non-activated		S9- activated	
	Solvent Control	1 ~ 2 μ M Mitomycin C	Solvent Control	20 ~ 40 μ M Cyclophosphamide
Mean	0	33	0	23
SD	0	10	0	6
Maximum	1	56	1	36
Minimum	0	14	0	11

Data Recording Period: Dec. 1998 – Dec. 2001

Solvent control (culture medium, distilled water, DMSO, vehicle supplied by Sponsor)

SD : Standard deviation