COELENTERAZINE: IN VITRO CHROMOSOMAL ABERRATION ASSAY IN CHINESE HAMSTER OVARY CELLS

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STUDY IDENTIFICATION

Coelenterazine: In Vitro Chromosomal Aberration Assay in Chinese Hamster Ovary Cells

Test Article: Coelenterazine

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Study Initiation Date: 2011-09-13
Experimental Start Date: 2011-09-13
First Day of Dosing: 2011-09-20
Experimental Completion Date: 2011-11-03
Study Completion Date: 2012-01-12
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QUALITY ASSURANCE STATEMENT

Study Director: Zhewen Hu

Test Article: Coelenterazine

Study Title: Coelenterazine: In Vitro Chromosomal Aberration Assay in Chinese Hamster Ovary Cells

Study Number: 139-0005

Pursuant to Good Laboratory Practice regulations this statement lists the dates of inspections and/or audits by the WuXi AppTec (Suzhou) Co., Ltd., GLP Quality Assurance Unit and reporting date(s) to the Study Director and Test Facility Management as appropriate according to QA SOPs. In addition, this statement confirms the final report accurately reflects the raw data.

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Robert J Coldreck, BS, RQAP-GLP
VP of GLP Quality Assurance

2012-01-12 Date
COMPLIANCE STATEMENT

The dose range-finding assay was non-GLP and conducted in full compliance with the study protocol and WuXi AppTec (Suzhou) Standard Operating Procedures (SOPs).

The definitive chromosome aberration assay performed at WuXi AppTec (Suzhou) Co., Ltd. adhered to the study protocol and WuXi AppTec Standard Operating Procedures (SOPs), and conformed to the most recent version of the Good Laboratory Practice (GLP) regulations listed below. The GLP terms (e.g., study protocol, test article, control article) used in this report are based on the US FDA Good Laboratory Practice regulations and are considered synonymous with equivalent terms used in the OECD Principles of Good Laboratory Practice.

♦ Principles on Good Laboratory Practice issued by the Organization for Economic Co-operation and Development (OECD), revised in 1997.
♦ The Nonclinical Laboratory Studies Good Laboratory Practice Regulations issued by the U.S. Food and Drug Administration (Title 21 of the Code of Federal Regulations, Part 58; effective June 20, 1979. and all subsequent amendments).
♦ Good Laboratory Practice for Non-clinical Laboratory Studies, issued by State Food and Drug Administration, P.R. China. Effective September 01, 2003.

Zhewen Hu, MS 胡哲文，硕士
Genetic Toxicology 遗传毒理
(Study Director) （专题负责人）

Date 日期 2012-01-12
COELENTERAZINE: IN VITRO CHROMOSOMAL ABERRATION ASSAY IN CHINESE HAMSTER OVARY CELLS

Study No. 实验编号: 139-0005

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Genetic Toxicology 遗传毒理
(Study Director) 专题负责人

Ying Jiang, PhD 姜颖，博士
Executive Director, Laboratory Science
实验科学部执行主任
WuXi AppTec (Suzhou) Co., Ltd.
苏州药明康德新药开发有限公司
(Facility Management 机构管理层)

2012-01-12  2012-01-12
SUMMARY

- The test article, Coelenterazine, was tested in the chromosome aberration assay using Chinese hamster ovary (CHO-WBL) cells in both the presence and absence of Aroclor 1254 induced rat liver S9 activation system. A dose range-finding assay was performed to establish the dose range for the definitive chromosome aberration assay. The definitive chromosome aberration assay was used to evaluate the clastogenic potential of the test article. The S9-activated 3 hours treatment series of the definitive chromosome aberration assay was repeated in this study.

- Ethanol was the solvent of choice based on the solubility of the test article and compatibility with the CHO-WBL cells. The test article formed suspension in ethanol at a concentration of approximately 211.73 mg/mL. The test article formed clear solution in ethanol at a concentration of approximately 6.2 mg/mL.

- In the dose range finding and definitive chromosome aberration assays, CHO-WBL cells were treated for 3 and 20 hours in the non-activated test systems and for 3 hours in the S9 activated test system. All cells were harvested 20 hours after initiation of test article treatment.

- In the dose range-finding assay, the maximum dose tested, 2117 µg/mL, was above the solubility limit of the test article after dosing into the culture medium.

- Selection of dose levels for the definitive chromosome aberration assay was based on the cell growth inhibition relative to the solvent control. Single culture of CHO cells was incubated with test article at 1, 2, 5, 10, 20, 40, 100, 250, 700 and 2117 µg/mL in S9 activated 3 hours exposure group and 1, 2, 5, 10, 20, 50, 100, 250, 700 and 2117 µg/mL in non-activated 3 and 20 hours exposure groups. For S9 activated 3 hours exposure group, substantial toxicity (ie, not greatly exceeding 50% cell growth inhibition, relative to the solvent control) was observed at dose levels ≥100 µg/mL, and the dose levels chosen for the definitive chromosome aberration assay were 20, 40, 50, 60, 70, 80 and 100 µg/mL; for non-activated 3 hours exposure group, substantial toxicity was observed at dose levels ≥100 µg/mL, and the dose levels chosen for the definitive chromosome aberration assay were 20, 40, 50, 60, 70, 80 and 100 µg/mL; for non-activated 20 hours exposure group, substantial toxicity was observed at dose levels ≥20 µg/mL, and the dose levels chosen for the definitive chromosome aberration assay were 5, 10, 15, 20, 30, 40 and 50 µg/mL.

- The definitive chromosome aberration assay was conducted in duplicate cultures and with concurrent solvent, blank and positive controls. Colcemid was added to the cultures at a final concentration of 0.1 µg/mL 2 hours prior to harvest. Precipitate was observed in the treatment medium at dose levels ≥40 µg/mL in the S9-activated treatment series, and ≥50 µg/mL in the non-activated treatment series. Selection of dose levels for the microscopic analysis (20, 40, and 60 µg/mL for the S9 activated 3 hours exposure group, 20, 40, and 60 µg/mL for the non-activated 3 hours exposure group, 5, 10, and 15 µg/mL for the non-activated 20 hours exposure group) was based on cytotoxicity (the lowest dose with not greatly exceeding 50% cell growth inhibition, relative to the solvent control, and 2 lower dose levels). One hundred well-spread metaphase cells for each culture and 200 for each dose were scored.

- There was no significantly increase of the percentage of cells with structural or numerical aberrations observed when compared to the solvent control at any dose level (p>0.05, Fisher’s exact test) in all three of the exposure groups. The validity of the assay was confirmed.
However, a SOP deviation occurred in the definitive chromosome aberration assay that the incomplete McCoy’s 5A medium was placed on ice after mixing with the S9 mixture. And the incidences of polyploid and endoreduplicated cells (3.38% and 9.50%, respectively) in cultures with S9 was significantly higher when compared to the cultures without S9 (2.44% and 0.99%, respectively) in this study and historical control data (0-2.91% and 1.48-6.54%, respectively). Therefore, the definitive chromosome aberration assay was repeated at dose levels of 20, 40, 60, and 70 µg/mL in 3-hour exposure with S9 group. And the dose levels selected for the microscopic analysis were 20, 40, and 60 µg/mL. There was no significantly increase of the percentage of cells with structural or numerical aberrations observed when compared to the solvent control at any dose level (p>0.05, Fisher’s exact test) and the incidence of polyploid and endoreduplicated cells was comparable with our laboratory historical data.

The results of the chromosome aberration assay are summarized in the following table:

<table>
<thead>
<tr>
<th>Treatment Time</th>
<th>Harvest Time</th>
<th>S9</th>
<th>Toxicity* at the Highest Dose Scored</th>
<th>Positive Dose(s) for Structural Aberrations</th>
<th>Positive Dose(s) for Numerical Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hours</td>
<td>20 hours</td>
<td>+</td>
<td>54%</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>3 hours</td>
<td>20 hours</td>
<td>-</td>
<td>53%</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>20 hours</td>
<td>20 hours</td>
<td>-</td>
<td>53%</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>3 hours#</td>
<td>20 hours</td>
<td>+</td>
<td>59%</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

* Cell growth inhibition; # Repeating test.

Based on the findings of this study, Coelenterazine was concluded to be negative for the induction of structural and numerical chromosome aberrations in CHO-WBL cells under the conditions of this GLP chromosome aberration study.
1 OBJECTIVE

This study was performed to evaluate the clastogenic potential of Coelenterazine by measuring its ability to induce chromosome aberrations in Chinese hamster ovary Cells with or without exogenous metabolic activation (Aroclor 1254 induced rat liver S9).

2 REGULATORY COMPLIANCE

The dose range-finding assay was non-GLP and conducted in full compliance with the study protocol and WuXi AppTec (Suzhou) Standard Operating Procedures (SOPs).

The definitive chromosome aberration assay performed at WuXi AppTec (Suzhou) Co., Ltd. adhered to the study protocol and WuXi AppTec Standard Operating Procedures (SOPs), and conformed to the most recent version of the Good Laboratory Practice (GLP) regulations listed below. The GLP terms (e.g., study protocol, test article, control article) used in this report are based on the US FDA Good Laboratory Practice regulations and are considered synonymous with equivalent terms used in the OECD Principles of Good Laboratory Practice.

- Principles on Good Laboratory Practice issued by the Organization for Economic Co-operation and Development (OECD), revised in 1997.
- The Nonclinical Laboratory Studies Good Laboratory Practice Regulations issued by the U.S. Food and Drug Administration (Title 21 of the Code of Federal Regulations, Part 58; effective June 20, 1979. and all subsequent amendments).
- Good Laboratory Practice for Non-clinical Laboratory Studies, issued by State Food and Drug Administration, P.R. China. Effective September 01, 2003.

3 TEST AND CONTROL ARTICLES

Certificate of Analysis (COA) of test and solvent control articles are presented in Appendices 1 and 2.

3.1 Test Article Description

<table>
<thead>
<tr>
<th>Identity:</th>
<th>Coelenterazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Code:</td>
<td>CTZ</td>
</tr>
<tr>
<td>CAS Number:</td>
<td>55779-48-1</td>
</tr>
<tr>
<td>Manufacturer:</td>
<td>WuXi AppTec (Tianjin) Co., Ltd.</td>
</tr>
<tr>
<td>Batch Number:</td>
<td>TH 03647-031</td>
</tr>
<tr>
<td>Purity:</td>
<td>93.57%</td>
</tr>
<tr>
<td>Molecular Weight:</td>
<td>423.46</td>
</tr>
<tr>
<td>Storage Conditions:</td>
<td>Store at -20°C, protect from light</td>
</tr>
<tr>
<td>Physical Characterization:</td>
<td>Yellow powder</td>
</tr>
<tr>
<td>Expiration Date</td>
<td>June 2021</td>
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</table>
3.2 Solvent/Vehicle Control Article Description

Identity: Ethanol
CAS Number: 64-17-5
Manufacturer: Sinopharm Chemical Reagent Co., Ltd.
Batch Number: 20100506
Concentration: 95%
Storage Conditions: Room temperature
Physical Characterization: Colorless liquid
Expiration Date: 2013-09-01

The solvent control article was evaluated concurrently with test article under each exposure group, at the same dose volume as was used to administer the test article in both the dose range-finding assay and definitive chromosome aberration assay.

As ethanol was a non-standard solvent used in this assay, untreated control cultures also were included to ensure that the vehicle had no deleterious effect upon cell growth or the endpoints measured.

3.3 Positive Control Articles Description

Cyclophosphamide monohydrate [CP; CAS 6055-19-2], an agent that requires metabolic activation to its clastogenic form, was evaluated concurrently at concentrations of 1.5 and 5.0 µg/mL in the S9 activated exposure group. Mitomycin C [MMC; CAS 50-07-7], a clastogen that does not require metabolic activation, was evaluated concurrently at concentrations of 0.17 and 0.5 µg/mL in the non-activated 3 hours exposure group, at concentrations of 0.05 and 0.25 µg/mL in the non-activated 20 hours exposure group. These positive control articles were obtained from Sigma Chemical Company, prepared in sterile distilled water as 100x stocks in advance, stored at -20°C, and thawed immediately before use. These positive controls were chosen because of the large data base available and they are recommended by OECD guideline 473.

Positive control articles were used only in the definitive chromosome aberration assay. Results obtained from the positive control articles were used to validate the responsiveness of the test system, but they were not used to provide a standard for comparison with the test article.

3.4 Sample Retention

One (1) g of the test article was collected by the Central Pharmacy Laboratory as the reserve sample. It was archived in the GLP archives according to WuXi AppTec (Suzhou) SOPs.

3.5 Inventory and Disposition

Bulk test article supplied by the Sponsor will be returned to the Sponsor following completion of the study, control article formulations supplied by WuXi AppTec (Suzhou) were discarded in accordance with testing facility SOPs.
4 DOSE FORMULATION PREPARATION AND ANALYSIS

4.1 Dose Formulation Preparation

A correction for potency was performed, as the concentrations in this assay were based on a theoretical potency of 93.57%.

For Coelenterazine dose formulations, which were clear solutions or suspensions, they were prepared separately, immediately prior to use by mixing a weighed amount of Coelenterazine with a measured volume of the solvent, and then diluting with solvent. Dose formulations were prepared under yellow light, and stored at room temperature in tightly closed sterile brown containers. Vortex mixing, and heating to 37°C±2°C were employed to aid in solubilization. Dose formulations were exposed to ambient temperature during transport. Details about dose formulation preparation were described and recorded in dose formulation instruction and preparation sheet.

4.2 Samples Collection

Dose formulations for the definitive chromosome aberration assay were analyzed. Dose formulations for the dose range-finding assay were not analyzed. No dose formulation analysis was performed on the positive control article dose formulations.

Upon preparation for use in definitive chromosome aberration assay, samples were collected from the solvent and all Coelenterazine dose formulations for analysis.

- For dose formulations that were clear solutions, 2 sets of 1 sample (1 mL each) were collected from the middle portion of the formulations for concentration analysis. One set was used for analysis and the other served as the backup.
- For dose formulations that were suspensions, 2 sets of 1 sample (1 mL each) each from the top, middle, and bottom portions of the formulations were collected. One set was used for analysis and the other served as the backup. The middle portion of the dose formulations were used to verify concentration and homogeneity. The top and bottom portions of the dose formulations were used to verify homogeneity along with the middle portion of the dose formulation.
- To assess the stability of the test article, 2 extra sets of 1 sample (1 mL each) were collected from the middle portion of the lowest and highest formulations for concentration analysis. One set was used for analysis and the other served as the backup.
- For the solvent, 2 sets of samples (1 mL each) were collected to confirm the absence of Coelenterazine. One set was used for analysis and the other served as the backup.

The volume of samples taken was adjusted per request of the Study Director. All samples collected for analysis were stored at room temperature and backups were stored at -20°C.
4.3 Dose Formulation Analysis

All analytical work was conducted by the Analytical Chemistry Laboratory using an analytical method developed and validated by WuXi AppTec (Suzhou) (Analytical Method: AM-A110818-01-01).

4.3.1 Concentration Verification

Immediately after the dose formulations were prepared, samples were collected and transported to the Analytical Chemistry Laboratory within the testing facility for concentration verification.

The collected dose formulation samples were analyzed for Coelenterazine concentration using a validated method.

4.3.2 Stability Assessment

For Coelenterazine dose formulation samples used for the high- and low-dose groups, one set of samples was analyzed for concentration verification. The extra set of samples was stored for 6 hours longer at room temperature and then analyzed to measure Coelenterazine concentration for evaluating Coelenterazine dose formulations’ stability up to 6 hours. The stability interval started with the baseline which was defined as when the first set of samples was analyzed.

4.3.3 Homogeneity Verification

For dose formulations that were suspensions, the concentrations of the samples from the top, middle, and bottom portions were all analyzed to verify the homogeneity.

4.3.4 Analysis of Concentration Data

Measured concentrations were compared to nominal concentrations to reach conclusions about the exposure of cells in the solvent control cultures to Coelenterazine, the accuracy of dose formulation preparation, and the stability of Coelenterazine in dose formulations.

The Coelenterazine concentration in the solvent formulation must be below the Limit of Quantification X Dilution Factor.

For clear solution formulations, measured Coelenterazine concentrations in dose formulation samples must be within 90% to 110% of their nominal concentrations.

For suspension formulations, measured Coelenterazine concentrations in dose formulation samples must be within 85% to 115% of their nominal concentrations.

The difference in % measured concentration between the six hours’ stability time point and the baseline must be within +/-10% of nominal value.

To reach conclusion of Coelenterazine homogeneity, measured Coelenterazine concentrations in dose formulation samples must be within 85% to 115% of their nominal concentrations with ≤10% relative standard deviation (RSD) of each concentration.
4.4 **Sample Disposition**

All unused dose formulation samples were discarded.

The residual neat test article used in the Analytical Chemistry Laboratory was discarded.

5 **MATERIALS AND METHODS**

The study design is based on the OECD Guideline 473, updated and adopted 21st July 1997.

5.1 **Test System Rationale**

Aberrations are a consequence of failure or mistakes in repair process such that breaks either do not rejoin or rejoin in abnormal configurations (Evans, 1962, 1976; Galloway et al., 1985, 1994).

Descriptions of the types of aberrations are provided in this report. Structural aberrations are of two types, chromosome or chromatid:

- **Chromosome aberration**: structural chromosome damage expressed as breakage, or breakage followed by reunion, of both sister chromatids at an identical site.
- **Chromatid aberration**: structural chromosome damage expressed as breakage of single chromatids or breakage followed by reunion between chromatids. This is the most common type of structural aberration.

Most known chemical clastogens (chromosome-breaking agents) require a period of DNA synthesis to convert initial DNA damage into chromosome alterations that become visible at mitosis. At predetermined intervals after exposure to the test article, the CHO cells will be treated with a metaphase-arresting substance, Colcemid, then harvested and stained, and metaphase cells are then analyzed microscopically for the presence of chromosomal aberrations.

Many mutagenic chemicals do not act directly on DNA but do so after being converted active intermediates by enzymes found in liver. CHO cells have only a limited capacity to metabolize some test articles, so an exogenous metabolic activation system (Aroclor 1254 induced rat liver S9) is included with a series of treatments to enhance the degree of conversion and the ability of the assay to detect clastogenic, metabolic intermediates.

Numerical aberrations are determined by the occurrence of polyploidy or endoreduplication, which may indicate that the test article has the potential to induce numerical aberrations.

5.2 **Test System**

The Chinese hamster ovary cell line, CHO-WBL with a modal chromosome number of 21 and a population doubling time of 12-13 hours was employed in this assay. CHO-WBL cells were originally obtained from Merck Research Laboratories, USA. The cell stocks were stored in liquid nitrogen. Every batch of the cell stocks was checked for the stability of the modal chromosome number and was tested and determined to be free from mycoplasma contamination. Cells were not used after the 15th passage from cloning.
5.3 Media and Cell Culture Condition

Complete medium was McCoy’s 5A medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 unit/mL penicillin, and 100 µg/mL streptomycin. Serum-free medium was McCoy’s 5A medium, supplemented with 2 mM L-glutamine, 100 unit/mL penicillin, and 100 µg/mL streptomycin.

CHO-WBL cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C.

5.4 Metabolic Activation System

The in vitro metabolic activation system (Maron and Ames, 1983) consisted of a Aroclor 1254 induced rat liver post-mitochondrial homogenate (hence referred to as S9) and an energy-producing system (NADP plus isocitric acid). The S9 homogenate was purchased commercially from Molecular Toxicology (Boone, NC) and stored frozen at -80°C until used. The S9 homogenate was prepared from male Sprague-Dawley rats that had been given a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. According to the quality control and production certificate of S9 supplied by Molecular Toxicology, dilutions of the sample S9, ranging from 0.2% to 10% in the S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates that were mutagenic in the Ames Reverse Mutation assay with Salmonella typhimurium tester strain TA100. Such documentation is presented in Appendix 3.

Immediately prior to use, the S9 was thawed and mixed with sterile cofactor solution. The components of S9 mixture were as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration in Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP (disodium salt)</td>
<td>0.8 mg/mL</td>
</tr>
<tr>
<td>Isocitric Acid (trisodium salt)</td>
<td>1.5 mg/mL</td>
</tr>
<tr>
<td>S9 Homogenate</td>
<td>15.0 µL/mL (1.5% v/v)</td>
</tr>
</tbody>
</table>

5.5 Solubility and pH Tests

A solubility test was conducted to determine the maximum soluble concentration or workable suspension using ethanol (CAS 64-17-5). The target concentration was 423.56 mg/mL (1 M).

Test article stocks were diluted 100-fold into culture medium to test for precipitate formation. The maximum final concentration of vehicle in the culture was 1% for organic vehicle.

The pH of the medium containing highest doses was checked using pH indicator paper.

5.6 Dose Range-finding Assay

In the dose range finding assay, 10 test article dose levels were tested in single cultures for each exposure group. The maximum dose level tested, 2117 µg/mL, was limited by the workable concentration of the test article in the solvent, and above the solubility limit of the test article after dosing into the culture medium.
5.6.1 Preparation of Target Cells

Exponentially growing CHO-WBL cells were seeded in complete medium for each treatment condition at approximately $1.2 \times 10^6$ cells/75 cm$^2$ flask with 10 mL medium. Two extra cultures were set up for determination of baseline cell counts at the time of treatment (time zero count, $X_0$; see “Cell Growth”, below). The flasks were incubated at 37°C in a humidified atmosphere of 5% CO$_2$ in air for 20 to 24 hours.

5.6.2 Treatment of Test System

Frequency and route of administration: target cells were treated for 3 and 20 hours in the non-activated test system and for 3 hours in the S9 activated test system, by incorporation of the test article-solvent mixture into the treatment medium. This technique has proven to be an effective method of detection of chemical clastogens in in vitro chromosome aberration assays (Evans, 1976).

All test article dosing was performed at room temperature under yellow light. Twenty to twenty-four hours after culture initiation, medium was aspirated. The cultures were then re-fed with appropriate volume of serum-free medium containing metabolic activation mix for the S9 activated 3 hours exposure group, and appropriate volume of complete medium for the non-activated 3 hours and 20 hours exposure groups. Treatment was carried out by adding 100 µL of dosing solution of test article in solvent or solvent alone to cultures. The final culture volume was 10 mL.

Cultures were incubated at 37°C in a humidified atmosphere of 5% CO$_2$ in air. For the 3 hours exposure with or without S9 groups, the cells were treated for 3 hours. For removal of the test article, the treatment medium was aspirated. The cells were washed with Hank's buffered salt solution (HBSS) twice, re-fed with complete medium, and cultured for an appropriate time until harvest, with Colcemid present for the last 2 hours. For non-activated 20 hours exposure group, the cells were treated for 20 hours with Colcemid present for the last 2 hours.

A baseline cell count was taken from the two parallel control cultures within 30 minutes of the beginning of treatment.

5.6.3 Cell Harvest

Cells were collected approximately 20 hours after initiation of treatment. This post-treatment harvest time represented approximately 1.5 normal cell cycles and was selected to ensure that the cells were analyzed in the first division metaphase after the initiation of treatment. Two hours prior to cell harvest, 100 µL Colcemid (10 µg/mL) was added to the cultures to give a final concentration of 0.1 µg/mL.

Cells were harvested by trypsinization, and an aliquot was removed for counting using automatic cell analysis system and trypan blue dye exclusion. The viable cell counts were used to determine cell growth inhibition relative to the solvent control (cytotoxicity).
5.6.4 Cell Growth (Population Doubling) and Cytotoxicity

Cell growth was expressed as the number of population doublings (PD) between the beginning of treatment and the time of harvest (Galloway et al 2004). The number of PD in the treated cultures was expressed as a percentage of the number of PD in the relevant solvent controls.

Population doublings (PD) = \[ \log \left( \frac{N}{X_0} \right) \div \log 2 \]

Where N = total cells harvested (final number; the viable cell count adjusted after assessing trypan blue exclusion, if applicable).

Xo = number of cells before treatment, that is, the baseline count obtained from the parallel control cultures at approximately the time treatment began for the test cultures.

Relative PD (expressed in terms of percentage) for each treated culture was calculated as follows:

Relative PD (%) = \[ \frac{\text{PD of treated cultures}}{\text{PD of solvent controls}} \times 100 \]

Cytotoxicity (%) was expressed as (100 - Relative PD).

5.7 Definitive Chromosome Aberration Assay

The definitive chromosome aberration assay was conducted using 3-hour exposures with and without S9 groups, and a 20-hour exposure without S9 group only, using duplicate cultures for each test and solvent/positive control article concentration.

The highest dose level tested in the definitive chromosome aberration assay was estimated to not greatly exceed 50% cytotoxicity (not greatly exceeding 50% cell growth inhibition relative to the solvent control). Six lower dose levels were included for each exposure group.

Because an SOP deviation occurred in the definitive chromosome aberration assay that the incomplete McCoy’s 5A medium was placed on ice after mixing with the S9 mixture, and the incidence of polyploid and endoreduplicated cells (3.38% and 9.50%, respectively) in cultures with S9 was significantly higher when compared to the cultures without S9 (2.44% and 0.99%, respectively) in this study and historical control data (0-2.91% and 1.48-6.54%, respectively), it was not sure if this had impact on the outcome of the 3 hours with S9 series in the definitive chromosome aberration assay. Then the definitive chromosome aberration assay was repeated at dose levels of 20, 40, 60, and 70 µg/mL using 3-hour exposures with S9 group.

5.7.1 Preparation of Target Cells and Treatment of Test System

Preparation of target cells and treatment of test system were performed as described in the dose range-finding assay. For positive controls treatment, 100 µL of solutions was dosed to the cultures.
5.7.2 Collection of Metaphase Cells

Cells were collected approximately 20 hours after the initiation of treatment. Two hours prior to cell harvest, 100 µL Colcemid (10 µg/mL) was added to the cultures to give a final concentration of 0.1 µg/mL.

Cells were harvested by trypsinization, and an aliquot was removed for counting using automatic cell analysis system and trypan blue dye exclusion. The remainder of the cells was swollen with 0.075M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight at approximately 4°C. The viable cell counts were used to determine cell growth inhibition relative to the solvent control (cytotoxicity). Cell counts were not performed for the positive control cultures. To prepare slides, the cells were collected by centrifugation and then resuspended in fresh fixative. The suspension of fixed cells was applied to glass microscope slides and air-dried. Two slides were prepared from each culture. The slides were stained with Giemsa and permanently mounted.

5.7.3 Slide Scoring for Mitotic Index

To ensure that a sufficient number of metaphase cells are present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) was determined and recorded for each coded treatment group.

Mitotic index (MI) was expressed as:

\[ MI = \frac{\text{The number of cells in metaphase observed}}{\text{The total number of cells observed}} \]

Relative MI (expressed in terms of percentage) for each treated culture was calculated as follows:

\[ \text{Relative MI (\%)} = \frac{\text{MI of treated cultures}}{\text{MI of solvent controls}} \times 100 \]

Reduction of mitotic index (%) was expressed as (100-Relative MI)

5.7.4 Cell Growth (Population Doubling) and Cytotoxicity

Cell growth and cytotoxicity were determined as described in the dose range-finding assay.

5.7.5 Selection of Dose Levels for Chromosome Aberration Analysis

The selection of dose levels for analysis of chromosome aberrations in CHO cells was based upon cytotoxicity of the test article. The highest dose level selected for evaluation was the dose which induced at least but not greatly exceeding 50% cytotoxicity, as measured by cell growth inhibition, relative to the solvent control, with sufficient number of scorable metaphase cells. Two additional lower dose levels were included in the evaluation.
5.7.6 Slide Scoring for Chromosome Aberration Analysis

Except those from the high doses of CP and MMC (positive control treatments), slides were coded using randomly generated numbers by an individual not involved in slide scoring.

For scoring chromosome aberration, the slides were oriented on the microscope stage with the frosted end up and on the left. Cells were analyzed at high power (1000×) under oil immersion. The slides were scanned methodically to locate good-quality metaphase spreads. The metaphase spreads were selected according to the following criteria:

a. The staining intensity must be adequate to give sufficient contrast.
b. The spread should appear unbroken and should not overlap with other nuclei nor be partially obscured by debris.
c. Chromosome morphology should be good, with little or no overlap of individual chromosomes, and centromeres should not be splitting.
d. The centric chromosomes were counted, and the cell was rejected if the number of centromeres differed by more than two from the diploid number (ie, 21).

200 metaphase cells from each dose level (100 per duplicate flask) were examined and scored. The nature of observed aberrations for each acceptable spread, the number of polyploid and endoreduplicated cells was recorded. A record was kept of the microscope stage locations (Vernier reading) for any metaphase cells with countable structural aberrations. Unless otherwise indicated, the slides will be discarded after the finalization of the report.

5.7.7 Aberration Definitions

Chromatid Type

TG (Chromatid Gap): “Tid Gap.” It is an achromatic (unstained) region in one chromatid. The size is equal to, or smaller than, the width of a chromatid. These are noted but not usually included in final totals of aberrations, as they may not all be true breaks.

IG (Isochromatid Gap): “Chromosome Gap.” The gaps are at the same locus in both sister chromatids. These are noted but are not usually included in final totals of aberrations as they may not all be true breaks.

TB (Chromatid Break): An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced, or missing.

ID (Chromatid Deletion): Length of chromatid “cut” from midregion of a chromatid resulting in a small fragment or ring lying beside a shortened chromatid or a gap in the chromatid.

TR (Triradial): An exchange between two chromosomes, which results in a three-armed configuration and may have an associated acentric fragment.

QR (Quadriradial): The same as the triradial, but resulting in a four-armed configuration.
CR (Complex Rearrangement): An exchange among more than two chromosomes which is the result of several breaks and exchanges.

TI (Chromatid Interchange): Exchange within a chromosome involving one or both arms.

**Chromosome Type**

SB (Chromosome Break): Terminal deletion. Chromosome has a clear break forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated and may remain associated or may appear anywhere in the cell. The broken ends of the chromosome and/or fragment may undergo sister chromatid union, forming a single ring and fragment. This is scored as a chromatid intrachange (Ti).

DM (Double Minute Fragment): Chromosome interstitial deletion. These appear as small double “dots” or may be paired rings. In some cases, they cannot be distinguished from acentric fragments that result from exchanges or terminal deletions.

D (Dicentric): An exchange between two chromosomes that results in a chromosome with two centromeres. This is often associated with an acentric fragment in which it is classified as Dicentric with Fragment (DF).

MC (Multi-centric Chromosome): An exchange among chromosomes that results in a chromosome with more than two centromeres.

R (Ring): A chromosome that forms a circle containing a centromere. This is often associated with an acentric fragment, in which case it is classified as a “Ring with Fragment” (RF). Acentric rings are also included in this category.

AB (Abnormal Monocentric Chromosome): This is a chromosome whose morphology is abnormal for the karyotype, and often the result of such things as a translocation or pericentric inversion. Classification used if abnormality cannot be ascribed to, e.g., a reciprocal translocation. These are noted but not included in aberration totals.

T (Translocation): Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes. When identifiable, it is scored as “T,” not as “2 AB.” These are noted but not included in aberration totals.

RT (Robertsonian Translocation): The result of a translocation which has occurred in the centromere region of two acrocentric chromosomes giving whole area exchanges. The resulting chromosome has the appearance of a metacentric chromosome but counts as two centromeres in the total chromosome count for the cell. These are noted but not included in aberration totals.

**Other**

SD (Severely Damaged Cell): A cell with 10 or more aberrations of any type. A heavily damaged cell should be analyzed to identify the type of aberrations and may not have 10 or more, e.g., because of multiple fragments such as those found associated with a tricentric.

PU (Pulverized Chromosome): Despiralized, or fragmented chromosome. This may simply be at a different stage of chromosome condensation. These are noted but not included in final totals of aberration.
**P+ (Pulverized Cell):** More than one chromosome, up to the whole nucleus, is “pulverized.” These are noted but not included in final totals of aberrations.

**Numerical Aberrations**

**PP (Polyploid Cell):** A cell containing multiple copies of the haploid number of chromosomes. Polyploid cells are occasionally observed in normal bone marrow or cell culture. These are recorded but are not included in final totals of structural aberrations.

**ER (Endoreduplication):** Chromosome reduplication occurring in the absence of any separation of centromeres forming diplochromosomes. Some of the chromosome pairs may separate due to the processing steps from cell harvest up to and including slide preparation. Endoreduplicated cells are not included in the final total of structural aberrations, and can result from different mechanisms, including perturbation of the cell cycle.

## 6 DATA

### 6.1 Data Presentation

In this final report, the following data are provided for each dose: cell growth inhibition in dose range-finding assay; cell growth inhibition, induction of mitotic index, the number and types of aberrations found, total spread cells scored, the percentage of structurally and numerically damaged cells (percent aberrant cells) in the total population of cells examined, and the mean aberrations per 100 cells are calculated and reported for each treatment group. Gaps are presented in the data but not included in the total percentage of aberrations or aberrant cells.

### 6.2 Statistical Method

Fisher’s exact test was used to identify significant differences, comparing aberrant cells frequency between treated group and solvent control group. The p value was adjusted manually by a Bonferroni correction for multiple comparisons. A difference is considered significant when the p value obtained is less than 0.05, but this p value referees to the value before Bonferroni adjustment.

### 6.3 Evaluation of Study Validity

The percentage of cells with aberrations for the solvent control must be comparable with the historical control data for solvent control cultures. The historical negative/positive data are presented in Appendix 7.

The high dose positive control culture must yield a level of aberrant cells that is increased significantly ($p \leq 0.05$) over the mean of the solvent controls. A low-dose positive control culture was included in the coded set of slides scored for aberrations, to improve the objectivity of the scoring. A statistically significant increase in aberrations in the low dose culture is not required for an acceptable assay.

There must be a minimum of three scorable doses, i.e., doses that yield at least 100 scorable metaphases, and show no evidence of microbial contamination. However, in cases of excessive cytotoxicity, fewer than 3 doses may be scored.
6.4 Evaluation of Clastogenic Potential

6.4.1 Criteria for a Positive Result

The conclusion is usually based on the statistical analysis of the proportion of cells with aberrations in treated cultures as compared with the concurrent controls, but this is not the only parameter used. It is also taken into account the types of chromosome aberrations, the occurrence of cells with multiple aberrations, and the historical control percentages of cells with aberrations. The tests with and without S9 and the different treatment lengths will be considered separately. The statistical method described above is used to analyze the data. The final evaluation of the test article is based upon scientific judgment.

A positive dose is a statistically significant increase of cells with structural aberration over concurrent solvent control. An assay is generally considered positive if there are two positive doses within a treatment group, without greatly exceeding a 50% reduction in growth. A single positive dose is considered equivocal until repeated in another assay. If any of the tests with S9 activation or without S9 is positive, the overall conclusion will be positive.

6.4.2 Criteria for a Negative Result

The test article is considered to be negative in the assay if it does not elicit a statistically significant increase in the frequency of cells with chromosome aberrations over concurrent solvent/negative control at any dose level.

When the percentages of cells with chromosome aberrations at all concentrations of test article are within the historical control range for negative and/or solvent control cultures, the test result is to be considered negative without further statistical analysis.

6.4.3 Criteria for an Equivocal Result

The test article is considered to induce an equivocal response if there is only one positive dose within a treatment schedule.

7 RESULTS AND DISCUSSION

7.1 Solubility and pH Tests

- Coelenterazine formed a suspension in ethanol at a concentration of approximately 211.73 mg/mL. The test article formed a clear solution in ethanol at a concentration of approximately 6.2 mg/mL and lower concentrations.
- Precipitate appeared when 50 µL test article formulation at concentrations ≥ 5 mg/mL was added to the 4.95 mL of complete McCoy’s 5A medium; Precipitate appeared when 50 µL test article formulation at concentrations ≥ 4 mg/mL was added to 4.95 mL of serum-free McCoy’s 5A medium.
- Finally, 211.7 mg/mL was determined to be the top concentration, and 2117 µg/mL was selected as the top dose level, for all the three exposure groups in the dose range-finding assay.
- The pH for the medium containing the highest dose in the solubility test, dose
range-finding assay and definitive chromosome aberration assay were all maintained at physiological pH (about 7.2), and consequently, no pH adjustment was required in the treatment medium.

7.2 Dose Formulation Analysis

In the definitive chromosome aberration assays (including the repeating test):

- The test article concentration in the solvent control formulation was below the Limit of Quantification \times Dilution Factor, which demonstrated the absence of Coelenterazine in the solvent control. Based on this result, it was concluded that cells in the solvent control groups were not exposed to Coelenterazine.

- Measured concentrations of the test article in the dose formulations (including suspensions and solutions) used in the definitive chromosome aberration assays were between 95% and 106% of nominal value. Based on these results, it was concluded that the dose formulations were accurately prepared.

- Measured concentrations (0.5 and 10mg/mL) of Coelenterazine in dose formulations stored for 6 hours at room temperature were -6% and 1% of nominal value. Based on these results, it was concluded that Coelenterazine was stable in formulations covered by the stability concentration range for at least 6 hours at room temperature.

- Measured test article concentrations in dose formulation samples which were suspensions were within 97% to 99% of their nominal concentrations with \leq 3\% relative standard deviation (RSD) of each concentration. Based on these results, it was concluded that the dose formulations were accurately prepared.

Dose formulation analysis report is presented in Appendix 6.

7.3 Dose Range-finding Assay

Dose levels for the definitive chromosome aberration assay were selected following a dose range-finding assay and were based on a reduction of cell growth (cell growth inhibition) relative to the solvent control. The CHO-WBL cells were exposed to solvent alone and to 10 concentrations of the test article at 1, 2, 5, 10, 20, 40, 100, 250, 700 and 2117 \mu g/mL in S9 activated 3 hours exposure group, and 1, 2, 5, 10, 20, 50, 100, 250, 700 and 2117 \mu g/mL in non-activated 3 and 20 hours exposure groups. Precipitate was observed at dose levels \geq 40 \mu g/mL in the S9 activated exposure group, and at dose levels \geq 50 \mu g/mL in the non-activated exposure groups. The results of the evaluation of cell growth inhibition are provided in Tables 1, 2, and 3. Substantial toxicity (i.e., not greatly exceeding 50\% cell growth inhibition, relative to the solvent control) was observed at dose levels \geq 100 \mu g/mL in the S9 activated exposure group, \geq 100 \mu g/mL in the non-activated 3 hours exposure group, and \geq 20 \mu g/mL in the non-activated 20 hours exposure group.

7.4 Definitive Chromosome Aberration Assay

Based on the results of the dose range-finding assay, the dose levels selected for testing in the definitive chromosome aberration assay and the repeating test were as follows:
In the definitive chromosome aberration assay, precipitate was observed at dose levels $\geq 40 \, \mu g/mL$ in the S9 activated exposure group, and at dose levels $\geq 50 \, \mu g/mL$ in the non-activated exposure groups.

**S9 activated 3 hours exposure group:**

Cytotoxicity data are presented in Table 4. Cytotoxicity of Coelenterazine (cell growth inhibition relative to the solvent control) in CHO-WBL cells was 54% at 60 $\mu g/mL$, the highest dose level selected for microscopic analysis (the cytotoxicity not greatly exceeding 50%). Two lower dose levels at 20 and 40 $\mu g/mL$ were also analyzed, and the cell growth inhibition, relative to the solvent control, was 4% and 13%, respectively. The results of the test article and solvent/blank/positive controls in the induction of chromosome aberrations are presented individually by treatment flask in Table 5 and summarized by treatment group in Table 12. The percentage of cells with structural aberrations in the solvent control treatment group was 1.50%, comparable with the blank control (1.50%) and historical control data. The increase in the average percentage of structurally damaged cells relative to the solvent control group in the high dose CP (positive control) treatment group (11.50%) was statistically significant ($p<0.01$ by Fisher’s Exact test). Based on these results, the assay was considered valid for the S9 activated 3 hours exposure group.

There was no significant increase in the percentage of cells with structural aberrations in the test article treated group observed at any dose level relative to the solvent control group ($p>0.05$ by Fisher’s Exact test). Also the percentages of cells with structural aberrations in the test article treated groups were in the range of historical negative control (see appendix 7).

There was no significant change of the percentage of cells with numerical aberrations (polyploidy or endoreduplication) in the test article treated group relative to the solvent control at any dose level (statistical analysis was not performed). However, the incidence of polyploid and endoreduplicated cells ($3.38\%$ and $9.50\%$, respectively) in cultures with S9 was significantly higher when compared to the cultures without S9 ($2.44\%$ and $0.99\%$, respectively) in this study and historical control data ($0-2.91\%$ and $1.48-6.54\%$, respectively). It was supposed that this phenomenon was caused by a SOP deviation that the incomplete McCoy’s 5A medium was placed on ice after mixing with the S9 mixture. Therefore the definitive chromosome aberration assay was repeated at dose levels of 20, 40, 60 and 70 $\mu g/mL$ in 3-hour exposure with S9 group.

**S9 activated 3 hours exposure group (repeat):**

Cytotoxicity data are presented in Table 6. Cytotoxicity of Coelenterazine (cell growth inhibition relative to the solvent control) in CHO-WBL cells was 59% at 60 $\mu g/mL$, the highest dose level selected for microscopic analysis (the

cytotoxicity not greatly exceeding 50%). Two lower dose levels at 20 and 40 µg/mL were also analyzed, and the cell growth inhibition, relative to the solvent control, was 4% and 18%, respectively. The results of the test article and solvent/blank/positive controls in the induction of chromosome aberrations are presented individually by treatment flask in Table 7 and summarized by treatment group in Table 12. The percentage of cells with structural aberrations in the solvent control treatment group was 1.00%, comparable with the blank control (1.00%) and historical control data. The increase in the average percentage of structurally damaged cells relative to the solvent control group in the high dose CP (positive control) treatment group (28.00%) was statistically significant (p<0.01 by Fisher’s Exact test). Based on these results, the assay was considered valid for the repeating S9 activated 3 hours exposure group.

There was no significant increase in the percentage of cells with structural aberrations in the test article treated group observed at any dose level relative to the solvent control group (p>0.05 by Fisher’s Exact test). Also the percentages of cells with structural aberrations in the test article treated groups were in the range of historical negative control (see appendix 7). Based on these results, Coelenterazine was concluded to be negative for the induction of structural chromosome aberrations in the test system of 3 hours exposure in the presence of S9 metabolic activation.

There was no significant change of the percentage of cells with numerical aberrations (polyploidy or endoreduplication) in the test article treated group relative to the solvent control at any dose level (statistical analysis was not performed). And the incidences of polyploid and endoreduplicated cells (0.99% and 1.96%, respectively) were comparable with our laboratory historical data.

- **Non-activated 3 hours exposure group:**

   Cytotoxicity data are presented in Table 8. Cytotoxicity of Coelenterazine (cell growth inhibition relative to the solvent control) in CHO-WBL cells was 53% at 60 µg/mL, the highest dose level selected for microscopic analysis (the cytotoxicity not greatly exceeding 50%). Two lower dose levels at 20 and 40 µg/mL were also analyzed, and the cell growth inhibition, relative to the solvent control, was 23% and 16%, respectively. However the test article showed little mitosis inhibition at dose levels of 20 µg/mL (6%) and 40 µg/mL (10%), compared to the solvent control. The results of Coelenterazine and solvent/blank/positive controls in the induction of chromosome aberrations are presented individually by treatment flask in Table 9 and summarized by treatment group in Table 12. The percentage of cells with structural aberrations in the solvent control treatment group was 1.50%, comparable with the blank control (2.00%) and historical control data. The increase in the average percentage of structurally damaged cells relative to the solvent control group in the high dose MMC (positive control) treatment group (30.00%) was statistically significant (p<0.01 by Fisher’s Exact test). Based on these results, the assay was considered valid for the non-activated 3 hours exposure group.

   There was no significant increase in the percentage of cells with structural aberrations in the test article treated group observed at any dose level relative to the solvent control group (p>0.05 by Fisher’s Exact test). Also the percentages of cells with structural aberrations in the test article treated groups
were in the range of historical negative control (see appendix 7). Based on these results, Coelenterazine was concluded to be negative for the induction of structural chromosome aberrations in the test system of 3 hours exposure in the absence of S9 metabolic activation.

There was no significant change of the percentage of cells with numerical aberrations (polyploidy or endoreduplication) in the test article treated group relative to the solvent control at any dose level (statistical analysis was not performed).

**Non-activated 20 hours exposure group:**

Cytotoxicity data are presented in Table 10. Cytotoxicity of Coelenterazine (cell growth inhibition relative to the solvent control) in CHO-WBL cells was 53% at 15 µg/mL, the highest dose level selected for microscopic analysis (the cytotoxicity not greatly exceeding 50%). Two lower dose levels at 5 and 10 µg/mL were also analyzed, and the cell growth inhibition, relative to the solvent control, was 33% and 32%, respectively. However the test article showed little mitosis inhibition at dose levels of 5 µg/mL (17%) and 10 µg/mL (18%), compared to the solvent control. The results of Coelenterazine and solvent/blank/positive controls in the induction of chromosome aberrations are presented individually by treatment flask in Table 11 and summarized by treatment group in Table 12. The percentage of cells with structural aberrations in the solvent control treatment group was 2.00%, comparable with the blank control (0.50%) and historical control data. The increase in the average percentage of structurally damaged cells relative to the solvent control group in the high dose MMC (positive control) treatment group (36.00%) was statistically significant (p<0.01 by Fisher’s Exact test). Based on these results, the assay was considered valid for the non-activated 20 hours exposure group.

There was no significant increase in the percentage of cells with structural aberrations in the test article treated group observed at any dose level relative to the solvent control group (p>0.05 by Fisher’s Exact test). Also the percentages of cells with structural aberrations in the test article treated groups were in the range of historical negative control (see appendix 7). Based on these results, Coelenterazine was concluded to be negative for the induction of structural chromosome aberrations in the test system of 20 hours exposure in the absence of S9 metabolic activation.

There was no significant change of the percentage of cells with numerical aberrations (polyploidy or endoreduplication) in the test article treated group relative to the solvent control at any dose level (statistical analysis was not performed).

8 CONCLUSIONS

The chromosome aberration assay is considered valid as discussed above.

Under the conditions of this GLP chromosome aberration study, Coelenterazine was conducted to be **negative** for the induction of structural/numerical chromosome aberrations both with and without S9.
9 DEVIATION

In SOP-GEN-0003 (In vitro chromosome aberration analysis using Chinese Hamster Ovary (CHO) Cells), it is requested that the medium should be warmed (37°C±2°C) before adding to the culture. However the incomplete medium was placed on ice after mixing with the S9 mixture in the first definitive chromosome aberration assay. The cold shock to cells led to high incidences of polyploid and endoreduplicated cells. After avoiding the cold shock condition in the repeating definitive chromosome aberration assay, the incidences of polyploid and endoreduplicated cells were in the range of historical negative data.

10 ARCHIVING

The raw data, study documentation generated at WuXi AppTec (Suzhou), the protocol and all amendments, and the original signed final report for this study will be archived in the GLP archives according to Test Facility SOPs for at least 1 year following report finalization. After one year, the test facility will contact the Sponsor to determine further disposition in accordance with their SOPs. No materials will be discarded without the prior approval by the Sponsor.

11 REFERENCES


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实验项目特征
肠腔素：中国仓鼠卵巢细胞体外染色体畸变实验

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摘要

该染色体畸变实验检测了供试品肠腔素在加或不加外源性代谢活化系统（Aroclor 1254 诱导的大鼠肝脏 S9）条件下诱发中国仓鼠卵巢细胞（CHO-WBL）产生染色体畸变的能力。其中，开展剂量探索实验是为染色体畸变实验主实验选择合适的剂量范围。染色体畸变实验主实验被用于评估供试品的致畸变潜力。其中染色体畸变实验主实验的 S9 活化给药 3 小时系列被重复了一次。

根据供试品的溶解度以及与 CHO-WBL 的相容性，选择乙醇为溶剂。肠腔素在乙醇中大约 211.73 mg/mL 可形成悬浮液。肠腔素在乙醇中大约 6.2 mg/mL 可形成澄清的溶液。

在剂量探索实验以及染色体畸变实验中，都为 CHO-WBL 细胞设立了以下给药处理系列：S9 代谢活化给药 3 小时系列、非代谢活化给药 3 小时系列和非代谢活化给药 20 小时系列。在给药 20 小时后收获所有细胞。

在剂量探索实验中，测试的最大剂量为 2117 μg/mL，该剂量大于供试品在培养基中溶解度的上限。

染色体畸变实验主实验的剂量选择取决于预实验中相对于溶剂对照组的细胞生长抑制情况。在 S9 代谢活化给药 3 小时系列中为供试品设立了以下剂量 1、2、5、10、20、40、100、250、700 和 2117 μg/mL，而在非代谢活化给药 3 和 20 小时两个系列中为供试品设立了以下剂量 1、2、5、10、20、50、100、250、700 和 2117 μg/mL；每个剂量组设 1 瓶细胞。S9 代谢活化给药 3 小时系列中，在≥100 μg/mL 的剂量组中发现了充分的毒性（即与相应溶剂对照组相比的细胞生长抑制不大超过 50%），因而在染色体畸变实验主实验中选择的剂量为 20、40、50、60、70、80 和 100 μg/mL；非代谢活化给药 3 小时系列中在≥100 μg/mL 的剂量组中也发现了充分的毒性，因而在染色体畸变实验主实验中选择的剂量为 20、40、50、60、70、80 和 100 μg/mL；非代谢活化给药 20 小时系列中在≥20 μg/mL 的剂量组中也发现了充分的毒性，因而在染色体畸变实验主实验中选择的剂量为 5、10、15、20、30、40 和 50 μg/mL。

在染色体畸变实验主实验中，每个供试品剂量组设 2 瓶细胞，并平行设立溶剂对照、空白对照和阳性对照。收获细胞前 2 小时加入秋水仙胺，在培养基中的终浓度为 0.1 μg/mL。S9 代谢活化给药 3 小时系列中≥40 μg/mL 的剂量组都观察到了沉淀，而在非代谢活化给药的两个系列中≥50 μg/mL 的剂量组都观察到了沉淀。读片的剂量（S9 代谢活化给药 3 小时系列为 20、40 和 60 μg/mL；非代谢活化给药 3 小时系列为 20、40 和 60 μg/mL；非代谢活化给药 20 小时系列为 5、10 和 15 μg/mL）是根据细胞毒性选择的（最高剂量为与相应溶剂对照组相比的细胞生长抑制不大超过 50%的最低剂量组，然后再选择两个较低的剂量组）。每瓶细胞计数 100 个形态良好的中期相细胞，而每个剂量组则一共计数 200 个。

在所有三个给药处理系列中都未发现，与相应的溶剂对照组比较，有供试品给药组的染色体结构畸变或数量畸变的比例显著增加（p>0.05，Fisher’s 确切概率法）。该实验的有效性也得到了确认。

然而，在染色体畸变实验主实验中发生了一个标准操作程序偏差，即 McCoy’s 5A 不完全培养基在与 S9 混合物混合后被放置在了冰上。而且在加 S9 的细胞中，多倍体和核内复制细胞的发生率（分别为 3.38%和 9.50%）与本实验中 3 小时不加 S9 系列组细胞的发生率（分别为 2.44%和 0.99%）以及历史数据（分别为 0.2-9.1%和 1.48-6.54%）相比，都显著增加。因此重复了染色体畸变实验主实验的 S9 代谢
活化给药 3 小时系列，给药剂量为 20, 40, 60 和 70 μg/mL。选择的读片剂量为 20, 40 和 60 μg/mL。与相应的溶剂对照组比较，未发现有供试品给药组的染色体结构畸变或数量畸变的比例显著增加（p>0.05, Fisher’s 确切概率法）。而且多倍体和核内复制细胞的发生率都与实验室历史数据具有可比性。

染色体畸变实验的结果总结在下表中：

<table>
<thead>
<tr>
<th>给药时间</th>
<th>收获时间</th>
<th>S9</th>
<th>选择最高计量的毒性*</th>
<th>染色体结构畸变 阳性剂量</th>
<th>染色体数量畸变 阳性剂量</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 小时</td>
<td>20 小时</td>
<td>+</td>
<td>54%</td>
<td>无</td>
<td>无</td>
</tr>
<tr>
<td>3 小时</td>
<td>20 小时</td>
<td>-</td>
<td>53%</td>
<td>无</td>
<td>无</td>
</tr>
<tr>
<td>20 小时</td>
<td>20 小时</td>
<td>-</td>
<td>53%</td>
<td>无</td>
<td>无</td>
</tr>
<tr>
<td>3 小时#</td>
<td>20 小时</td>
<td>+</td>
<td>59%</td>
<td>无</td>
<td>无</td>
</tr>
</tbody>
</table>

* 细胞生长抑制；# 重复实验

根据本次实验的结果，在本次GLP染色体畸变实验条件下，推断肠腔素在CHO-WBL细胞中诱导染色体结构畸变和数量畸变的潜能为阴性。
1 目的

本实验的目的是通过检测供试品肠腔素在加或不加外源性代谢活化系统（Aroclor 1254 诱导的大鼠肝脏 S9）条件下诱发中国仓鼠卵巢细胞 (CHO) 产生染色体畸变的能力，以评价其致畸变的潜力。

2 依从法规

剂量探索实验部分是本次染色体畸变的预实验，不属于 GLP 范畴，其实施严格遵照了实验方案和公司相关标准操作规程的规定。

染色体畸变主实验部分的实施严格遵照了实验方案、苏州药明康德公司相关标准操作规程以及下面所列的良好实验室规范准则（GLP）的最新版本。实验报告中的一些 GLP 词汇（如实验方案、供试品、对照品）是基于美国 FDA 良好实验室规范准则的描述，可以认为与 OECD 良好实验室规范中所使用的相应词汇是一致的。

♦ 经济合作与发展组织（OECD）良好实验室规范准则，1997 年修订。
♦ 美国 FDA 非临床研究质量管理规范 (美国联邦法规第 21 条, 58 部分, 1979 年 6 月 20 日生效，及所有补充修正案)。
♦ 中国 SFDA《药物非临床研究质量管理规范》（GLP），2003 年 9 月 1 日生效。

3 供试品和对照品

供试品和溶剂对照品的质检证明（COA）被列在附件 1 和 2。

3.1 供试品描述

名称： 肠腔素
代码： CTZ
CAS 编号： 55779-48-1
制造商： 天津药明康德新药开发有限公司
批号： TH 03647-031
纯度： 93.57%
分子量： 423.46
保存条件： 储存在-20°C 条件，避光
理化性质： 黄色粉末
失效日期： 2021 年 6 月
3.2 溶剂/阴性对照品描述

名称：乙醇
CAS 编号：64-17-5
制造商：国药集团化学试剂有限公司
批号：20100506
浓度：95%
保存条件：室温
理化性质：无色液体
失效日期：2013-09-01

在剂量探索实验和染色体畸变实验主实验中，每个暴露处理组中都将分别设立溶剂对照，给药体积与供试品所用给药体积一致。

因为乙醇在本实验中是不典型的溶剂，实验中设立了空白对照以确定溶媒对细胞生长或染色体畸变没有不利影响。

3.3 阳性对照品描述

环磷酰胺一水合物[CP: CAS 6055 19-2]，需要代谢活化才具有致突变作用，在 S9 活化给药组中平行设立 CP 给药，给药剂量为 1.5 和 5.0 µg/mL。丝裂霉素 C [MMC: CAS 50-07-7]，不需要代谢活化，本身即具有致突变作用，在非活化 3 小时给药组中平行设立 MMC 给药，给药剂量为 0.17 和 0.5 µg/mL；在非活化 20 小时给药组中平行设立 MMC 给药，给药剂量为 0.05 和 0.25 µg/mL。这些阳性对照品被事先在无菌去离子水中配制成 100×储备液，保存在-20°C 条件下，临用前融化。选用这些阳性对照品，是由于这些化合物有大量数据库可供参考，而且 OECD 指导原则 473 也推荐使用这些化合物。

只在主实验中使用了阳性对照。从阳性对照所得的结果，只是用来验证实验系统的反应性，不与供试品作比较。

3.4 留样

供试品中心实验室取出 1 克供试品作为留样。留样供试品被按照苏州药明康德新药开发有限公司相应标准操作规程归档在 GLP 档案室中。

3.5 编目和处置

原料供试品在实验结束后将返还给委托单位。由苏州药明康德公司提供的对照品按照研究机构相应的标准操作规程丢弃。

4 制剂配制及制剂分析

4.1 制剂配制

制剂配制时，供试品纯度以 93.57%计算，进行校正。
供试品制剂均现配现用，供试品制剂的澄清溶液和悬浊液分开配制，都是称取一定质量的供试品，加入一定体积的溶剂，得到最高浓度母液，较低剂量供试品制剂则通过稀释等较高浓度制备获得。在黄光灯条件配制供试品制剂，制剂配制后分装在无菌棕色容器中，盖紧后保存在室温下。配制制备时通过涡旋以及 37±2°C 水浴来促进供试品溶解。制剂在从配制场所运输到给药场所或制剂分析场所过程中暴露于环境温度。供试品制剂配制的详细步骤在《供试品制剂配制指南及配制记录表》中进行描述并在此表记录配制过程。

4.2 制剂取样

用于染色体畸变实验主实验的制剂进行了制剂分析，而用于剂量探索实验的制剂则未分析，阳性对照物制剂也没有进行制剂分析。

主实验中完成制剂配制后立即从溶剂和所有供试品制剂中取出一部分用于制剂分析。

◆ 供试品制剂为澄清的溶液的，每个剂量取两套样品，每套样品 1 mL，取样点为制剂的中间层。其中一套用来制剂分析，另一套作为备份。

◆ 供试品制剂为悬浊的，从每个剂量的上中下三部分分别取两套样品，每套样品 1 mL。其中一套用来制剂分析，另一套作为备份。中间部分的制剂用作浓度测定和均一性确认。上、下部分的制剂则与中层部分的制剂一起用来确认制剂的均一性。

◆ 为了评估供试品的稳定性，从最高和最低浓度制剂的中层外取两套样品分析浓度，每套样品 1 mL。其中一套用来制剂分析，另一套作为备份。

◆ 溶剂取样两套，每套 1 mL，其中一套用来分析以确定溶剂中不含有供试品，另一套作为备份。

取样体积根据专题负责人的要求作了改动。所有取样用于分析的样品都保存在室温下，而备份则保存在-20°C。

4.3 制剂分析

采用苏州药明康德新药开发有限公司开发并验证过的分析方法（分析方法编号：AM-A110818-01-01），由分析化学实验室实施制剂分析。

4.3.1 浓度测定

制剂配制后立即取样并转运至公司内化验化学实验室，用于浓度测定。

取送检样品的中间层，并用验证了的分析方法测定供试品的浓度。

4.3.2 稳定性评估

高剂量和低剂量的送检样品，一份样品用于供试品浓度分析，另一份室温放置 6 小时后，再次进行供试品浓度分析。以这两份样品进行的浓度分析为基线来评价供试品 6 小时的稳定性。
4.3.3 均一性确认

分析比较每个送检混悬液样品的上、中和下层部分的浓度来评价供试品制剂的均一性。

4.3.4 制剂分析数据的可接受标准

比较测定浓度与标识浓度以判定溶剂对照组培养基中的细胞暴露于供试品的水平，制剂配制的准确度和制剂中供试品的稳定度。

溶剂中供试品的浓度值必须低于“定量限”乘以“稀释因子”。

供试品制剂为澄清溶液的，送检样品的浓度测出值必须是标识浓度的 90%到 110%。

供试品制剂为混悬液的，送检样品的浓度测出值必须是标识浓度的 85%到 115%。

供试品制剂在 6 小时稳定性测试点所测浓度和基线时浓度之间的差异性百分比必须在理论值的±10%。

为了判定供试品均一性（若供试品制剂有混悬液），送检样品的浓度测出值必须是标识浓度的 85%到 115%，并且每个样品浓度的相对标准差 (RSD) 必须≤10%。

4.4 样品处置

所有未用完的供试品制剂在给药后都被废弃。

分析化学实验室使用后剩余的少量供试品，作为废弃物处理。

5 材料和方法

该实验设计基于 OECD 指导原则 473，1997 年 7 月 21 日修订生效。

5.1 实验系统基本原理

畸变是染色体修复过程失败或发生错误的结果，以致于染色体断裂后不重新结合或者以不正常的结构形式结合（Evans, 1962, 1976; Galloway, 1985, 1994）。

本报告中提供了详尽的畸变类型的描述。结构畸变包括两种类型，染色体型或染色单体型：

◆ 染色体型畸变：染色体结构损伤，表现为两条姐妹染色单体在同一位置发生断裂或断裂后重新联接。

◆ 染色单体型畸变：染色体结构损伤，表现为单条染色单体的断裂或断裂后染色单体之间重新联接。这种畸变类型是最常见的结构畸变。

大多数已知的化学断裂剂（也称为染色体断裂剂）需经过一段时间的DNA 合成后，将最初的 DNA 损伤转变为 DNA 的结构改变，并在有丝分裂期时可以直观地用显微镜进行观察。经供试品处理后一定时间，将 CHO 细胞用一种中期相阻断剂（秋水仙胺）处理，然后收获细胞并染色，在显微镜下分析中期相细胞是否存在染色体畸变。
许多致突变化合物不直接造成 DNA 损伤，但是经肝代谢酶活化后的中间产物可以造成损伤。CHO 细胞对供试品的代谢活化能力有限，所以在实验中需特意设立一系列给药组，在该组中添加外源性代谢活化系统（Aroclor 1254 诱导的大鼠肝脏 S9），以强化化合物向其代谢中间产物的转化，从而增加实验检测中间代谢产物为断裂剂的化合物的能力。

染色体数量畸变将取决于多倍体和核内复制的发生，若实验中观察到多倍体和核内复制发生频率显著增加，则说明该供试品有产生数量畸变的潜力。

5.2 实验系统

本实验使用中国仓鼠卵巢细胞（CHO-WBL 细胞），核型具有 21 条染色体，生长周期为 12 至 13 小时。CHO-WBL 细胞最初由美国默克研究实验室赠送，后在本实验室扩增。细胞储备液储存在液氮中。每一批次的细胞储备液都鉴定了细胞核型，并经检测证明未被支原体污染。细胞单克隆后传代不得超过 15 次。

5.3 培养基和细胞生长条件

全培养基为 McCoy’s 5A 培养基，补充加入 10%胎牛血清、2 mM L-谷氨酸、100 unit/mL 青霉素和 100 μg/mL 链霉素。不含血清培养基为 McCoy’s 5A 培养基，补充加入 2 mM L-谷氨酸、100 unit/mL 青霉素和 100 μg/mL 链霉素。

CHO-WBL 细胞生长条件为温度 37℃、CO₂ 浓度为 5%的高湿度环境。

5.4 代谢活化系统

本实验中使用的代谢活化系统（Maron 和 Ames，1983）包含 Aroclor 1254 诱导的大鼠肝脏微粒体匀浆（简称 S9）和一个“能量产生系统”（NADP 二钠加异柠檬酸三钠）。S9 匀浆购自美国 Molecular Toxicology（Boone, NC）公司，使用前一直低温储存在-80℃条件下。该 S9 匀浆的制备方法为：将 Aroclor 1254 单次腹腔注射雌性 Sprague Dawley 大鼠，剂量为 500 mg/kg，5 天后解剖取材。根据 Molecular Toxicology 公司提供的 S9 质检报告，该公司利用 Ames 细菌回复突变实验的沙门氏菌 TA100，测试了 S9 在 S9 混合物中浓度为 0.2%至 10%时，S9 活化苯并芘和 2-氨基蒽的能力。该质检报告列在附件 3 中。

临用前，将 S9 匀浆融化，并与配制的“能量产生系统”无菌溶液混匀。S9 混合物的组分如下：

<table>
<thead>
<tr>
<th>组分</th>
<th>培养基中的终浓度</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP（二钠盐）</td>
<td>0.8 mg/mL</td>
</tr>
<tr>
<td>异柠檬酸（三钠盐）</td>
<td>1.5 mg/mL</td>
</tr>
<tr>
<td>S9 匀浆</td>
<td>15.0 μL/mL (1.5% v/v)</td>
</tr>
</tbody>
</table>

5.5 溶解度和 pH 测试

以乙醇(CAS 64-17-5)为溶剂测试了供试品的最大溶解度或可给药浓度。测试的目标浓度为 423.56 mg/mL（1 M）。
供试品母液被 100 倍稀释到培养基中以观察沉淀形成情况。对于有机溶剂来说，溶媒在培养基中的比例为 1%（v/v）。

使用 pH 试纸检测含有最高浓度供试品母液的培养基 pH 值。

5.6 剂量探索实验

在剂量探索实验中，每个处理系列中设 10 个剂量水平，供试品每个剂量设一瓶细胞。受限于供试品在溶剂中的可使用浓度，测试的最大剂量为 2117 μg/mL，该剂量大于供试品在培养基中形成沉淀的下限浓度。

5.6.1 接种细胞

将处于指数生长期的 CHO-WBL 细胞按照大约 1.2×10^6 个细胞 /10 mL 培养基接种于 75 cm^2 培养瓶中。另外接种两瓶细胞用于测试在给药时的基线细胞数（零点细胞计数，X0；见下文“细胞生长”）。接种好细胞后的培养瓶放置在温度 37℃、CO₂ 浓度为 5%的高湿度环境培养 20 到 24 小时。

5.6.2 实验系统处置

给药频率和途径：在非代谢活化实验系统中，细胞给药处理 3 小时
和 20 小时；而 S9 代谢活化实验系统中，细胞给药处理 3 小时；给药途径
为将供试品与溶剂的混合物加入到细胞培养基中。这种技术已在许多
体外染色体畸变实验中被证明是一种检测化学致断裂剂的有效方法
(Evans, 1976)。

所有供试品给药均在室温及黄光条件下进行。

接种细胞 20 至 24 小时后，吸掉培养瓶中的培养基。然后向 S9 代
谢活化处理系列中加入合适体积的含有代谢活化 S9 混合物的不含血清
培养基，而向两个非代谢活化处理系列中加入合适体积的全培养基。给药时，向培养基中加入 100 μL 供试品制剂或溶剂。培养基的终体积为 10 mL。

将细胞培养瓶放回到温度 37℃、CO₂ 浓度为 5%的高湿度环境中。对
于 S9 代谢活化 3 小时给药系列和非活化 3 小时给药系列，细胞接触
供试品的时间均 3 小时。为了除去供试品，先将瓶中含有供试品的培养基
吸掉，然后用 Hank's 缓冲液（HBSS）洗细胞 2 次，加入全培养基后接着
培养直至收获。在收获细胞前 2 小时，将向培养瓶中加入秋水仙胺以聚
集中期相细胞。对于非活化 20 小时给药系列，细胞接触供试品的时间为
20 小时，在收获细胞前 2 小时，将向培养瓶中加入秋水仙胺以聚集中期
相细胞。

在给药后 30 分钟内，计数分外接种的两瓶细胞的基线细胞数。

5.6.3 收获细胞

给药后大约 20 小时收获细胞。该时间段大约是 CHO 细胞生长周期
的 1.5 倍，选择该时间段的目的是为了确保可以分析到给药后经历第一次
分裂形成的中相细胞。收获细胞前两小时，将 100 μL 秋水仙胺（浓度
为 10 μg/mL）加入到培养基中，秋水仙胺的终浓度为 0.1 μg/mL。
通过消化收获细胞，取其中一部分细胞悬液用于细胞计数。计数的方法为自动细胞计数仪加血细胞计数板台盼蓝染色判断活细胞率，自动细胞计数仪所得的细胞总数乘以台盼蓝染色所得的活细胞率即为活细胞数。而活细胞被用来计算供试品相对于溶剂对照对细胞生长的抑制（即细胞毒性）。

### 5.6.4 细胞生长（倍增）和细胞毒性

细胞生长表达为给药开始至收获细胞的这段时间内细胞倍增数目（PD）（Galloway, 2004）。计算给药组细胞倍增数目与相应溶剂对照组细胞倍增数目的比值，以百分比形式表达。

\[
PD = [\log (N + Xo)] + \log 2
\]

其中 N=收获细胞时的细胞数（最终数目；经台盼蓝染色检查活细胞率后所得的活细胞数）。

\(Xo\)=给药时的起始细胞数，即给药时计数分外接种的两瓶细胞的基线细胞数。

每一给药组的相对 PD(RPD，以百分比形式表示)按下式计算：

\[
RPD(\%) = \frac{供试品给药组 PD}{溶剂对照组 PD} \times 100
\]

细胞毒性（%）表达为（100-RPD）。

### 5.7 染色体畸变实验主实验

染色体畸变实验主实验设立了以下 3 个处理系列：S9 代谢活化给药 3 小时系列（3 hours+S9）、非代谢活化给药 3 小时系列（3 hours-S9）和非代谢活化给药 20 小时系列（20 hours-S9）。每一供试品/溶剂对照/阳性对照组均测试 2 瓶细胞。

染色体畸变实验主实验中测试的最高剂量估计诱导大于 50%的细胞毒性（与相应溶剂对照组相比的细胞生长抑制）。每一系列中另外测试 6 个较低浓度的剂量。

因为在染色体畸变实验主实验中发生了一个标准操作规程偏离，即 McCoy’s 5A 不完全培养基在与 S9 混合物混合后被放置在了冰上，而且在加 S9 的细胞中，多倍体和核内复制细胞的发生率（分别为 3.38%和 9.50%）与本实验中 3 小时不加 S9 系列组细胞的发生率（分别为 2.44%和 0.99%）以及历史数据（分别为 0.21%和 1.48-6.54%）相比，都显著增加。不能够确定这种情况是否对主实验中的 S9 代谢活化给药 3 小时系列结果是否有影响。因此重复了染色体畸变实验主实验的 S9 代谢活化给药 3 小时系列，给药剂量为 20、40、60 和 70 μg/mL。

### 5.7.1 接种细胞及实验系统处置

接种细胞及实验系统处置将参照剂量探索实验中的描述操作。阳性对照的给药体积为 100 μL。
5.7.2 中期相细胞的收集

给药后大约 20 小时收获细胞。收获细胞前两小时，将 100 μL 秋水仙胺（浓度为 10 μg/mL）加入到培养基中，秋水仙胺的终浓度为 0.1 μg/mL。

通过消化收获细胞，取其中一部分细胞悬液用于细胞计数，计数方法参照试剂探索实验中的描述。活细胞被用来计算供试品相对于溶剂对照对细胞生长的抑制（即细胞毒性），而阳性对照组不需要做细胞计数。余下的细胞用 0.075M KCl 溶液低糖处理，用固定液（甲醇: 酒精，3:1 v/v）洗 2 次，盖上盖保存在大约 4℃ 条件下过夜。制片前，离心细胞悬液，再次用固定液洗一次细胞并将细胞重悬在固定液中。将固定好的细胞悬液滴到载玻片上并晾干。每一瓶细胞做 2 张片子。将载玻片用吉姆萨染液染色，封片。

5.7.3 有丝分裂指数的计数

为了确保载玻片上有足够数目的中期相细胞，将计数有丝分裂指数。每张载玻片上计数至少 500 个细胞，记录处于有丝分裂期细胞的比例。

有丝分裂指数（MI）计算方法如下：

\[ MI = \frac{\text{观察到的中期相细胞数目}}{\text{观察到细胞总数}} \]

每一给药组的相对 MI（RMI，以百分比形式表示）按照下式计算：

\[ \text{RMI} (\%) = \frac{\text{给药处理组 MI}}{\text{溶剂对照组 MI}} \times 100 \]

有丝分裂减少（%）表达为（100-RMI）。

5.7.4 细胞生长(倍增)和细胞毒性

细胞生长和细胞毒性按照试剂探索实验中所述方法计算。

5.7.5 选择染色体畸变分析的剂量

CHO 细胞染色体畸变分析剂量的选择取决于供试品的细胞毒性。选择用于评估的最高剂量应诱导至少但不超过 50% 太多的细胞毒性，细胞毒性与溶剂对照相比细胞生长抑制来判断，但是应保证具有足够数目的中期相细胞。至少还应评估 2 个更低的剂量组。

5.7.6 染色体畸变分析片

除了 CP 和 MMC（阳性对照）高剂量组，所有载玻片均应用随机生成的盲码标示，以减少读片的主观性，盲码应由不参与读片的人员制作。

读片时，将载玻片平放在显微镜载物台上，使毛边端在左边且向上。在油镜（1000×）下分析中期相细胞。调整载玻片位置寻找质量好的中期相细胞。选择中期相细胞的标准如下：

a. 染色充分，便于观察。
b. 中期相没有破裂，与其它细胞核没有重叠，也没有被碎片覆盖。

c. 染色体形态良好，单条染色体很少重叠，且着丝粒没有裂开。

d. 计数着丝粒数，若着丝粒数目与标准数目（即 21）相差超过 2，则不分析该中期相细胞。

每一副离子组分析至少 200 个中期相细胞（每瓶 100 个）。记录观察到的畸变的类型以及多倍体和核内复制的数目。记录具有染色体结构畸变的中期相细胞在显微镜上的坐标。除非有其它指示，实验报告完成后载玻片将被废弃。

5.7.7 畸变类型

染色单体型

TG（染色单体裂隙）：也称为“Tid 裂隙”，在一条染色单体上出现一段无色（未染色）区段，其大小小于等于染色单体的宽度。这些只做标记，通常不包括在最终畸变总数里，因为它们不全是真的断裂。

IG（等位染色单体裂隙）：也称为“染色体裂隙”，裂隙发生在两条姐妹染色单体的同一位置。这些只做标记，通常不包括在最终畸变总数里，因为它们不全是真的断裂。

TB（染色单体断裂）：在一条染色单体上出现一段无色区段，其大小于染色单体的宽度。产生的断片可能部分或全部移开，或消失了。

ID（染色单体删除）：一定长度的染色单体从染色单体的中间区域“切除”，形成一条小的断片或环，位于变短的染色单体附近，或者在染色单体上形成一个裂隙。

TR（三辐射体）：两条染色体之间的交换，形成一个三臂的结构。它可能会产生一个断片。

QR（四辐射体）：与三辐射体相同，只是产生了一个四臂的结构。

CR（复杂重排）：两条以上染色体之间的交换，会几次断裂和交换形成的。

TI（染色单体交换）：在一条染色体内的交换，包括一条臂和两条臂交换。

染色体系型

SB（染色体断裂）：终端删除。染色体有一明显的断裂，形成一条不正常（删除了的）的染色体，带着一脱位的无着丝粒断片，该断片可能与染色体位置对应，也可能在细胞内任何位置。

DM（微体断片）：染色体断裂删除。表现为小的双点或成对的环。在某些情况下，它们很难与交换或终端删除形成的无着丝粒断片相区分。

D（双着丝粒染色体）：两条染色体之间的交换，形成一条有两个着丝粒的染色体。这经常或有一个相应的无着丝粒断片。在这种情况下，分类为双重着丝粒染色体带断片(DF)。

MC（多着丝粒染色体）：多条染色体之间交换，形成一条带有多于两个着丝粒的染色体。
R（环）：一条染色体形成一个圈，包含一个着丝粒。这通常会有一条相应的无着丝粒断片，在这种情况下分类为环带断片(RF)。无着丝粒的环也通常包含在这一类中。

AB（不正常的单着丝粒染色体）：指一条染色体，它的形态与细胞染色体中的一条染色体不正常，通常是易位或单线性导致的。当不正常染色体出现，但又不能归结于某种形如臂间倒位时，归入这一分类。仅作记录但不归入畸变总数中。

T（易位）：两条染色体间有明显的片段转移，形成两条不正常的染色体。鉴定后，记作“T’，而不是“2 AB”。仅作记录但不归入畸变总数中。

RT（罗宾逊易位）：两条端着丝粒染色体在着丝粒区域整条臂交换形成的结果，形成的染色体表现为一条等臂染色体，在计数细胞染色体总数时作两个着丝粒。仅作记录但不归入畸变总数中。

其它

SD（严重损伤细胞）：一个细胞中有大于等于 10 个任一类型的畸变。一个严重损伤细胞应分析其畸变的类型，分析后可能不是大于等于 10 个畸变，例如由于一个三着丝粒可以发现很多的断片。

PU（粉碎染色体）：粉碎的或断片化的染色体。这可能只是由于染色体浓缩的不同阶段导致的。仅作记录但不归入畸变总数中。

P+（粉碎细胞）：多于一条染色体，甚至整个细胞核，都是粉碎的。仅作记录但不归入畸变总数中。

数量畸变

PP（多倍体细胞）：一个细胞中含有很多份单倍体数目的染色体。在正常骨髓或培养细胞中会偶尔见到多倍体。仅作记录但不归入结构畸变总数中。

ER（核内复制）：当形成双分染色体时缺少着丝粒分离，就形成染色体核内复制。有些染色体对可能会由于细胞分裂到制片过程中的一些影响而分离。核内复制细胞不包含在结构畸变总数中，它可能是由于不同机制产生，包括细胞周期受到干扰。

6 数据

6.1 数据陈述

本实验报告为每一剂量组提供以下数据：剂量探索实验的细胞生长抑制，染色体畸变实验主实验中的细胞生长抑制、有丝分裂指数减少、染色体结构畸变的数目和类型、计数中期相细胞总数、计数细胞中畸变细胞百分比和 100 个细胞中畸变染色体的平均数，裂隙在数据中陈述但不包括在畸变染色体百分比或畸变细胞百分比的计算中。

6.2 统计学分析

使用 Fisher’s 确切概率法比较给药组与溶剂对照组畸变细胞发生频率差异的显著性。作多重比较时，P 值将经过 Bonferroni 调整。当 P 值小于等于 0.05 时，可认为差异具有显著性，这里的 P 值指的是未经 Bonferroni 调整前的 P 值。
6.3 实验有效性评估

溶液对照组的畸变细胞百分比应与历史数据具有可比性。阴性/阳性的历史数据列于附件 7 中。

高剂量阳性对照组的畸变细胞百分比与相应的溶剂对照组相比应显著提高（经统计分析后 \( p \leq 0.05 \)）。实验中设立了低剂量阳性对照组，且与供试品给药组载玻片放在一起盲码读片，这都是为了提高读片的客观性。不需要低剂量阳性对照组必需做出阳性结果。

一般至少要有三个可供读片的剂量，即每瓶细胞中至少有 100 个可读的中期相细胞。但是若供试品毒性太大，计数少于 3 个剂量也是可以接受的。

6.4 实验结果的评判标准

6.4.1 阳性结果

通常根据供试品给药组发生畸变细胞比例与相应溶剂对照组发生畸变细胞比例的统计分析结果来判断，但这也不是唯一的考量参数。也可以将一些生物学因素考虑在内，如畸变的类型、一个细胞中有多条染色体畸变的发生频率以及阴性对照畸变细胞频率的历史数据。实验的三个给药处理系列结果会分开考虑。应用上文所述的统计分析方法分析实验数据。对结果的最终评判应基于科学的判断。

畸变细胞比例统计学上显著增加且在阴性和/或溶剂对照历史数据范围之外，则可认为该剂量为阳性剂量。若在同一个给药处理系列中有两个阳性剂量，且这两个剂量诱导的细胞毒性都不应超过 50%太多。若只出现一个阳性剂量，只可判断为可疑阳性，若该阳性剂量结果可以在另一次实验中重复，则可以判断为阳性。只要三个给药处理系列中任一个是阳性，则实验整体上即可判断为阳性。

6.4.2 阴性结果

如果任一剂量组的畸变细胞频率与阴性/溶剂对照组相比都没有显著增加，则可认为该供试品为阴性。

当所有供试品畸变细胞百分比都在阴性和/或溶剂对照历史数据范围内时，这个系列可以不进行统计学分析即判断为阴性。

6.4.3 可疑阳性结果

如果在某一给药处理系列中只出现一个阳性剂量，可认为供试品诱导了可疑阳性反应。

7 结果和讨论

7.1 溶解度和pH值测试

- 肠腔素在乙醇中于大约 211.73 mg/mL 浓度时形成悬浊液。肠腔素在乙醇中于大约 6.2 mg/mL 浓度时形成澄清溶液，并且在更低剂量时依旧可溶。
- 当将 50 μL 的≥5mg/mL 的供试品制剂加入到 4.95 mL 的 McCoy’s 5A 全培
养基中或将 50 μL 的≥4mg/mL 的供试品制剂加入到 4.95 mL 不含血清 McCoy’s 5A 培养基中时，都有沉淀形成。

- 最终，剂量探索实验中三个暴露处理系列都选择 211.7 mg/mL 为最大给药浓度，2117 μg/mL 为最大给药剂量。
- 在溶解度测试，剂量探索实验及染色体畸变实验主实验中，含有最高剂量供试品的培养基 pH 值都维持在胜利水平（约 7.2），因此给药后不需要调整 pH 值。

### 7.2 制剂分析

在染色体畸变实验主实验中（包括重复实验）：

- 在溶剂对照制剂中的供试品浓度低于定量限×稀释因子，这证明在溶剂对照中不含有肠腔素。根据这个结果，可以推断在溶剂对照组中的细胞没有暴露于肠腔素。
- 测得供试品制剂（包括悬浊液和溶液）的浓度在标识浓度的 95%至 106%之间。根据这个结果，可以推断，各浓度的供试品制剂配制准确。
- 肠腔素的低高两浓度制剂（分别为 0.5 和 10mg/mL）在室温下放置 6 小时后测得的浓度与初始值相比相差为标识浓度的-6%和 1%。根据这个结果，可以推断，本实验中各个浓度的肠腔素制剂在室温至少稳定 6 个小时。
- 测得各悬浊液供试品制剂的浓度在标识浓度的 97%到 99%之间，且每一浓度的相对标准差（RSD）都小于等于 3%。根据这个结果可以推断，本实验中各浓度的悬浊液是均一的。

制剂分析报告被列在附件 6 中。

### 7.3 剂量探索实验

在选定染色体畸变实验主实验的剂量水平前先开展剂量探索实验，并根据相对于溶剂对照组细胞生长减少（细胞生长抑制）情况选定剂量。在三个给药系列中，CHO-WBL 细胞暴露于溶剂对照和以下 10 个供试品剂量水平，

在 S9 代谢活化给药 3 小时系列中为 1, 2, 5, 10, 20, 40, 100, 250, 700
和 2117 μg/mL，在非代谢活化给药 3 小时和 20 小时系列中为 1, 2, 5, 10, 20, 50, 100, 250, 700 和 2117 μg/mL。在 S9 代谢活化给药 3 小时系列中
≥40 μg/mL 剂量组的培养基中都可以观察到沉淀；在非代谢活化给药 3 小时和
20 小时系列中≥50 μg/mL 剂量组的培养基中都可以观察到沉淀。细胞生长抑制的具体结果列在表 1.2 和 3.2 中。在 S9 代谢活化给药 3 小时系列中≥100 μg/mL
剂量组中可见明显毒性（即与溶剂对照组相比细胞生长抑制不超 50%太多）；在非代谢活化给药 3 小时系列中≥100 μg/mL 剂量组中可见明显毒性，
在非代谢活化给药 20 小时系列中≥20 μg/mL 剂量组中可见明显毒性。

### 7.4 染色体畸变实验主实验

根据剂量探索实验结果，染色体畸变实验主实验及重复实验的剂量水平
选择如下：

Confidential
在染色体畸变实验主实验中，S9 代谢活化给药 3 小时系列中≥40 μg/mL 剂量组的培养基中都可以观察到沉淀；在非代谢活化给药 3 小时和 20 小时系列中≥50 μg/mL 剂量组的培养基中都可以观察到沉淀。

**S9 代谢活化给药 3 小时系列**

细胞毒性数据列在表 4 中。肠腔素在 60 μg/mL 剂量组中对 CHO-WBL 细胞的毒性（相对于溶剂对照组的细胞生长抑制）为 54%，选择该剂量作为读片的最高剂量（细胞毒性不超过 50%太多）。此外也选择读片了 20 和 40 μg/mL 两个较低剂量组，这三个剂量下，相对于溶剂对照，细胞生长抑制分别为 4% 和 13%。供试品及溶剂/空白/阳性对照诱导染色体畸变的结果按照每瓶细胞为单位详细列在表 5 中，并按照剂量组为单位总结在表 12 中。溶剂对照组的染色体结构畸变百分比为 1.50%，与空白对照组 (1.50%) 及历史数据具有可比性。高剂量 CP 阳性对照组诱导的染色体结构畸变百分比 (11.50%) 与溶剂对照组相比显著增加（p<0.01, Fisher’s 确切概率法）。根据以上结果，可以认为本实验的 S9 活化 3 小时给药系列是有效的。

与溶剂对照组比较，供试品各剂量处理组的染色体结构畸变百分比都没有显著增加（p>0.05, Fisher’s 确切概率法）。而且供试品各剂量诱导的染色体结构畸变百分比都在阴性历史数据范围之内（见附件 7）。

供试品各剂量处理组的染色体数量畸变百分比 (多倍体和核内复制) 与溶剂对照组比较，都没有增加（未作统计学分析）。然而 S9 活化 3 小时给药系列中，多倍体和核内复制的发生率 (分别为 3.38% 和 9.50%) 与本实验中非代谢活化给药 3 小时系列的发生率 (分别为 2.44% 和 0.99%) 以及历史数据 (分别为 0-2.91% 和 1.48-6.54%) 相比都有显著增加。我们认为这一现象是由于一个 SOP 偏离造成的，即不含血清 McCoy’s 5A 培养基在 S9 混合物混合后被放置在了冰上。因此重复了一次染色体畸变实验主实验的 S9 活化 3 小时处理系列，剂量为 20、40、60 和 70 μg/mL。

**S9 代谢活化给药 3 小时系列 (重复实验)**

细胞毒性数据列在表 6 中。肠腔素在 60 μg/mL 剂量组中对 CHO-WBL 细胞的毒性（相对于溶剂对照组的细胞生长抑制）为 59%，选择该剂量作为读片的最高剂量（细胞毒性不超过 50%太多）。此外也选择读片了 20 和 40 μg/mL 两个较低剂量组，在这两个剂量下，相对于溶剂对照，细胞生长抑制分别为 4% 和 18%。供试品及溶剂/空白/阳性对照诱导染色体畸变的结果按照每瓶细胞为单位详细列在表 7 中，并按照剂量组为单位总结在表 12 中。溶剂对照组的染色体结构畸变百分比为 1.00%，与空白对照组 (1.00%) 及历史数据具有可比性。高剂量 CP 阳性对照组诱导的染色体结构畸变百分比 (28.00%) 与溶剂对照组相比显著增加 (p<0.01, Fisher’s 确
根据以上结果，可以认为本实验的 S9 活化 3 小时给药系列是有效的。

与溶剂对照组比较，供试品各剂量处理组的染色体结构畸变百分比都没有显著增加（p>0.05，Fisher’s 确切概率法）。而且供试品各剂量组诱导的染色体结构畸变百分比都在阴性历史数据范围之内（见附件 7）。根据以上结果，可以推断肠腔素在 S9 活化 3 小时处理条件下诱导染色体结构畸变的结果为阴性。

供试品各剂量处理组的染色体数量畸变百分比（多倍体和核内复制）与溶剂对照组比较，都没有增加（未作统计学分析）。而且多倍体和核内复制细胞的发生率（分别为 0.99%和 1.96%）与实验室历史数据具有可比性。

♦ 非代谢活化给药 3 小时系列：

细胞毒性数据列在表 8 中。肠腔素在 60 µg/mL 剂量组中对 CHO-WBL 细胞的毒性（相对于溶剂对照组的细胞生长抑制）为 53%，选择该剂量作为读片的最高剂量（细胞毒性不超过 50%太多）。此外也选择读片了 20 和 40 µg/mL 两个较低剂量组，这在两个剂量下，相对于溶剂对照，细胞生长抑制分别为 23%和 16%。然而供试品在较低两个剂量组中，与溶剂对照组相比，都没有显示出有丝分裂抑制，在 20 µg/mL 时为 6%，在 40 µg/mL 时为 10%。供试品及溶剂/空白/阳性对照诱导染色体畸变的结果按照每瓶细胞为单位详细列在表 9 中，并按照剂量组为单位总结在表 12 中。溶剂对照组的染色体结构畸变百分比为 1.50%，与空白对照组（2.00%）及历史数据具有可比性。高剂量 MMC 阳性对照组诱导的染色体结构畸变百分比（30.00%）与溶剂对照组相比显著增加（p<0.01，Fisher’s 确切概率法）。根据以上结果，可以认为本实验的非活化 3 小时给药系列是有效的。

与溶剂对照组比较，供试品各剂量处理组的染色体结构畸变百分比都没有显著增加（p>0.05，Fisher’s 确切概率法）。而且供试品各剂量组诱导的染色体结构畸变百分比都在阴性历史数据范围之内（见附件 7）。根据以上结果，可以推断肠腔素在非活化 3 小时处理条件下诱导染色体结构畸变的结果为阴性。

供试品各剂量处理组的染色体数量畸变百分比（多倍体和核内复制）与溶剂对照组比较，都没有增加（未作统计学分析）。

♦ 非代谢活化给药 20 小时系列：

细胞毒性数据列在表 10 中。肠腔素在 15 µg/mL 剂量组中对 CHO-WBL 细胞的毒性（相对于溶剂对照组的细胞生长抑制）为 53%，选择该剂量作为读片的最高剂量（细胞毒性不超过 50%太多）。此外也选择读片了 5 和 10 µg/mL 两个较低剂量组，这在两个剂量下，相对于溶剂对照，细胞生长抑制分别为 33%和 32%。然而供试品在较低两个剂量组中，与溶剂对照组相比，显示出很少的有丝分裂抑制，在 5 µg/mL 时为 17%，在 10 µg/mL 时为 18%。供试品及溶剂/空白/阳性对照诱导染色体畸变的结果按照每瓶细胞为单位详细列在表 11 中，并按照剂量组为单位总结在表 12 中。溶剂对照组的染色体结构畸变百分比为 2.00%，与空白对照组（0.50%）及历史数据具有可比性。高剂量 MMC 阳性对照组诱导的染色体结构畸变百分比（36.00%）与溶剂对照组相比显著增加（p<0.01，Fisher’s 确切概率法）。根据以上结果，可以认为本实验的非活化 20 小时给药系
列是有效的。

与溶剂对照组比较，供试品各剂量处理组的染色体结构畸变百分比都没有显著增加（p>0.05, Fisher's 确切概率法）。而且供试品各剂量组诱导的染色体结构畸变百分比都在阴性历史数据范围之内（见附件 7）。根据以上结果，可以推断肠腔素在非活化 3 小时处理条件下诱导染色体结构畸变的结果为阴性。

供试品各剂量处理组的染色体数量畸变百分比（多倍体和核内复制）与溶剂对照组比较，都没有增加（未作统计学分析）。

与溶剂对照组比较，供试品各剂量处理组的染色体结构畸变百分比都没有显著增加（p>0.05, Fisher's 确切概率法）。而且供试品各剂量组诱导的染色体结构畸变百分比都在阴性历史数据范围之内（见附件 7）。根据以上结果，可以推断肠腔素在非活化 20 小时处理条件下诱导染色体结构畸变的结果为阴性。

供试品各剂量处理组的染色体数量畸变百分比（多倍体和核内复制）与溶剂对照组比较，都没有增加（未作统计学分析）。

8 结论

根据以上讨论，可以认为该染色体畸变实验是有效的。

在该GLP染色体畸变实验条件下，推断肠腔素在加和不加S9 代谢活化系统条件下诱导染色体结构/数量畸变的结果为阴性。

9 偏离

在 SOP-GEN-0003（中国仓鼠卵巢（CHO）细胞体外培养染色体畸变实验）中，要求培养基在加入到细胞中去之前应加热到 37±2°C。然而在第一次染色体畸变实验主实验中，不含血清培养基在与 S9 混合物混合后被放置在了冰上。

该冷休克造成多倍体和核内复制细胞的发生率增高。在重复实验中避免冷休克后，多倍体和核内复制细胞的发生率都在阴性历史数据范围内。

10 归档

实验产生的原始数据、相关文件、实验方案及其修正案，以及总结报告原始版将根据苏州药明康德新药开发有限公司的标准操作规程在总结报告签署当日归档保存，时限至少 1 年。1年后，苏州药明康德新药开发有限公司将根据相关 SOP 的要求与委托单位沟通决定这些原始记录如何处理。没有委托单位的同意，任何材料均不会被丢弃。

11 参考文献

cytogenetics testing with Chinese hamster ovary cells: Comparison of results of 22 compounds in two laboratories. Environ. Mutagen. 7:1 51.


# TABLES
Table 1  Assessment of Cytotoxicity in Dose Range-finding Assay, S9 Activated 3 Hours Exposure

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Viable Cells/Flask (x10^6)</th>
<th>Population Doublings</th>
<th>Relative PD (%)</th>
<th>Cytotoxicity (%)</th>
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<td>-240</td>
<td>340</td>
</tr>
<tr>
<td>2117</td>
<td>1.44</td>
<td>-1.54</td>
<td>-159</td>
<td>259</td>
</tr>
</tbody>
</table>

NA-Not applicable  \[X_0=4.18 \times 10^6\]

Treatment: CHO cells were treated in the presence of an exogenous metabolic activation for 3 hours.

Viable cells were determined by an automatic cell analysis system with trypan blue dye exclusion for viability.

Population doublings (PD) = the log of the ratio of the final count to the starting count (Xo), divided by the log of 2.

Relative PD = (PD of treated cultures / PD of solvent controls) X 100

Cytotoxicity (%) = 100 - Relative PD

& The reported values were rounded off to two significant numbers.
Table 2  Assessment of Cytotoxicity in Dose Range-finding Assay,
Non-activated 3 Hours Exposure

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Viable Cells/Flask (x10^6)</th>
<th>Population Doublings</th>
<th>Relative PD (%)</th>
<th>Cytotoxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>11.09</td>
<td>1.36</td>
<td>100</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>10.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank control</td>
<td>11.26</td>
<td>1.43</td>
<td>105</td>
<td>NA</td>
</tr>
<tr>
<td>Coelenterazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10.39</td>
<td>1.31</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>10.60</td>
<td>1.34</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>10.50</td>
<td>1.33</td>
<td>98</td>
<td>2</td>
</tr>
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<td>10</td>
<td>10.02</td>
<td>1.26</td>
<td>93</td>
<td>7</td>
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<tr>
<td>20</td>
<td>9.46</td>
<td>1.18</td>
<td>87</td>
<td>13</td>
</tr>
<tr>
<td>50</td>
<td>8.05</td>
<td>0.95</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>100</td>
<td>4.33</td>
<td>0.05</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>250</td>
<td>0.55</td>
<td>-2.93</td>
<td>-215</td>
<td>315</td>
</tr>
<tr>
<td>700</td>
<td>0.03</td>
<td>-7.12</td>
<td>-524</td>
<td>624</td>
</tr>
<tr>
<td>2117</td>
<td>0.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA-Not applicable  \[ X_0=4.18 \times 10^6 \]
Treatment: CHO cells were treated in the absence of an exogenous metabolic activation for 3 hours.
Viable cells were determined by an automatic cell analysis system with trypan blue dye exclusion for viability.
Population doublings (PD) = the log of the ratio of the final count to the starting count (Xo), divided by the log of 2.
Relative PD = (PD of treated cultures / PD of solvent controls) × 100
Cytotoxicity (%) = 100 - Relative PD
& The reported values were rounded off to two significant numbers.
### Table 3  Assessment of Cytotoxicity in Dose Range-finding Assay, Non-activated 20 Hours Exposure

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Viable Cells/Flask (x10⁶) &amp;</th>
<th>Population Doublings</th>
<th>Relative PD (%)</th>
<th>Cytotoxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>11.42</td>
<td>1.48</td>
<td>100</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>11.87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank control</td>
<td>12.32</td>
<td>1.56</td>
<td>105</td>
<td>NA</td>
</tr>
<tr>
<td>Coelenterazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10.73</td>
<td>1.36</td>
<td>92</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>9.86</td>
<td>1.24</td>
<td>84</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>9.42</td>
<td>1.17</td>
<td>79</td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td>8.51</td>
<td>1.03</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>20</td>
<td>6.54</td>
<td>0.65</td>
<td>44</td>
<td>56</td>
</tr>
<tr>
<td>50</td>
<td>4.78</td>
<td>0.19</td>
<td>13</td>
<td>87</td>
</tr>
<tr>
<td>100</td>
<td>2.81</td>
<td>-0.57</td>
<td>-39</td>
<td>139</td>
</tr>
<tr>
<td>250</td>
<td>2.56</td>
<td>-0.71</td>
<td>-48</td>
<td>148</td>
</tr>
<tr>
<td>700</td>
<td>0.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2117</td>
<td>0.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA-Not applicable

X₀ = 4.18 x 10^6

Treatment: CHO cells were treated in the absence of an exogenous metabolic activation for 24 hours. Viable cells were determined by an automatic cell analysis system with trypan blue dye exclusion for viability.

Population doublings (PD) = the log of the ratio of the final count to the starting count (X₀), divided by the log of 2.

Relative PD = (PD of treated cultures / PD of solvent controls) X 100

Cytotoxicity (%) = 100 - Relative PD

& The reported values were rounded off to two significant numbers.
### Table 4  Assessment of Cytotoxicity in Definitive Chromosome Aberration Assay, S9 Activated 3 Hours Exposure

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Flask</th>
<th>Viable Cells/Flask (x10^6)</th>
<th>PD</th>
<th>Relative PD (%)</th>
<th>Cell Growth Inhibition (%)</th>
<th>Mitotic Index (individual, %)</th>
<th>Mitotic Index (mean, %)</th>
<th>Mitotic Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>A</td>
<td>8.61</td>
<td>0.91</td>
<td>100</td>
<td>NA</td>
<td>13.4</td>
<td>12.8</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank Control</td>
<td>A</td>
<td>8.62</td>
<td>0.88</td>
<td>97</td>
<td>NA</td>
<td>13.2</td>
<td>13.2</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coelenterazine</td>
<td>20#</td>
<td>A</td>
<td>8.39</td>
<td>0.87</td>
<td>96</td>
<td>4</td>
<td>12.2</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8.58</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40#P</td>
<td>A</td>
<td>8.41</td>
<td>0.79</td>
<td>87</td>
<td>13</td>
<td>15.8</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 P</td>
<td>A</td>
<td>7.17</td>
<td>0.67</td>
<td>74</td>
<td>26</td>
<td>11.8</td>
<td>14.2</td>
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<td>B</td>
<td>7.59</td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
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<td>A</td>
<td>6.47</td>
<td>0.42</td>
<td>46</td>
<td>54</td>
<td>6.0</td>
<td>5.6</td>
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<td></td>
<td>B</td>
<td>6.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>70 P</td>
<td>A</td>
<td>5.33</td>
<td>0.15</td>
<td>16</td>
<td>84</td>
<td>1.8</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.02</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>80 P</td>
<td>A</td>
<td>4.51</td>
<td>-0.08</td>
<td>-9</td>
<td>109</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>4.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 P</td>
<td>A</td>
<td>4.12</td>
<td>-0.15</td>
<td>-16</td>
<td>116</td>
<td>0.2</td>
<td>0.4</td>
</tr>
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<td></td>
<td>B</td>
<td>4.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA-Not applicable, $X_0=4.65 \times 10^6$

Treatment: CHO cells were treated in the presence of an exogenous metabolic activation for 3 hours

Viable cells were determined by an automatic cell analysis system with trypan blue dye exclusion for viability

Population doublings (PD) = the log of the ratio of the final count to the starting count ($X_0$), divided by the log of 2

Relative PD = (PD of treated cultures / PD of solvent controls) X 100

Cell growth inhibition (%) = 100 - Relative PD

Mitotic index (MI, %) = (Cells in mitosis / 500 cells scored) X 100

Mitotic inhibition (%) = 100 - (MI of treated cultures/MI of solvent controls) X 100

& The reported values were rounded off to two significant numbers

P=Precipitate was observed in the medium/test article mixer

#=Highlighted concentrations were selected for chromosome aberration analysis
Table 5  **Chromosome Aberrations in Definitive Chromosome Aberration Assay, S9 Activated 3 Hours Exposure**

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Flask</th>
<th>Total Cells Scored</th>
<th># Polyploid Cells</th>
<th># Endoreduplicated Cells</th>
<th>Total Aberrations - Gaps</th>
<th>Total Aberrant Cells - Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>A</td>
<td>100</td>
<td>2</td>
<td>10</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>100</td>
<td>5</td>
<td>11</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>200</td>
<td>7</td>
<td>21</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Blank Control</td>
<td>A</td>
<td>100</td>
<td>0</td>
<td>9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>100</td>
<td>3</td>
<td>11</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>200</td>
<td>3</td>
<td>20</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Coelenterazine</td>
<td>A</td>
<td>100</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>B</td>
<td>100</td>
<td>3</td>
<td>8</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>200</td>
<td>8</td>
<td>14</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>40</td>
<td>A</td>
<td>100</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>100</td>
<td>3</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
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<td>200</td>
<td>4</td>
<td>12</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>60</td>
<td>A</td>
<td>100</td>
<td>3</td>
<td>9</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>100</td>
<td>3</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>200</td>
<td>6</td>
<td>18</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>CP 1.5</td>
<td>A</td>
<td>100</td>
<td>2</td>
<td>9</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>100</td>
<td>3</td>
<td>16</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>200</td>
<td>5</td>
<td>25</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>CP 5.0</td>
<td>A</td>
<td>100</td>
<td>2</td>
<td>15</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>100</td>
<td>0</td>
<td>16</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>200</td>
<td>2</td>
<td>31</td>
<td>7</td>
<td>13</td>
</tr>
</tbody>
</table>

**Treatment:** CHO cells were treated for 3 hours in the presence of an exogenous metabolic activation. Dose levels of 50, 70, 80 and 100 µg/mL were not analyzed for chromosome aberration.

Polyplloid and endoreduplicated cells are not included in the total cells scored as they are numerical aberrant cells.

CP=Cyclophosphamide monohydrate

Confidential
### Table 6  Assessment of Cytotoxicity in Definitive Chromosome Aberration Assay, S9-activated 3 Hours Exposure (Repeating Test)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Flask</th>
<th>Viable Cells/Flask (x10^6)</th>
<th>PD</th>
<th>Relative PD (%)</th>
<th>Cell Growth Inhibition (%)</th>
<th>Mitotic Index (individual, %)</th>
<th>Mitotic Index (mean, %)</th>
<th>Mitotic Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>A</td>
<td>9.76</td>
<td>1.11</td>
<td>100</td>
<td>NA</td>
<td>14.4</td>
<td>14.1</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>9.74</td>
<td></td>
<td></td>
<td></td>
<td>13.8</td>
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</tr>
<tr>
<td>Blank</td>
<td>A</td>
<td>10.16</td>
<td>1.10</td>
<td>99</td>
<td>NA</td>
<td>14.0</td>
<td>14.2</td>
<td>14.1</td>
</tr>
<tr>
<td>Control</td>
<td>B</td>
<td>9.29</td>
<td></td>
<td></td>
<td></td>
<td>14.1</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Coelenterazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20#</td>
<td>A</td>
<td>9.64</td>
<td>1.07</td>
<td>96</td>
<td>4</td>
<td>13.6</td>
<td>14.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>9.42</td>
<td></td>
<td></td>
<td></td>
<td>14.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40#P</td>
<td>A</td>
<td>8.60</td>
<td>0.91</td>
<td>82</td>
<td>18</td>
<td>12.2</td>
<td>12.6</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8.42</td>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60#P</td>
<td>A</td>
<td>6.45</td>
<td>0.46</td>
<td>41</td>
<td>59</td>
<td>6.6</td>
<td>6.5</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.03</td>
<td></td>
<td></td>
<td></td>
<td>6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 P</td>
<td>A</td>
<td>5.36</td>
<td>0.26</td>
<td>23</td>
<td>77</td>
<td>2.4</td>
<td>2</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5.47</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA-Not applicable, $X_0=4.53 \times 10^6$

Treatment: CHO cells were treated in the presence of an exogenous metabolic activation for 3 hours.

Viable cells were determined by an automatic cell analysis system with trypan blue dye exclusion for viability.

Population doublings (PD) = the log of the ratio of the final count to the starting count ($X_0$), divided by the log of 2.

Relative PD = (PD of treated cultures / PD of solvent controls) X 100

Cell growth inhibition (%) = 100 - Relative PD

Mitotic index (MI, %)= (Cells in mitosis / 500 cells scored) X 100

Mitotic inhibition (%)= 100 - (MI of treated cultures/MI of solvent controls) X 100

& The reported values were rounded off to two significant numbers.

P=Precipitate was observed in the medium/test article mixer

#=#Highlighted concentrations were selected for chromosome aberration analysis.
Table 7  Chromosome Aberrations in Definitive Chromosome Aberration Assay, S9-activated 3 Hours Exposure (Repeating Test)

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Flask</th>
<th>Total Cells Scored</th>
<th>Polyploid Cells</th>
<th>Endoreduplicated Cells</th>
<th># Chromatid Deletions</th>
<th># Chromosome Deletions</th>
<th>Total Aberrations - Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>A</td>
<td>100</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>100</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>200</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
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<td>A</td>
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<td>2</td>
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<tr>
<td>Control</td>
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</tr>
<tr>
<td></td>
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<td>4</td>
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</tbody>
</table>

| Coelenterazine   | A     | 100                | 1               | 1                      | 0                    | 0                  | 2                       |
|                  | B     | 100                | 0               | 1                      | 0                    | 0                  | 2                       |
|                  | Total | 200                | 1               | 2                      | 1                    | 0                  | 4                       |
|                  | A     | 100                | 1               | 3                      | 0                    | 0                  | 3                       |
|                  | B     | 100                | 1               | 3                      | 0                    | 0                  | 3                       |
|                  | Total | 200                | 2               | 6                      | 1                    | 0                  | 6                       |
|                  | A     | 100                | 1               | 3                      | 0                    | 0                  | 3                       |
|                  | B     | 100                | 1               | 3                      | 0                    | 0                  | 3                       |
|                  | Total | 200                | 2               | 6                      | 1                    | 0                  | 6                       |

| CP 1.5           | A     | 100                | 1               | 1                      | 0                    | 0                  | 2                       |
|                  | B     | 100                | 2               | 6                      | 0                    | 0                  | 8                       |
|                  | Total | 200                | 3               | 11                     | 6                    | 0                  | 16                      |
|                  | A     | 100                | 1               | 3                      | 9                    | 0                  | 3                       |
|                  | B     | 100                | 0               | 1                      | 0                    | 0                  | 6                       |
|                  | Total | 200                | 1               | 4                      | 9                    | 0                  | 9                       |

| CP 5.0           | A     | 100                | 1               | 3                      | 9                    | 0                  | 3                       |
|                  | B     | 100                | 2               | 6                      | 1                    | 0                  | 6                       |
|                  | Total | 200                | 3               | 9                      | 1                    | 0                  | 9                       |

Treatment: CHO cells were treated for 3 hours in the presence of an exogenous metabolic activation. Dose level of 70 µg/mL were not analyzed for chromosome aberration.

Polyploid and endoreduplicated cells are not included in the total cells scored as they are numerical aberrant cells.

CP=Cyclophosphamide monohydrate
### Table 8: Assessment of Cytotoxicity in Definitive Chromosome Aberration Assay, Non-activated 3 Hours Exposure

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Treatment</th>
<th>Viable Cells/Flask (x10^6)&amp;</th>
<th>PD</th>
<th>Relative PD (%)</th>
<th>Cell Growth Inhibition (%)</th>
<th>Mitotic Index (individual, %)</th>
<th>Mitotic Index (mean, %)</th>
<th>Mitotic Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
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<td>10.86</td>
<td>1.22</td>
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<td>A</td>
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<td>1.17</td>
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<td>11.4</td>
<td>11.3</td>
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</tr>
</tbody>
</table>

NA-Not applicable, $X_0=4.65 \times 10^6$

Treatment: CHO cells were treated in the absence of an exogenous metabolic activation for 3 hours.

Viable cells were determined by an automatic cell analysis system with trypan blue dye exclusion for viability.

Population doublings (PD) = the log of the ratio of the final count to the starting count (Xo), divided by the log of 2.

Relative PD = (PD of treated cultures / PD of solvent controls) X 100

Cell growth inhibition (%) = 100 - Relative PD

Mitotic index (MI, %)= (Cells in mitosis / 500 cells scored) X 100

Mitotic inhibition (%)= 100 - (MI of treated cultures/MI of solvent controls) X 100

& The reported values were rounded off to two significant numbers.

P=Precipitate was observed in the medium/test article mixer

#-Highlighted concentrations were selected for chromosome aberration analysis
Table 9  Chromosome Aberrations in Definitive Chromosome Aberration Assay, Non-activated 3 Hours Exposure

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Flask</th>
<th>Total Cells Scored</th>
<th>Total Polyploid Cells</th>
<th>Total Endoreduplicated Cells</th>
<th>Number of structural aberrations</th>
<th>Total Aberrant Cells - Gaps</th>
<th>Total Aberrant Gaps</th>
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<tbody>
<tr>
<td></td>
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<td></td>
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<td></td>
<td>Chromatid</td>
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<td></td>
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<td>Deletions</td>
<td>Exchanges</td>
<td>Deletions</td>
</tr>
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<td></td>
<td>TB</td>
<td>ID</td>
<td>TR</td>
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<td>0</td>
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</tr>
<tr>
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<tr>
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<td>6</td>
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<td>6</td>
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<td>17</td>
<td>1</td>
<td>10</td>
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<td></td>
<td>Total</td>
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<td>6</td>
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<td>34</td>
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<td>16</td>
</tr>
</tbody>
</table>

Treatment: CHO cells were treated for 3 hours in the absence of an exogenous metabolic activation. Dose levels of 50, 70, 80 and 100 µg/mL were not analyzed for chromosome aberration.

Polyploid and endoreduplicated cells are not included in the total cells scored as they are numerical aberrant cells.

MMC=Mitomycin C
**Table 10 Assessment of Cytotoxicity in Definitive Chromosome Aberration Assay, Non-activated 20 Hours Exposure**

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Viable Cells/Flask (x10^6) &amp; Flam</th>
<th>PD</th>
<th>Relative PD (%)</th>
<th>Cell Growth Inhibition (%)</th>
<th>Mitotic Index (individual, %)</th>
<th>Mitotic Index (mean, %)</th>
<th>Mitotic Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ethanol</strong></td>
<td>A: 10.82</td>
<td>1.22</td>
<td>100</td>
<td>NA</td>
<td>8.8</td>
<td>9.0</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>B: 10.86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Blank Control</strong></td>
<td>A: 10.90</td>
<td>1.24</td>
<td>102</td>
<td>NA</td>
<td>9.6</td>
<td>9.8</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>B: 11.01</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Coelesterazine</strong></td>
<td>5 A: 8.07</td>
<td>0.82</td>
<td>67</td>
<td>33</td>
<td>7.2</td>
<td>7.6</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>B: 8.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 A: 8.42</td>
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<td>7</td>
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</tr>
<tr>
<td></td>
<td>20 A: 6.79</td>
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<td></td>
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<td>72</td>
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<tr>
<td></td>
<td>40 A: 5.70</td>
<td>0.24</td>
<td>20</td>
<td>80</td>
<td>1.6</td>
<td>1.8</td>
<td>81</td>
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<tr>
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<td>10</td>
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<td>0.8</td>
<td>90</td>
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</tbody>
</table>

NA-Not applicable, \( X_0 = 4.65 \times 10^6 \)

Treatment: CHO cells were treated in the absence of an exogenous metabolic activation for 20 hours

Viable cells were determined by an automatic cell analysis system with trypan blue dye exclusion for viability.

Population doublings (PD) = the log of the ratio of the final count to the starting count (Xo), divided by the log of 2

Relative PD = (PD of treated cultures / PD of solvent controls) X 100

Cell growth inhibition (%) = 100 - Relative PD

Mitotic index (MI, %) = (Cells in mitosis / 500 cells scored) X 100

Mitotic inhibition (%) = 100 - (MI of treated cultures/MI of solvent controls) X 100

& The reported values were rounded off to two significant numbers

P=Precipitate was observed in the medium/test article mixer

#=#Highlighted concentrations were selected for chromosome aberration analysis
### Table 11 Chromosome Aberrations in Definitive Chromosome Aberration Assay, Non-activated 20 Hours Exposure

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>Flask</th>
<th>Total Cells Scored</th>
<th># Polyploid Cells</th>
<th># Endoreduplicated Cells</th>
<th>Chromatid Deletions</th>
<th>Chromatid Exchanges</th>
<th>Chromosome Deletions</th>
<th>Chromosome Exchanges</th>
<th>Total Aberrations - Gaps</th>
<th>Total Aberrant Cells - Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>A</td>
<td>100</td>
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<td>1</td>
<td>0</td>
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<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>B</td>
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<td>0</td>
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</tr>
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<td>10</td>
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<tr>
<td>Blank Control</td>
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<td>0</td>
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<td>0</td>
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<td>9</td>
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</tbody>
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Treatment: CHO cells were treated for 3 hours in the presence of an exogenous metabolic activation. Dose levels of 20, 30, 40 and 50 µg/mL were not analyzed for chromosome aberration.

Polyplloid and endoreduplicated cells are not included in the total cells scored as they are numerical aberrant cells.

MMC=Mitomycin C

An SD cell counts as 10 in the total aberrations per cell, but as one in the percentage of cells with aberrations.
Table 12 Summary of Definitive Chromosome Aberration Assay

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>+/- S9</th>
<th>Treatment Time (hours)</th>
<th>Cell Growth Inhibition (%)</th>
<th>Mitosis Inhibition (%)</th>
<th>Total Cells Scored</th>
<th>Cells with Polyploidy (%)#</th>
<th>Cells with Endoreduplication (%)#</th>
<th>% Gaps</th>
<th>% Aberrations -Gaps</th>
<th>% Aberrant Cells -Gaps</th>
</tr>
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<tbody>
<tr>
<td>Ethanol</td>
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<td>+S9</td>
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**Coelenterazine**

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<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>+/- S9</th>
<th>Treatment Time (hours)</th>
<th>Cell Growth Inhibition (%)</th>
<th>Mitosis Inhibition (%)</th>
<th>Total Cells Scored</th>
<th>Cells with Polyploidy (%)#</th>
<th>Cells with Endoreduplication (%)#</th>
<th>% Gaps</th>
<th>% Aberrations -Gaps</th>
<th>% Aberrant Cells -Gaps</th>
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<td>NA</td>
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<tr>
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**Coelenterazine (repeating test)**

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<th>Cell Growth Inhibition (%)</th>
<th>Mitosis Inhibition (%)</th>
<th>Total Cells Scored</th>
<th>Cells with Polyploidy (%)#</th>
<th>Cells with Endoreduplication (%)#</th>
<th>% Gaps</th>
<th>% Aberrations -Gaps</th>
<th>% Aberrant Cells -Gaps</th>
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**Coelenterazine**

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<th>Cell Growth Inhibition (%)</th>
<th>Mitosis Inhibition (%)</th>
<th>Total Cells Scored</th>
<th>Cells with Polyploidy (%)#</th>
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<th>% Gaps</th>
<th>% Aberrations -Gaps</th>
<th>% Aberrant Cells -Gaps</th>
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**Coelenterazine**

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<td>60.50</td>
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Treatment: CHO-WBL cells from all treatment conditions were harvested 20 hours after the initiation of the treatments.

% Aberrant Cells-Gaps: *, p≤0.05; **, p≤0.01; using Fisher's Exact test.

#Polyploid and endoreduplicated cells are not included in the total cells scored as they are numerical aberrant cells.
APPENDICES
APPENDIX 1  CERTIFICATE OF ANALYSIS OF COELENTERAZINE

Certificate of Analysis

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<td>TH 03647-031</td>
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<tr>
<td>CAS Number:</td>
<td>55779-48-1</td>
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<tr>
<td>MDL Number:</td>
<td>MFCD00467176</td>
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<td>Formula:</td>
<td>C_{26}H_{21}N_{3}O_{3}</td>
</tr>
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<td>Formula Weight:</td>
<td>423.46 g/mol</td>
</tr>
<tr>
<td>Storage Temperature:</td>
<td>Store at -20 °C, sealed, keep in dark place</td>
</tr>
<tr>
<td>Quality Release Date:</td>
<td>28 JUN 2011</td>
</tr>
<tr>
<td>Recommended Retest Date:</td>
<td>JUN 2021</td>
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</table>

<table>
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<th>Specification</th>
<th>Result</th>
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<td>Appearance (Color)</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>Appearance (Form)</td>
<td>Powder</td>
<td>Powder</td>
</tr>
<tr>
<td>Melting Point</td>
<td>176-181°C</td>
<td>178-180°C</td>
</tr>
<tr>
<td>Purity (By HPLC)</td>
<td>&gt;90%</td>
<td>93.57%</td>
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<td>Identification</td>
<td>Spectrum is consistent with structure</td>
<td>By NMR, Conforms</td>
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<tr>
<td></td>
<td>Spectrum is consistent with structure</td>
<td>By MS, Conforms</td>
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Analyst: Guang Yang
Checker: [Signature]
Supervisor: [Signature]
## APPENDIX 2  CERTIFICATE OF ANALYSIS OF SOLVENT

### Certification of Analysis

**Commodity No.** 10009164  
**Name** Ethanol 95%  
**Property condition** 无色透明，易挥发，易燃液体。  
**Formula** \( \text{CH}_3\text{CH}_2\text{OH} \)  
**Molecular Weight** 46.07  
**Packing** 500mL  
**Standard of Analysis** GB/T 679-2002

### Specification

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<th>Standard</th>
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<td>95.2%</td>
</tr>
<tr>
<td>Evaporating loss</td>
<td>≤0.001%</td>
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</tr>
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<td>Acid (as H&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>≤0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Alkalinity (as OH&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>≤0.01</td>
<td>0.006</td>
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<tr>
<td>Methanol</td>
<td>≤0.003%</td>
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<tr>
<td>Acetone &amp; Isopropyl alcohol</td>
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<td>&lt;0.0005%</td>
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<tr>
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<tr>
<td>Sulfur</td>
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**Date of Analysis** 2010-05-08  
**Conclusion** 合格 To pass test
APPENDIX 3  PROTOCOL AND PROTOCOL AMENDMENT
FINAL PROTOCOL
实验方案

STUDY TITLE: Coelenterazine: In Vitro Chromosomal Aberration Assay in Chinese Hamster Ovary Cells

实验名称: 肠腔素：中国仓鼠卵巢细胞体外染色体畸变实验

STUDY NO.: 139-0005

TESTING FACILITY: WuXi AppTec (Suzhou) Co., Ltd.
1318 Wuzhong Avenue, Wuzhong District
Suzhou 215104, China

研究机构：苏州药明康德新药开发有限公司
中国江苏省苏州市吴中区吴中大道 1318 号，邮编：215104

SPONSORS:

Prolume Ltd. POB 2746, Pinetop, AZ 85935
Office Tel: 1-928-367-1200
www.prolume.com

and 和

BioLume Inc. 6325 Old Mill Farm Drive, Wendell, NC 27591
Office Tel: 1-919-824-9299
www.biolume.net
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1 OBJECTIVE

The objective of this study is to evaluate the clastogenic potential of Coelenterazine by measuring its ability to induce chromosome aberrations in Chinese hamster ovary cells (CHO) with or without exogenous metabolic activation (Aroclor 1254 induced rat liver S9).

2 REGULATORY COMPLIANCE

The dose range-finding assay will be non-GLP and conducted in full compliance with the study protocol and WuXi AppTec Standard Operating Procedures (SOPs).

The definitive chromosome aberration assay performed at WuXi AppTec (Suzhou) Co., Ltd. will adhere to the study protocol and WuXi AppTec Standard Operating Procedures (SOPs) and will conform to the most recent version of the Good Laboratory Practice (GLP) regulations listed below. The GLP terms, (eg, study protocol, test article, control article) used in this protocol are based on the US FDA Good Laboratory Practice Regulations and are considered synonymous with equivalent terms used in the OECD Principles of Good Laboratory Practice.

♦ Principles on Good Laboratory Practice issued by the Organization for Economic Co-operation and Development (OECD), revised in 1997.
♦ The Nonclinical Laboratory Studies Good Laboratory Practice Regulations issued by the U.S. Food and Drug Administration (Title 21 of the Code of Federal Regulations, Part 58; effective June 20, 1979. and all subsequent amendments).
♦ Good Laboratory Practice for Non-clinical Laboratory Studies, issued by State Food and Drug Administration, P.R. China. Effective September 01, 2003.

3 SPONSOR REPRESENTATIVE

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4 TESTING FACILITY

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5.3 Related Laboratory Personnel

<table>
<thead>
<tr>
<th>Responsibility</th>
<th>Personnel</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose Formulation</td>
<td>Shushu Li, BA</td>
<td>Central Pharmacy Laboratory</td>
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<tr>
<td></td>
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<td>Email: <a href="mailto:li_shushu@wuxiapptec.com">li_shushu@wuxiapptec.com</a></td>
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<tr>
<td>Formulation Analysis</td>
<td>Xiaosan Rong, BS</td>
<td>Analytical Chemistry Laboratory</td>
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<td>Email: <a href="mailto:jiang_jinjin@wuxiapptec.com">jiang_jinjin@wuxiapptec.com</a></td>
</tr>
</tbody>
</table>
6 PROPOSED STUDY SCHEDULE

Proposed Experimental Initial Date: 2011-09-13
Proposed Experimental Completion Date: 2011-10-28
Proposed Audited Draft Report Date: 2011-11-11

7 TEST AND CONTROL ARTICLES

Determination and documentation of the identity, strength, purity, stability, and uniformity of the test article, as defined in the Good Laboratory Practice (GLP) regulations, is the responsibility of the Sponsor. The Sponsor will provide these test article characterization data in a Certificate of Analysis [COA] or equivalent for inclusion in the final report. The GLP compliance statement in the final report will note any exceptions if any of these test article characterization data not available prior to the issuance of the final report.

The neat positive control articles and the solvent used to prepare the test article dose formulations will be characterized by COAs provided by the Supplier(s) which will be kept in WuXi AppTec (Suzhou). An exact copy of the solvent’s COA will be included in the final report.

7.1 Test Article Description

Identity: Coelenterazine
Code: CTZ
CAS Number: 55779-48-1
Supply: WuXi AppTec (Tianjin) Co., Ltd.
Batch Number: TH 03647-031
Purity: 93.57%
Molecular Weight: 423.46
Storage Conditions: Store at -20°C, protect from light
Physical Characterization: Yellow powder
Expiration Date: June 2021
7.2 Solvent/Negative Control Description

Solvent control article will be ethanol, with a concentration of 95%. The solvent control article will be evaluated concurrently with test article for each exposure group, at the same dosing volume as is used to administer the test article.

As ethanol is an atypical solvent used in this assay, untreated control cultures also will be included to ensure that the vehicle has no deleterious effect upon cell growth or the endpoints measured.

7.3 Positive Control Articles Description

Cyclophosphamide monohydrate [CP; CAS 6055-19-2], an agent that requires metabolic activation to its clastogenic form, will be evaluated concurrently at concentrations of 1.5 and 5.0 µg/mL in the S9-activated exposure group. Mitomycin C [MMC; CAS 50-07-7], a clastogen that does not require metabolic activation, will be evaluated concurrently at concentrations of 0.17 and 0.5 µg/mL in the non-activated 3 hours exposure group, at concentrations of 0.05 and 0.25 µg/mL in the non-activated 20 hours exposure group. These positive control articles will be prepared in sterile distilled water as 100× stocks in advance, stored at -20°C, and thawed immediately before use. These positive controls are chosen because of the large database available for these compounds and they are recommended by OECD guideline 473.

Positive control articles will be used only in the definitive chromosome aberration assay. Results obtained from the positive control articles will be used to validate the responsiveness of the test system, but they will not provide a standard for comparison with the test article.

7.4 Sample Retention

Refer to SOP-CPL-0001

A test article reserve sample of 1 g will be collected by the Central Pharmacy Laboratory.

7.5 Inventory and Disposition

Refer to SOP-CPL-0001 and SOP-GEN-0023

A test article inventory will be maintained. Bulk test article supplied by the Sponsor will be returned to the Sponsor following completion of this study, unless otherwise instructed. Control articles supplied by WuXi AppTec (Suzhou) will be retained in accordance with Testing Facility SOPs.
7.6 Handling/Safety Precautions

Refer to SOP-CPL-0011

The Testing Facility will use standard laboratory safety procedures for handling the test and control articles. Appropriate personal protective equipments will be worn while preparing and administering doses. The Sponsor will provide safety information about the test article in the form of a Material Safety Data Sheet (MSDS), if applicable.

8 DOSE FORMULATION PREPARATION AND ANALYSIS

8.1 Dose Formulation Preparation

Refer to SOP-CPL-0004

A correction for potency will be needed, as the concentrations in this assay will be based on a theoretical potency of 93.57%.

For Coelenterazine dose formulations, which are clear solutions or suspensions, they will be prepared separately, immediately prior to use by mixing a weighed amount of Coelenterazine with a measured volume of the solvent, and then diluting with solvent. Dose formulations will be prepared under yellow light, and stored at room temperature in tightly closed sterile brown containers. Vortex mixing, sonication and heating to $37\pm2^\circ$C may be employed to aid in solubilization. Dose formulations can be exposed to ambient temperature during transport. Details about dose formulation preparation will be described and recorded in dose formulation instruction and preparation sheet.

8.2 Dose Formulation Samples Collection

Dose formulations for the definitive chromosome aberration assay will be analyzed. Dose formulations for the dose range-finding assay will not be analyzed. No dose formulation analysis will be performed on the positive control article dose formulations.

Upon preparation for use in definitive chromosome aberration assay, samples will be collected from the solvent and all Coelenterazine dose formulations for analysis.

♦ For dose formulations that are clear solutions, 2 sets of 1 sample (1 mL each) will be collected from the middle portion of the formulations for concentration analysis. One set will be used for analysis and the other will serve as the backup.

♦ For dose formulations that are suspensions, 2 sets of 1 sample (1 mL each) each from the top, middle, and bottom portions of the formulations will be collected. One set will be used for analysis and the other will serve as the backup.
For the solvent, 2 sets of samples (1 mL each) will be collected to confirm the absence of Coelenterazine. One set will be used for analysis and the other will serve as the backup.

The volume of samples taken may be adjusted per request of the Study Director. All samples collected for analysis will be stored at room temperature and backups will be stored at -20°C.

In case that the nominal concentrations of some doses are below the Limit of Quantification determined by the analytical method, only doses which are above the Limit of Quantification will be collected, analyzed and reported.

8.3 Dose Formulation Analysis

Refer to SOP-ACL-0010

All analytical work will be conducted by the Analytical Chemistry Laboratory using an analytical method developed and validated by WuXi AppTec (Suzhou).

8.3.1 Concentration Verification

Immediately after the dose formulations are prepared, samples will be collected and transported to the Analytical Chemistry Laboratory within the testing facility for concentration verification.

Aliquots will be collected from the middle portion of the collected dose formulation samples and analyzed for Coelenterazine concentration using a validated method.

8.3.2 Stability Assessment

For Coelenterazine dose formulation samples used for the high- and low-dose groups (above the Limit of Quantification), a second aliquot will be collected at the same time of collecting the first aliquot (for concentration verification). The second aliquot will be stored for 6 hours at room temperature and then analyzed to measure Coelenterazine concentration for evaluating Coelenterazine dose formulations’ stability up to 6 hours. The stability interval will start with the baseline which is defined as when the first analytical aliquot is taken.

8.3.3 Homogeneity Verification

If all the dose formulations in the definitive chromosome aberration assay are clear solutions, homogeneity will not be tested.

If some dose formulations are suspensions in the definitive chromosome aberration assay, homogeneity will be tested and the concentrations of the samples from the top, middle, and bottom portions will all be analyzed.
8.3.4 Acceptance Criteria for Dose Formulation Analysis

Measured concentrations will be compared to nominal concentrations to reach conclusions about the exposure of cells in the solvent control cultures to Coelenterazine, the accuracy of dose formulation preparation, and the stability of Coelenterazine in dose formulations.

The Coelenterazine concentration in the solvent formulation must be below the Limit of Quantification X Dilution Factor.

For clear solution formulations, measured Coelenterazine concentrations in dose formulation samples must be within 90% to 110% of their nominal concentrations.

For suspension formulations, measured Coelenterazine concentrations in dose formulation samples must be within 85% to 115% of their nominal concentrations.

The difference in % nominal concentration between the six hours’ stability time point and the baseline must be within +/-10%.

To reach conclusion of Coelenterazine homogeneity (if performed), measured Coelenterazine concentrations in dose formulation samples must be within 85% to 115% of their nominal concentrations with ≤10% relative standard deviation (RSD) of each concentration.

In the event that a sample is outside of the acceptable specification range, the Study Director will justify the acceptability of the results or suggest re-analysis of the backup samples.

8.4 Sample Disposition

All unused dose formulation samples will be discarded after dosing. The residual neat Coelenterazine used in the Analytical Chemistry Laboratory will be discarded after finalizing the study at the Study Director’s direction.

9 MATERIALS AND METHODS

Refer to SOP-GEN-0003, SOP-GEN-0007, SOP-GEN-0009 and SOP-GEN-0016

This study design is based on the OECD Guideline 473, updated, and adopted 21st July 1997.

9.1 Test System Rationale

Aberrations are a consequence of failure or mistakes in repair process such that breaks either do not rejoin or rejoin in abnormal configurations (Evans, 1962, 1976; Galloway et al., 1985, 1994).
Descriptions of the types of aberrations are provided in this protocol. Structural aberrations are of two types, chromosome, or chromatid:

- **Chromosome aberration**: structural chromosome damage expressed as breakage, or breakage followed by reunion, of both sister chromatids at an identical site.

- **Chromatid aberration**: structural chromosome damage expressed as breakage of single chromatids or breakage followed by reunion between chromatids. This is the most common type of structural aberration.

Most known chemical clastogens (chromosome-breaking agents) require a period of DNA synthesis to convert initial DNA damage into chromosome alterations that become visible at mitosis. At predetermined intervals after exposure to the test article, the CHO cells will be treated with a metaphase-arresting substance, Colcemid, then harvested, and stained, and metaphase cells will be analyzed microscopically for the presence of chromosomal aberrations.

Many mutagenic chemicals do not act directly on DNA but do so after being converted active intermediates by enzymes found in liver. CHO cells have only a limited capacity to metabolize some test article, so an exogenous metabolic activation system (Aroclor 1254 induced rat liver S9) will be included with a series of treatments to enhance the degree of conversion and the ability of the assay to detect clastogenic, metabolic intermediates.

Numerical aberrations will be determined by the occurrence of polyploidy or endoreduplication, which may indicate that the test article has the potential to induce numerical aberrations.

### 9.2 Test System

The Chinese hamster ovary cell line, CHO-WBL with a modal chromosome number of 21 and a population doubling time of 12-13 hours will be employed in this assay. CHO-WBL cells were originally obtained from Merck Research Laboratories, USA. The cell stocks are stored in liquid nitrogen. Every batch of the cell stocks will be checked for the stability of the modal chromosome number and will be tested and determined to be free from mycoplasma contamination. Cells will not be used after the 15th passage from cloning.

### 9.3 Media and Cell Culture Condition

Complete medium will be McCoy’s 5A medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 unit/mL penicillin, and 100 µg/mL streptomycin. Serum-free medium will be McCoy’s 5A medium, supplemented with 2 mM L-glutamine, 100 unit/mL penicillin, and 100 µg/mL streptomycin.

CHO-WBL cells will be cultured in a standard condition: a humidified atmosphere of 4%-8% CO₂ in air at 37°C±1°C.
9.4 Metabolic Activation System

The in vitro metabolic activation system (Maron and Ames, 1983) consists of a Aroclor 1254 induced rat liver post-mitochondrial homogenate (hence referred to as S9) and an energy-producing system (NADP plus isocitric acid). The S9 homogenate will be purchased commercially from Molecular Toxicology (Boone, NC) and stored frozen at -80°C until used. The S9 homogenate is prepared from male Sprague Dawley rats that have been given a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. According to the quality control and production certificate of S9 supplied by Molecular Toxicology, dilutions of the sample S9, ranging from 0.2% to 10% in the S9 mix, are tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates that are mutagenic in the Ames Reverse Mutation assay with Salmonella typhimurium tester strain TA100. Such documentation will be presented in final report.

Immediately prior to use, the S9 will be thawed and mixed with sterile cofactor solution. The components of S9 mixture are as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration in Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP (disodium salt)</td>
<td>0.8 mg/mL</td>
</tr>
<tr>
<td>Isocitric Acid (trisodium salt)</td>
<td>1.5 mg/mL</td>
</tr>
<tr>
<td>S9 Homogenate</td>
<td>15.0 µL/mL (1.5% v/v)</td>
</tr>
</tbody>
</table>

9.5 Identification of Test System

The treatment flasks will be identified by the study number, cell identification, test/control article identification, treatment series, dose level, and culture number.

9.6 Solubility and pH Tests

Coelenterazine may be unstable in dimethylsulfoxide (DMSO; CAS 67-68-5), so DMSO will not be selected as the vehicle.

Preliminary solubility tests were carried out with water (CAS 7732-18-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1) before the initiation of this study at WuXi AppTec (Suzhou). In the tests, Coelenterazine was insoluble in water or acetone, soluble in ethanol up to 5.1 mg/mL. Ethanol will be selected as the vehicle.

A solubility test will be carried out with ethanol to confirm the solubility information. The target concentration will be 423.56 mg/mL (1 M).

Test article stocks will be diluted 100-fold into culture medium to test for precipitate formation. The maximum final concentrations of vehicle in the culture will be, unless specified differently by the Sponsor, up to 1% for organic vehicle.
The pH of the medium containing highest doses will be checked using pH indicator paper. PH adjustment will be done with 1 to 5 N NaOH or HCl, if a pH change occurs after stocks are added to culture medium.

9.7 Dose Range-Finding Assay

In the dose range finding assay, a range of Coelenterazine dose levels covering about 2 to 4 orders of magnitude in a series of 2-fold to 3.16-fold series dilutions will be tested (about 10 dose levels) in single cultures for each exposure group. The cultures for solvent control will be in duplicate. The maximum dose level will be determined on a case by case basis. In the absence of any toxicity information, the highest dose level evaluated will be 5 mg/mL or 10 mM, whichever is lower, for freely soluble test article. However, if limited by solubility, the maximum dose level evaluated will be the highest dose able to be administered.

9.7.1 Preparation of Target Cells

Exponentially growing CHO-WBL cells will be seeded in complete medium for each treatment condition at approximately $1.2 \times 10^6$ cells/75 cm$^2$ flask with 10 mL medium. Two extra cultures will be set up for determination of baseline cell counts at the time of treatment (time zero count, Xo; see “Cell Growth”, below). The flasks will be incubated in a humidified atmosphere of 4%-8% CO$_2$ in air at 37°C±1°C for 20 to 24 hours.

9.7.2 Treatment of Test System

Frequency and route of administration: target cells will be treated for 3 and 20 hours in the non-activated test system and for 3 hours in the S9-activated test system, by incorporation of the test article-solvent mixture into the treatment medium. This technique has proven to be an effective method of detection of chemical clastogens in in vitro chromosome aberration assays (Evans, 1976).

All test article dosing will be performed at room temperature under yellow light.

Twenty to twenty-four hours after culture initiation, medium will be aspirated. The cultures will then be re-fed with an appropriate volume of serum-free medium containing metabolic activation mix for the S9-activated group, and an appropriate volume of complete medium for the non-activated groups. Treatment will be carried out by adding 100 µL of dose formulations of Coelenterazine in solvent or solvent alone to cultures. The final culture volume will be 10 mL.

Cultures will be incubated in a humidified atmosphere of 4%-8% CO$_2$ in air at 37°C±1°C. For the S9-activated 3-hour exposure group and non-activated 3-hour exposure group, the cells will be treated for 3 hours. For removal of Coelenterazine, the treatment medium will be aspirated. The cells will be
washed twice with Hank's buffered salt solution (HBSS), re-fed with complete medium, and cultured for an appropriate time until harvest, with Colcemid present for the last 2 hours. For the non-activated 20-hour exposure group, the cells will be treated for 20 hours with Colcemid present for the last 2 hours.

A baseline cell count will be taken from the two parallel control cultures within 30 minutes of the beginning of treatment.

### 9.7.3 Cell Harvest

Cells will be collected approximately 20 hours after initiation of treatment. This post-treatment harvest time represents approximately 1.5 normal cell cycles and is selected to ensure that the cells are analyzed in the first division metaphase after the initiation of treatment. Two hours prior to cell harvest, 100 µL Colcemid (10 µg/mL) will be added to the cultures to give a final concentration of 0.1 µg/mL.

Cells will be harvested by trypsinization, and an aliquot will be removed for counting using automatic cell analysis system and trypan blue dye exclusion. The viable cell counts will be used to determine cell growth inhibition relative to the solvent control (cytotoxicity).

### 9.7.4 Cell Growth (Population Doubling) and Cytotoxicity

Cell growth will be expressed as the number of population doublings (PD) between the beginning of treatment and the time of harvest (Galloway et al. 2004). The number of PD in the treated cultures will be expressed as a percentage of the number of PD in the relevant solvent controls.

Population doublings (PD) = the log of the ratio of the final count (N) to the starting count (Xo), divided by the log of 2. That is:

\[
PD = \left[ \log (N + Xo) \right] / \log 2
\]

Where \( N \) = number of cells harvested (final number; the viable cell count adjusted after assessing trypan blue dye exclusion, if applicable).

\( Xo \) = starting cells before treatment, that is, the baseline count obtained from the parallel control cultures at approximately the time treatment began for the test cultures.

Relative PD (expressed in terms of percentage) for each treated culture will be calculated as follows:

\[
\text{Relative PD} \times 100 = \frac{\text{PD of treated cultures}}{\text{PD of solvent controls}}
\]

Cytotoxicity (%) will be expressed as (100-Relative PD).
9.7.5 Repetition of the Dose Range-finding Assay

In the event dose levels can not be selected for the definitive chromosome aberration assay according to the toxicity data, complete or part of the dose range-finding assay will be repeated and dose levels will be decreased at the discretion of the study director and sponsor.

9.8 Definitive Chromosome Aberration Assay

The definitive chromosome aberration assay will be conducted using 3-hour exposures with and without S9 groups, and a 20-hour exposure without S9 group only, using duplicate cultures for each test and solvent/positive control article concentration.

Whenever possible, the highest dose level to evaluate chromosome aberrations will be estimated to exceed 50% cytotoxicity (cell growth inhibition relative to the solvent control) irrespective of solubility but not to exceed 5mg/mL or 10 mM, whichever is lower. At least two additional lower dose levels will be included, with no more than 3.16-fold intervals between. In the event Coelenterazine cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, then the highest dose to be tested in the definitive chromosome aberration assay will be the concentration resulting in minimum precipitation in test medium. Precipitation will be determined by direct visual inspection. In the event Coelenterazine demonstrates a dose-responsive increase in cytotoxicity at concentrations that exceed solubility in treatment medium, then the highest dose to be tested will be the maximum concentration that results in exceeding 50% cytotoxicity. In the event that neither cytotoxicity nor insolubility is observed in the dose range-finding assay, the highest dose level in the definitive chromosome aberration assay will be 5 mg/mL or 10 mM, whichever is lower. The dose levels once determined for the definitive chromosome aberration assay will be documented in the raw data and presented in the report.

9.8.1 Preparation of Target Cells and Treatment of Test System

Preparation of target cells and treatment of test system will be performed as described in the dose range-finding assay. For positive controls treatment, 100 µl of solutions will be dosed to the cultures.

9.8.2 Collection of Metaphase Cells

Cells will be collected approximately 20 hours after the initiation of treatment. Two hours prior to cell harvest, 100 µL Colcemid (10 µg/mL) will be added to the cultures to give a final concentration of 0.1 µg/mL.

Cells will be harvested by trypsinization, and an aliquot will be removed for counting using automatic cell analysis system and trypan blue dye exclusion. The viable cell counts will be used to determine cell growth inhibition relative
to the solvent control (cytotoxicity). Cell counts will not be performed for the positive control cultures. The remainder of the cells will be swollen with 0.075M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and may be stored overnight or longer at approximately 2° to 8°C. To prepare slides, the cells will be collected by centrifugation and if necessary, the cells will be resuspended in fresh fixative. The suspension of fixed cells will be applied to glass microscope slides and air-dried. At least two slides will be prepared from each culture. The slides will be stained with Giemsa and permanently mounted.

9.8.3 Slide Scoring for Mitotic Index

To ensure that a sufficient number of metaphase cells are present on the slides, the percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined and recorded for each coded treatment group.

Mitotic index (MI) will be expressed as:

\[
MI = \frac{\text{The number of cells in metaphase observed}}{\text{The total number of cells observed}}
\]

Relative MI (RMI, expressed in terms of percentage) for each treated culture will be calculated as follows:

\[
RMI (%) = \frac{\text{MI of treated cultures}}{\text{MI of solvent controls}} \times 100
\]

Reduction of mitotic index (%) will be expressed as (100-RMI)

9.8.4 Cell Growth (Population Doubling) and Cytotoxicity

Cell growth and cytotoxicity will be determined as described in the dose range-finding assay.

9.8.5 Selection of Dose Levels for Chromosome Aberration Analysis

The selection of dose levels for analysis of chromosome aberrations in CHO cells will be based upon cytotoxicity of Coelenterazine. The highest dose level selected for evaluation will be the dose which induces at least but not greatly exceeding 50% cytotoxicity, as measured by cell growth inhibition, relative to the solvent control, with sufficient number of scorable metaphase cells. In treatment groups with lack of a dose level with close to 50% reduction in cell growth, selection of doses for microscopic analysis will be based on mitotic index (the lowest dose level with at least 50% reduction in mitotic index). At least two additional lower dose levels will be included in the evaluation.
If cytotoxicity is not applicable in the selection of dose levels for microscopic analysis, for example, there is very little or no cytotoxicity observed in all dose levels tested, the highest dose level selected for chromosome aberration analysis will be highest dose level tested.

9.8.6 Slide Scoring for Chromosome Aberration Analysis

Except those from the high doses of CP and MMC (positive control treatments), slides will be coded using randomly generated numbers by an individual not involved in slide scoring.

For scoring chromosome aberration, the slides will be oriented on the microscope stage with the frosted end up and on the left. Cells will be analyzed at high power (1000×) under oil immersion. The slide will be scanned methodically to locate good-quality metaphase cells. The metaphase cells will be selected according to the following criteria:

a. The staining intensity must be adequate to give sufficient contrast.

b. The spread should appear unbroken and should not overlap with other nuclei nor be partially obscured by debris.

c. Chromosome morphology should be good, with little or no overlap of individual chromosomes, and centromeres should not be splitting.

d. The centric chromosomes will be counted, and the cell will be rejected if the number of centromeres differs by more than two from the diploid number (ie, 21).

Whenever possible, a minimum of 200 metaphase cells from each dose level (100 per duplicate flask) will be examined and scored. If there are not enough metaphase spreads, as many spreads as possible will be scored and the reason for the lack of spreads will be recorded. Chromatid-type aberrations include chromatid breaks, chromatid deletion, and exchange figures such as triradial, quadriradial, complex rearrangement, and chromatid interchange. Chromosome-type aberrations include chromosome break, double minute fragment, and exchange figures such as dicentric, multi-centric chromosome and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells, and severely damaged cells (≥10 aberrations) will also be recorded. The nature of observed aberrations for each acceptable spread, the number of polyploid and endoreduplicated cells will be recorded. A record will be kept of the microscope stage locations (Vernier reading) for any metaphase cells with countable structural aberrations. Unless otherwise indicated, the slides will be discarded after the finalization of the report.
10 DATA

10.1 Data Presentation

In the final report, the following data will be provided for each dose: cell growth inhibition in dose range-finding assay; cell growth inhibition, induction of mitotic index, the number and types of aberrations found, total spread cells scored, the percentage of structurally and numerically damaged cells (percent aberrant cells) in the total population of cells examined, and the mean aberrations per 100 cells will be calculated and reported for each treatment group. Gaps will be presented in the data but will not be included in the total percentage of aberrations or aberrant cells.

10.2 Statistical Method

Fisher’s exact test will be used to identify significant differences, comparing aberrant cells frequency between treated group and solvent control group. The p value will be adjusted manually by a Bonferroni correction for multiple comparisons. A difference is considered significant when the p value obtained is less than or equal to 0.05, but this p value refers to the value before Bonferroni adjustment.

10.3 Evaluation of Study Validity

The percentage of cells with aberrations for the solvent control must be comparable with the historical control data for solvent control cultures.

The high dose positive control culture must yield a level of aberrant cells that is statistically significantly increased over the mean of the solvent controls (p≤0.05). A low-dose positive control culture is included in the coded set of slides scored for aberrations, to improve the objectivity of the scoring. A statistically significant increase in aberrations in the low dose culture is not required for an acceptable assay.

Typically, there must be a minimum of three scorable doses, ie, doses that yield at least 100 scorable metaphase spreads, and show no evidence of microbial contamination. However, in cases of excessive cytotoxicity, fewer than 3 doses may be scored.

10.4 Evaluation of Clastogenic Potential

10.4.1 Positive Result

The conclusion is usually based on the statistical analysis of the proportion of cells with aberrations in treated cultures as compared with the concurrent controls, but this is not the only parameter used. It is also taken into account
the types of chromosome aberrations, the occurrence of cells with multiple aberrations, and the historical control percentages of cells with aberrations. The tests with and without S9 and the different treatment lengths will be considered separately. The statistical method described above is used to analyze the data. The final evaluation of the test article is based upon scientific judgment.

A positive dose is a statistically significant increase. An assay is considered positive if there are two positive doses within a treatment group, without greatly exceeding a 50% reduction in growth. A single positive dose is considered equivocal until repeated in another assay. If any of the tests with S9 activation or without S9 is positive, the overall conclusion will be positive.

10.4.2 Negative Result

The test article is considered to be negative in the assay if it does not elicit a statistically significant increase in the frequency of cells with chromosome aberrations over concurrent solvent/negative control at any dose level.

When the percentages of cells with chromosome aberrations at all concentrations of test article are within the historical control range for negative and/or solvent control cultures, the test result is to be considered negative without further statistical analysis.

10.4.3 Equivocal Result

The test article is considered to induce an equivocal response if there is only one positive dose within a treatment type.

11 QUALITY ASSURANCE

The Quality Assurance Unit (QAU) at WuXi AppTec (Suzhou) Co., Ltd. will audit the protocol and final report, and it will inspect and/or audit the conduct of the study in accordance with its SOPs.

12 PROTOCOL AMENDMENTS AND DIVIATIONS

Changes to the protocol may be made as the study progresses; however, no changes to the protocol will be made without the consent of the Sponsor. If necessary, the Sponsor may authorize protocol amendments verbally or by telephone, facsimile, or other electronic means. Authorization for any change must be documented and followed by a protocol amendment signed and dated by the Study Director, facility management [and the Sponsor Representative, if required]. The amendment will clearly describe the change and the reason(s) for the change.

All protocol and/or SOP deviations will be documented in the study records as in accordance with Testing Facility SOPs. The Study Director will notify the Sponsor as soon as possible of deviations that could result in a significant impact on the study.
13 REPORTING

The final report will include the dates of study initiation and completion, the purpose, changes to, and deviations from the approved protocol, identification of the test system and description of the methods, all findings and study results, and the conclusions. An electronic audited draft report will be submitted to the Sponsor as a Word document for review.

If within 6 months of receiving the audited draft report the Sponsor has not requested revisions or communicated instructions to finalize, WuXi AppTec (Suzhou) Co., Ltd. will issue the final report (unless prior arrangements are made with the Sponsor). The final report will be provided to the Sponsor as a PDF copy.

14 RETENTION OF RECORDS

The raw data, study documentation generated at WuXi AppTec (Suzhou), the protocol and all amendments, and the original signed final report for this study will be archived in the GLP archives according to Test Facility SOPs for at least 1 year following report finalization. After one year or directed otherwise, the test facility will contact the Sponsor to determine further disposition in accordance with their SOPs. No materials will be discarded without the prior approval by the Sponsor.

15 REFERENCE

♦ OECD guideline for testing chemicals: In vitro mammalian chromosome aberration test. 473 adopted 1997.

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1 目的

本实验的目的是通过检测供试品肠腔素在加或不加外源性代谢活化系统（Aroclor 1254 诱导的大鼠肝脏 S9）条件下诱导中国仓鼠卵巢细胞（CHO）产生染色体畸变的能力，以评价其致畸变的潜力。

2 依从法规

剂量探索实验部分是本次染色体畸变的预实验，不属于 GLP 范畴，其实施将格遵照实验方案和公司相关标准操作规程的规定。

染色体畸变实验主实验部分的实施将严格遵照实验方案、苏州药明康德公司相关标准操作规程以及下面所列的良好实验室规范准则 (GLP) 的最新版本。实验方案中的一些 GLP 词汇（如实验方案、供试品、对照品）是基于美国 FDA 良好实验室规范准则的描述，可以认为与 OECD 良好实验室规范中所使用的相应词汇是一致的。

◆ 经济合作与发展组织 (OECD) 良好实验室规范准则，1997 年修订。
◆ 美国 FDA 非临床研究质量管理规范 (美国联邦法规第 21 条，58 部分，1979 年 6 月 20 日生效，及所有补充修正案)。
◆ 中国 SFDA《药物非临床研究质量管理规范》（GLP），2003 年 9 月 1 日生效。

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<th>主要人员</th>
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</table>
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预期实验计划

预期实验开始日期：  2011-09-13
预期实验结束日期：  2011-10-28
预期实验报告草案完成日期：  2011-11-11

供试品和对照品

委托单位有责任向研究单位提供有关于供试品 理化性质的文件材料，如质检报告（COA）或同等文件，以供专题负责人参考。如果在总结报告定案之前没有获得关于供试品相关理化性质的数据和/或制剂分析的结果，那么应该在法规依从性声明中指出这些例外情况。

阳性对照物和用于配制供试品制剂的溶剂对照将由供应商的 COA 描述，这些 COA 都保存在苏州药明康德公司。最终实验报告中将包含溶剂的 COA 复印件。

7.1  供试品描述

名称：  肠腔素
代码：  CTZ
CAS 编号：  55779-48-1
制造商：  天津药明康德新药开发有限公司
批号：  TH 03647-031
纯度：  93.57%
分子量：  423.46
保存条件：  储存在-20℃ 条件，避光
理化性质：黄色粉末
失效日期：2021年6月

7.2 溶剂/阴性对照品描述

溶剂对照品为乙醇，浓度为95%。在每个暴露处理组中都将分别设立溶剂对照，给药体积与供试品所用给药体积一致。

因为乙醇在本实验中是不典型的溶剂，将设立空白对照以确定溶媒对细胞生长或染色体畸变没有不利影响。

7.3 阳性对照品描述

环磷酰胺一水合物[CP; CAS 6055-19-2]，需要代谢活化才具有致突变作用，在S9活化给药组中平行设立CP给药，给药剂量为1.5和5.0μg/mL。丝裂霉素C [MMC; CAS 50-07-7]，不需要代谢活化，本身即具有致突变作用，在非活化3小时给药组中平行设立MMC给药，给药剂量为0.17和0.5μg/mL；在非活化20小时给药组中平行设立MMC给药，给药剂量为0.05和0.25μg/mL。这些阳性对照品将事先在无菌去离子水中配制成100×储备液，保存在-20℃条件下，临用前融化。选用这些阳性对照品，是由于这些化合物有大量数据库可供参考，而且OECD指导原则473也推荐使用这些化合物。

只在主实验中使用阳性对照。从阳性对照所得的结果，只是用来验证实验系统的反应性，不与供试品作比较。

7.4 留样

参考SOP-CPL-0001

供试品中心实验室将取出1克供试品作为留样。

7.5 额外和处置

参考SOP-CPL-0001和SOP-GEN-0023

为供试品建立目录。如果委托单位未作其它指示，原料供试品在实验结束后将返还给委托单位。由苏州药明康德公司提供的对照品将按照研究机构相应的标准操作规程保管。
7.6 安全操作及预防措施

参考 SOP-CPL-0011

研究机构将使用标准的实验安全流程来操作供试品及对照品。当进行药物配制和给药时，应穿戴合适的个人防护设施。委托单位如果有，应以化学品安全说明书（MSDS）形式提供供试品的安全信息。

8 制剂配制及制剂分析

8.1 制剂配制

参考 SOP-CPL-0004

制剂配制时，供试品纯度以 93.57% 计算，需进行校正。

供试品制剂应现配现用，供试品制剂的澄清溶液和混浊液应分开配制，都是称取一定质量的供试品，加入一定体积的溶剂，得到最高浓度母液，较低剂量供试品制剂则通过稀释较高浓度制剂获得。在黄光灯条件配制供试品制剂，制剂配制后分装在无菌棕色容器中，盖紧后保存在室温下。配制制剂时可以通过涡旋、超声、以及 37±2℃ 水浴等方法促进供试品溶解。允许制剂在从配制场所转运到给药场所或制剂分析场所过程中暴露于环境温度。供试品制剂配制的详细步骤将在“供试品制剂配制指南及配制记录表”中进行描述并在每呈表记录配制过程。

8.2 制剂取样

用于染色体畸变实验室实验的制剂需要制剂分析，而用于剂量探索实验的制剂则不需要，阳性对照物亦无需进行制剂分析。

主实验中完成制剂配制后立即从溶剂和所有供试品制剂中取出一部分用于制剂分析。

◆ 如果供试品制剂为澄清的溶液，每个剂量取两套样品，每套样品 1 mL，取样点为制剂的中间层。其中一套用来制剂分析，另一套作为备份。

◆ 如果供试品制剂为浑悬液，每个剂量取两套样品，每套样品 1 mL，每套样品均由制剂的顶层、中间和底层取三个样品。其中一套用来分析，另一套作为备份。

◆ 溶剂同样取样两套，每套 1 mL，其中一套用来分析以确定溶剂中不含有供试品，另一套作为备份。

取样体积可根据专项负责人要求调整。用于制剂分析的样品放置在室温以待分析，而备份样品保存在-20℃。
8.3 制剂分析

参考 SOP-ACL-0010

采用苏州药明康德新药开发有限公司开发并验证过的分析方法，由分析化学实验室实施制剂分析。

8.3.1 浓度测定

制剂配好后立即取样并转运至公司内的分析化学实验室，用于浓度测定。

取送检样品的中间层，并用验证了的分析方法测定供试品的浓度。

8.3.2 稳定性评估

高剂量和低剂量（高于“定标线”的最低浓度）的送检样品同时采样两份，一份如上立刻进行供试品浓度分析，另一份室温放置 6 小时后，再次进行供试品浓分析。以首份样品进行的浓度分析为基线来评价供试品 6 小时的稳定性。

8.3.3 均一性确认

供试品制剂如果都是澄清溶液，则不需进行均一性分析。

供试品制剂若含有混悬液，则需要进行均一性分析。通过分析比较每个送检样品的上层、中间层和下层部分的浓度而评价供试品制剂的均一性。

8.3.4 制剂分析数据的可接受标准

比较测定浓度与标识浓度以判定溶剂对照组培养基中的细胞暴露于供试品的水平、制剂配制的准确度和制剂中供试品的稳定度。

溶剂中供试品的浓度值必须低于“定标线”乘以“稀释因子”。

供试品制剂为澄清溶液的，送检品的浓度测出值必须是标识浓度的 90%到 110%。

供试品制剂为混悬液的，送检品的浓度测出值必须是标识浓度的 85%到 115%。
供试品制剂在 6 小时稳定性测试点所测浓度和基线时浓度之间的差异性百分比必须在±10%。

为了判定供试品均一性（若供试品制剂有混悬液），送检样品的浓度测出值必须是标识浓度的 85% 到 115%，并且每个样品浓度的相对标准差（RSD）必须≤10%。

如果出现偏离上述标准范围的情况，将由专题负责人判断结果的接受度或者建议对备份样品进行重新测试。

8.4 样品处理

所有未用完的供试品制剂在给药后都将被废弃。少量用于制剂分析方法开发的供试品，在实验结束后经过专题负责人同意作为废弃物处理。

9 材料和方法

参考 SOP-GEN-0003、SOP-GEN-0007、SOP-GEN-0009 和 SOP-GEN-0016。

该实验设计基于 OECD 指导原则 473，1997 年 7 月 21 日修订生效。

9.1 实验系统基本原理


实验报告中将会提供详尽的畸变类型的描述。结构畸变包括两种类型，染色体型或染色单体型：

♦ 染色体型畸变：染色体结构损伤，表现为两条姐妹染色单体在同一位置发生断裂或断裂后重新联接。
♦ 染色单体型畸变：染色体结构损伤，表现为单条染色单体的断裂或断裂后染色单体之间重新联接。这种畸变类型最常见。

大多数化学断裂剂（也称为染色体断裂剂）经过一段时间进行 DNA 合成后，可将最初的 DNA 损伤转变为 DNA 的结构改变，并在有丝分裂期时可以直观地用显微镜进行观察。经供试品处理后一定时间，将 CHO 细胞用一种中期相阻断剂（秋水仙胺）处理，然后收获细胞并染色，在显微镜下分析中期相细胞是否存在染色体畸变。

许多致突变化合物不直接造成 DNA 损伤，但是经肝代谢酶活化后的中间产物可以造成损伤。CHO 细胞对供试品的代谢活化能力有限，所以在实验中需特意设立一系列给药组，于该组中添加外源性代谢活化系统（Aroclor...
1254 诱导的大鼠肝脏 S9），以强化化合物向其代谢中间产物的转化，从而增加实验检测中间代谢产物为断裂剂的化合物的能力。

染色体数量畸变将取决于多倍体和核内复制的发生，若实验中观察到多倍体和核内复制发生频率显著增加，则说明该供试品有产生数量畸变的潜力。

9.2 实验系统

本实验使用中国仓鼠卵巢细胞（CHO-WBL 细胞），核型具有 21 条染色体，生长周期为 12 到 13 小时。CHO-WBL 细胞最初由美国默克研究实验室赠送，后在本实验室扩增。细胞储备液储存在液氮中。每一批次的细胞储备液都鉴定了细胞核型，并经检测证明未被支原体污染。细胞单克隆后传代不得超过 15 次。

9.3 培养基和细胞生长条件

全培养基为 McCoy’s 5A 培养基，补充加入 10%胎牛血清、2 mM L-谷氨酰胺、100 unit/mL 青霉素和 100 µg/mL 链霉素。不含血清培养基为 McCoy’s 5A 培养基，补充加入 2 mM L-谷氨酰胺、100 unit/mL 青霉素和 100 µg/mL 链霉素。

CHO-WBL 细胞生长标准条件为：温度 37℃±1℃、CO₂浓度为 4%-8%的高湿度环境。

9.4 代谢活化系统

本实验中使用的代谢活化系统（Maron 和 Ames，1983）包含 Aroclor 1254 诱导的大鼠肝脏微粒体匀浆（简称 S9）和一个“能量产生系统”（NADP 二钠加异柠檬酸三钠）。S9 匀浆购自美国 Molecular Toxicology（Boone，NC）公司，使用前一直低温储存在 -80℃ 条件下。该 S9 匀浆的制备方法为：将 Aroclor 1254 单次腹腔注射雄性 Sprague Dawley 大鼠，剂量为 500 mg/kg，5 天后解剖取材。根据 Molecular Toxicology 公司提供的 S9 质检报告，该公司利用 Ames 细菌回复突变实验的沙门氏菌 TA100，测试了 S9 在 S9 混合物中浓度为 0.2%至 10%时，S9 活化苯并芘和 2-氨基蒽的能力。该质检报告将附在最终实验报告中。

临用前，将 S9 匀浆融化，并与配好的“能量产生系统”无菌溶液混匀。S9 混合物的组分如下：

--- Proprietary Information ---
1318 Wuzhong Avenue  •  Wuzhong District  •  Suzhou 215104  •  China  •  Tel: 86-512-6883-7321  •  Fax: 86-512-6883-7303
<table>
<thead>
<tr>
<th>组分</th>
<th>培养基中的终浓度</th>
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<tbody>
<tr>
<td>NADP（二钠盐）</td>
<td>0.8 mg/mL</td>
</tr>
<tr>
<td>异柠檬酸（三钠盐）</td>
<td>1.5 mg/mL</td>
</tr>
<tr>
<td>S9 匀浆</td>
<td>15.0 μL/mL (1.5% v/v)</td>
</tr>
</tbody>
</table>

### 9.5 实验系统标记

为了区分不同给药组细胞，细胞培养瓶上将标记以下信息：实验编号、细胞名称、供试品/对照品名称、给药处理系列、给药剂量和培养瓶编号。

### 9.6 溶解度和 pH 测试

肠腔素在二甲基亚砜（DMSO; CAS 67-68-5）中可能不稳定，因此不选择 DMSO 为溶媒。

本实验开始之前，苏州药明康德新药开发有限公司开展了肠腔素在水（CAS 7732-18-5）、乙醇（CAS 64-17-5）和丙酮（CAS 67-64-1）的溶解度预实验。在实验中，肠腔素不溶于水和丙酮，在乙醇中的溶解度可以达到 5.1 mg/mL。本实验中将选用乙醇作为溶媒。

正式实验中将以乙醇为溶媒做溶解度测试，以确认其溶解度信息。测试的目标浓度值为 423.56 mg/mL (1 M)。

供试品母液将以 100 倍稀释到培养基中，以测试沉淀形成情况。除非委托单位特意指出，溶媒在培养基中的 最大终浓度都不超过 1%（有机溶媒）。

使用 pH 试纸检测含有最高浓度供试品母液的培养基 pH 值。若母液加入到培养基中后发生 pH 值改变，可用 1 到 5 N NaOH 或 HCl 溶液调节。

### 9.7 剂量探索实验

在剂量探索实验中，每个处理系列中将设大约 10 个剂量水平，而剂量之间的间距为 2 到 3.16 倍之间。供试品每个剂量设一瓶细胞，而溶剂对照则设 2 瓶。最高剂量的选择将视情况而定。在没有毒性数据情况下，对于无溶解度限制的供试品，测试的最高剂量选择 5mg/mL 或 10 mM，取其较低者。然而，对于难溶的供试品，测试的最高剂量将是配制成可用于给药的最大浓度。

#### 9.7.1 接种细胞

将处于指数生长期的 CHO-WBL 细胞按照大约 1.2×10^6 个细胞 /10 mL 培养基接种于 75 cm² 培养瓶中。另外接种两瓶细胞用于测试在给
药时的基线细胞数（零点细胞计数，X0；见下文“细胞生长”）。接种好细胞后的培养瓶放置在温度 37°C±1°C、CO₂浓度为 4%-8%的高湿度环境培养 20 到 24 小时。

9.7.2 实验系统处置

给药频率和途径：在非代谢活化实验系统中，细胞给药处理 3 小时和 20 小时；而 S9 代谢活化实验系统中，细胞给药处理 3 小时；给药途径为将供试品与溶剂的混合物加入到细胞培养基中。这种技术已在许多体外染色体畸变实验中被证明是一种检测化学致突变剂的有效方法（Evans, 1976）。

所有供试品给药均应在室温及黄光条件下进行。

接种细胞 20 至 24 小时后，吸掉培养瓶中的培养基。然后向 S9 代谢活化处理系列中加入合适体积的含有代谢活化 S9 混合物的不含血清培养基，而向两个非代谢活化处理系列中加入合适体积的全培养基。给药时，向培养基中加入 100 µL 供试品制剂或溶剂。培养基的终体积为 10 mL。

将细胞培养瓶放回到温度 37°C±1°C、CO₂浓度为 4%-8%的高湿度环境中。对于 S9 代谢活化 3 小时给药系列和非活化 3 小时给药系列，细胞接触供试品的时间均为 3 小时。为了防止供试品，先将瓶中含有供试品的培养基吸掉，然后用 Hank's 缓冲液 (HBSS) 洗细胞 2 次，加入全培养基后接着培养直至收获，在收获细胞前 2 小时，将向培养瓶中加入秋水仙胺以聚集中期相细胞。对于非活化 20 小时给药系列，细胞接触供试品的时间为 20 小时，在收获细胞前 2 小时，将向培养瓶中加入秋水仙胺以聚集中期相细胞。

在给药后 30 分钟内，计数分外接种的两瓶细胞的基线细胞数。

9.7.3 收获细胞

给药后大约 20 小时收获细胞。该时间段大约是 CHO 细胞生长周期的 1.5 倍，选择该时间段的目的是为了确保可以分析到给药后经历第一次分裂形成的不同期相细胞。收获细胞前两小时，将 100 µL 秋水仙胺（浓度为 10 µg/mL）加入到培养基中，秋水仙胺的终浓度为 0.1 µg/mL。

通过消化收获细胞，取其中一部分细胞悬液用于细胞计数，计数的方法为自动细胞计数仪加血细胞计数板台盼蓝染色判断活细胞率，自动细胞计数仪所得的细胞总数乘以台盼蓝染色所得的活细胞率即为活细胞数。而活细胞将被用来计算供试品相 对于溶剂对照对细胞生长的抑制（即细胞毒性）。
9.7.4 细胞生长(倍增)和细胞毒性

细胞生长表达为给药开始至收获细胞的这段时间内细胞倍增数目（PD）（Galloway, 2004）。计算给药组细胞倍增数目与相应溶剂对照组细胞倍增数目的比值，以百分比形式表达。

\[
PD = \frac{\log (N + Xo)}{\log 2}
\]

其中，N=收获细胞时的细胞数（最终数目；经台盼蓝染色检查活细胞率后所得的活细胞数）。

Xo=给药时的起始细胞数，即给药时计数分外接种的两瓶细胞的基数细胞数。

每一给药组的相对PD(RPD，以百分比形式表示)按下式计算:

\[
RPD(\%) = \frac{\text{供试品给药组PD}}{\text{溶剂对照组PD}} \times 100
\]

细胞毒性（%）表达为（100-RPD）。

9.7.5 剂量探索实验的重复

若实验中未找到无毒剂量，经委托单位同意，专题负责人可以重新设计剂量，部分或全部重做剂量探索实验。

9.8 染色体畸变实验主实验

染色体畸变实验主实验将设立以下3个处理系列：S9代谢活化给药3小时系列（3 hours+S9）、非代谢活化给药3小时系列（3 hours－S9）和非代谢活化给药20小时系列（20 hours－S9）。每一供试品/溶剂对照/阳性对照组均包测试2瓶细胞。

根据剂量探索实验的毒性数据，如果供试品溶解度不是限制因素，主实验中的最高剂量应诱导出大于50%的细胞毒性（与相应溶剂对照组相比的细胞生长抑制）且不超过5mg/mL或10mM，取其较低者。主实验还需包含至少2个低剂量，剂量之间的间距应小于3.16倍。若由于供试品溶解度太低而没有一个足够高的剂量诱导出细胞毒性，那么主实验中的最高剂量为可以在培养基中产生少量沉淀的剂量，沉淀靠肉眼观察判断：若供试品的细胞毒性在不超过30%后仍不能诱导出现细胞毒性，那么主实验中的最高剂量为诱导出大于50%细胞毒性的剂量，可以含有多个产生沉淀的浓度；若在剂量探索实验中还没有发现细胞毒性也没有溶解度限制，则主实验中的最高剂量为5mg/mL或10mM，取其较低者。主实验剂量一旦选定，将记录在原始数据里，并在实验报告中陈述。
9.8.1 接种细胞及实验系统处置

接种细胞及实验系统处置将参照剂量探索实验中的描述操作。阳性对照的给药体积为 100 µL。

9.8.2 中期相细胞的收集

给药后大约 20 小时收获细胞。收获细胞前两小时，将 100 µL 秋水仙胺（浓度为 10 µg/mL）加入到培养基中，秋水仙胺的终浓度为 0.1 µg/mL。

通过消化收获细胞，取其中一部分细胞悬液用于细胞计数，计数方法参照剂量探索实验中的描述。活细胞将被用来计算供试品相对于溶剂对照对细胞生长的抑制（即细胞毒性），而阳性对照组不需要做细胞计数。余下的细胞用 0.075M KCl 溶液低渗处理，用固定液（甲醇： 醋酸，3:1 v/v）洗 2 次，盖上盖保存在 2°至 8°C 条件下过夜或更长时间。制片前，离心细胞悬液，可以再次用固定液洗一次细胞并将细胞重悬在固定液中。将固定好的细胞悬液滴到载玻片上并晾干。每一瓶细胞至少做 2 张片子。将载玻片用吉姆萨染液染色，封片。

9.8.3 有丝分裂指数的计数

为了确保载玻片上有足够数目的中期相细胞，将计数有丝分裂指数。每张载玻片上计数至少 500 个细胞，记录处于有丝分裂期细胞的比例。

有丝分裂指数（MI）计算方法如下：

\[
MI = \frac{\text{观察到的中期相细胞数目}}{\text{观察到细胞总数}}
\]

每一给药组的相对 MI（RMI，以百分比形式表示）按照下式计算：

\[
RMI (%) = \frac{\text{给药处理组 MI}}{\text{溶剂对照组 MI}} \times 100
\]

有丝分裂减少（%）表达为（100-RMI）

9.8.4 细胞生长(倍增)和细胞毒性

细胞生长和细胞毒性按照剂量探索实验中所述方法计算。
9.8.5 选择染色体畸变分析的剂量

CHO 细胞染色体畸变分析剂量的选择取决于供试品的细胞毒性。选择用于评估的最高剂量应诱导至少但不超过 50% 多的细胞毒性，细胞毒性高与溶剂对照相比细胞生长抑制来判断，但是应保证具有足够数目的中期相细胞。若没有接近 50% 细胞生长抑制的剂量，选择染色体畸变分析的剂量将取决于有丝分裂指数（选择的最高剂量为与溶剂对照组相比，有丝分裂指数减少至少 50%）。至少还应评估 2 个更低的剂量组。

若细胞毒性不适用于选择染色体畸变分析的剂量，例如所有测试的剂量组都没有细胞毒性，则测试的最高剂量将作为染色体畸变分析的最高剂量。

9.8.6 染色体畸变分析读片

除了 CP 和 MMC（阳性对照）高剂量组，所有载玻片均应用随机生成的盲码标注，以减少读片的主观性，盲码应由不参与读片的人员制作。

读片时，将载玻片平放在显微镜物台上，使毛边端在左边且向上。在油镜（1000×）下分析中期相细胞。调整载玻片位置寻找质量好的中期相细胞。选择中期相细胞的标准如下：

a. 染色充分，便于观察。

b. 中期相没有破裂，与其他细胞核没有重叠，也没有被碎片覆盖。

c. 染色体形态良好，单条染色体很少重叠，且着丝粒没有裂开。

d. 计数着丝粒数。若着丝粒数目与标准数目（即 21）相差超过 2，则不分析该中期相细胞。

每一剂量组分析至少 200 个中期相细胞（每瓶 100 个）。若没有足够的中期相细胞，则分析尽可能多的中期相细胞，并记录中期相细胞缺少的原因。染色单体型畸变包括染色单体断裂、染色单体缺失和染色单体互换，例如三射体、四射体、复杂重排和染色单体交换。染色体型畸变包括染色体断裂、双微体断片和交换，例如双着丝粒染色体、多着丝粒染色体和环。若中期相细胞中未发现染色体交换，却出现了断片（染色单体或无着丝粒断片），应记作断裂（染色单体型或染色体型）。而在中期相细胞中同时观察到染色体交换和断片时，断片不应记作畸变而是被认作染色体交换不完全的产物。粉碎染色体、粉碎细胞和严重损伤细胞（≥10 条染色体畸变）也应做记录。记录观察到的畸变的类型以及多倍体和核内复制的数目。记录具有染色体结构畸变的中期相细胞在显微镜上的坐标。除非有其它指示，实验结束后载玻片将被废弃。
10 数据

10.1 数据陈述

实验报告中将为每一剂量组提供以下数据：剂量探索实验的细胞生长抑制，染色体畸变实验主实验中的细胞生长抑制、有丝分裂指数减少、染色体结构畸变的数目和类型、计数中期相细胞总数、计数细胞中结构畸变和数量畸变细胞百分比和 100 个细胞中畸变染色体的平均数，裂隙会在数据中陈述但不包含在畸变染色体百分比或畸变细胞百分比的计算中。

10.2 统计学分析

将使用 Fisher’s 确切概率法比较给药组与溶剂对照组畸变细胞发生频率差异的显著性。作多重比较时，P 值将经过 Bonferroni 调整。当 P 值小于等于 0.05 时，可认为差异具有显著性，这里的 P 值指的是未经 Bonferroni 调整前的 P 值。

10.3 实验有效性

溶剂对照组的畸变细胞百分比应与历史数据具有可比性。

高剂量阳性对照组的畸变细胞百分比与相应的溶剂对照组相比应显著提高（经统计分析后 \( p \leq 0.05 \)）。实验中设立了低剂量阳性对照组，且与供试品给药组载玻片放在一起盲码读片，这只是为了提高读片的客观性。不需要低剂量阳性对照组必需做出阳性结果。

一般至少要有三个可供读片的剂量，即每瓶细胞中至少有 100 个可读的中期相细胞。但是若供试品毒性太大，计数少于 3 个剂量也是可以接受的。

10.4 实验结果的评判标准

10.4.1 阳性结果

通常根据供试品给药组发生畸变细胞比例与相应溶剂对照组发生畸变细胞比例的统计分析结果来判断，但这也只是唯一的考量参数。也可以将一些生物学因素考虑在内，如畸变的类型、一个细胞中有多条染色体畸变的发生频率以及阴性对照畸变细胞频率的历史数据。实验的三个给药处理系列结果会分开考虑。应用上文所述的统计分析方法分析实验数据，对结果的最终评判应基于科学的判断。

畸变细胞比例统计学上显著增加且在阴性和/或溶剂对照历史数据范围之外，则可认为该剂量为阳性剂量。若在同一个给药处理系列中有两
个阳性剂量，且这两个剂量诱导的细胞毒性都不应超过 50% 太少。若只出现一个阳性剂量，只可判断为可疑阳性，若该阳性剂量结果可以在另一次实验中重复，则可以判断为阳性。只要三个给药处理系列中任一个是阳性，则实验整体上即可判断为阳性。

10.4.2 阴性结果

如果任一剂量组的畸变细胞频率与阴性/溶剂对照组相比都没有显著增加，则可认为该供试品为阴性。

当所有供试品畸变细胞百分比都在阴性和/或溶剂对照历史数据范围内时，这个系列可以不进行统计学分析即判断为阴性。

10.4.3 可疑结果

如果在某一给药处理系列中只出现一个阳性剂量，可认为供试品诱导了可疑阳性反应。

11 质量保证

苏州药明康德新药开发有限公司的质量保证部（QAU）将根据其标准操作规程的要求对实验方案、总结报告进行审查，并将对实验施行进行检查和/或审查。

12 实验方案修正案与偏离记录

随着实验进展，可能会对实验方案进行修改，然而对实验方案的修改必须征得委托单位的同意。若需要，委托单位可通过口头、电话、传真或其它电子途径授权批准实验方案修正案。对实验方案修正的授权必须记录且专题负责人和机构管理层应在实验方案修正案上签字签字日期（若要求，委托单位代表也应在实验方案修正案上签字签字日期）。修正案中应清除描述修改之处以及修改的原因。

发生的所有实验方案偏离和/或标准操作规程（SOP）偏离都应根据研究单位的标准操作规程进行记录。如果发生严重影响实验结果的偏离，实验专题负责人应及时通知委托单位。

13 总结报告

总结报告将包含但不仅限于以下内容：实验研究的起止日期、实验目的、实验方案及修正案、所有实验方案偏离、实验系统的鉴定、实验方法、实验结果以及结论等。质量保证部门审查过的总结报告草案将以 Word 版本提交给委托单位复审。
如果委托单位收到总结报告草案 6 个月内，没有提出修改意见或者没有提出关于总结报告定案的指示，苏州药明康德 新药开发有限公司将定案总结报告（除非与委托单位预先有其它约定）。定案的总结报告将以 PDF 版本提交给委托单位。

14 实验记录和记录归档

原始数据、相关文件、实验方案及其修正案，以及总结报告原始版将根据苏州药明康德新药开发有限公司的标准操作规程在总结报告签署当日归档保存，时限至少 1 年。1年后，苏州药明康德新药开发有限公司将根据 SOP 的要求与委托单位沟通这些原始记录是否继续保存。没有委托单位的同意，任何材料均不可丢弃。

15 参考文献

♦ OECD guideline for testing chemicals: In vitro mammalian chromosome aberration test. 473 adopted 1997.
17 PROTOCOL APPROVAL SIGNATURES 方案批准签名

I have read WuXi AppTec's protocol: Coelenterazine: In Vitro Chromosomal Aberration Assay in Chinese Hamster Ovary Cells and I approve the test methods described.

我已阅读苏州药明康德新药开发有限公司的实验方案，同意上述实验方案。

Zhewen Hu, MS 胡哲文，硕士
Genetic Toxicology 遗传毒理
(Study Director) (专题负责人)

Date 日期 2011-09-13

Bruce Bryant, MD
CEO Prolume/Majolight
(Sponsor representative) (委托方代表)

Date 日期 9 Sep 2011

Ying Jiang, PhD 姜颖，博士
Executive Director, Laboratory Science
(实验科学部执行主任)
WuXi AppTec (Suzhou) Co., Ltd.
苏州药明康德新药开发有限公司
(Facility Management 机构管理层)

Date 日期 2011-09-13

Robert J Coldrick, BS, 学士 RQAP-GLP
Vice President, GLP QA 副总裁，GLP QA
QA Acknowledgement Signature /确认签名
(SFDA GLP Chapter 6 Article 27)
(SFDA GLP 第 6 章第 27 条)

Date 日期 2011-09-13
PROTOCOL AMENDMENT NO. 01

STUDY TITLE: Coelenterazine: In Vitro Chromosomal Aberration Assay in Chinese Hamster Ovary Cells

STUDY NO.: 139-0005

SPONSOR STUDY NO.: NA

TESTING FACILITY: WuXi AppTec (Suzhou) Co., Ltd.
1318 Wuzhong Avenue, Wuzhong District
Suzhou 215104, China

SPONSORS:
Prolume Ltd. POB 2746, Pinetop, AZ 85935
Office Tel: 1-928-367-1200
www.prolume.com

and

BioLume Inc. 6325 Old Mill Farm Drive, Wendell, NC 27591
Office Tel: 1-919-824-9299
www.biolume.net
1 SECTION TO BE AMENDED

7.1 Test Article Description

Original: Supply

Replaced With: Manufacturer

Reason: Correction for wording error

2 SECTION TO BE AMENDED

7.2 Solvent/Negative Control Description

Add:

Identity: Ethanol

CAS Number: 64-17-5

Manufacturer: Sinopharm Chemical Reagent Co., Ltd.

Batch Number: 20100506

Concentration: 95%

Storage Conditions: Room temperature

Physical Characterization: Colorless liquid

Expiration Date: 2013-09-01

Reason:

Add descriptions of solvent control article according to the Good Laboratory Practice for Non-clinical Laboratory Studies, issued by State Food and Drug Administration, P.R. China. Effective September 01, 2003.
3 SECTION TO BE AMENDED

8.2 Dose Formulation Samples Collection

Original:

♦ For dose formulations that are suspensions, 2 sets of 1 sample (1 mL each) each from the top, middle, and bottom portions of the formulations will be collected. One set will be used for analysis and the other will serve as the backup.

Replaced With/Add:

♦ For dose formulations that are suspensions, 2 sets of 1 sample (1 mL each) each from the top, middle, and bottom portions of the formulations will be collected. One set will be used for analysis and the other will serve as the backup. The middle portion of the dose formulations will be used to verify concentration and homogeneity. The top and bottom portions of the dose formulations will be used to verify homogeneity along with the middle portion of the dose formulation.

♦ To assess the stability of the test article, 2 extra sets of 1 sample (1 mL each) will be collected from the middle portion of the lowest and highest formulations for concentration analysis. One set will be used for analysis and the other will serve as the backup.

Reason:

Clarify the procedures for dose formulation samples collection

4 SECTION TO BE AMENDED

8.3 Dose Formulation Analysis

Original:

8.3.1 Concentration Verification

Immediately after the dose formulations are prepared, samples will be collected and transported to the Analytical Chemistry Laboratory within the testing facility for concentration verification.

Aliquots will be collected from the middle portion of the collected dose formulation samples and analyzed for Coelenterazine concentration using a validated method.

8.3.2 Stability Assessment

For Coelenterazine dose formulation samples used for the high- and low-dose groups (above the Limit of Quantification), a second aliquot will be collected at the same time of collecting the first aliquot (for concentration verification). The second aliquot will
be stored for 6 hours at room temperature and then analyzed to measure Coelenterazine concentration for evaluating Coelenterazine dose formulations’ stability up to 6 hours. The stability interval will start with the baseline which is defined as when the first analytical aliquot is taken.

8.3.3 Homogeneity Verification

If all the dose formulations in the definitive chromosome aberration assay are clear solutions, homogeneity will not be tested.

If some dose formulations are suspensions in the definitive chromosome aberration assay, homogeneity will be tested and the concentrations of the samples from the top, middle, and bottom portions will all be analyzed.

**Replaced With:**

8.3.1 Concentration Verification

Immediately after the dose formulations are prepared, samples will be collected and transported to the Analytical Chemistry Laboratory within the testing facility for concentration verification.

The collected dose formulation samples will be analyzed for Coelenterazine concentration using a validated method.

8.3.2 Stability Assessment

For Coelenterazine dose formulation samples used for the high- and low-dose groups (above the Limit of Quantification), one set of samples will be analyzed for concentration verification as described in 8.3.1. The extra set of samples will be stored for 6 hours longer at room temperature and then analyzed to measure Coelenterazine concentration for evaluating Coelenterazine dose formulations’ stability up to 6 hours. The stability interval will start with the baseline which is defined as when the first set of samples is analyzed.

8.3.3 Homogeneity Verification

For dose formulations that are suspensions, the concentrations of the samples from the top, middle, and bottom portions will all be analyzed to verify the homogeneity.

**Reason:**

Clarify the procedures for dose formulation samples collection
5 SECTION TO BE AMENDED

16 APPENDIX: TRANSLATED PROTOCOL

Original:

8.2 制剂取样

如果供试品制剂为混悬液，每个剂量取两套样品，每套样品 1 mL，每套样品均由制剂的顶层、中间和底层取三个样品。其中一套用来分析，另一套作为备份。

8.3 制剂分析

8.3.1 浓度测定

制剂配好后立即取样并转运至公司内的分析化学实验室，用于浓度测定。

取送检样品的中间层，并用验证了的分析方法测定供试品的浓度。

8.3.2 稳定性评估

高剂量和低剂量（高于“定标线”的最低浓度）的送检样品同时采样两份，一份如上立刻进行供试品浓度分析，另一份室温放置 6 小时后，再次进行供试品浓度分析。以首份样品进行的浓度分析为基线来评价供试品 6 小时的稳定性。

8.3.3 均一性确认

供试品制剂如果都是澄清溶液，则不需进行均一性分析。

供试品制剂若含有混悬液，则需要进行均一性分析。通过分析比较每个送检样品的上层、中间层和下层部分的浓度而评价供试品制剂的均一性。

Add/Replaced With:

7.2 溶剂/阴性对照品描述

名称： 乙醇
CAS 编号： 64-17-5
制造商： 国药集团化学试剂有限公司
批号： 20100506
浓度： 95%
保存条件： 室温
理化性质： 无色液体
失效日期： 2013-09-01
8.2 制剂取样

- 如果供试品制剂为混悬液，每个剂量取两套样品，每套样品 1 mL，每套样品均由制剂的顶层、中间和底层取三个样品。其中一套用来分析，另一套作为备份。制剂取样的中间层用来确认浓度和均一性。而制剂取样的顶层和底层则和制剂取样的中间层一起用于确认供试品均一性。
- 为了评估供试品的稳定性，从最高和最低浓度制剂的中间层分外取两套样品，每套样品 1mL，用于浓度分析其中一套用来分析，另一套作为备份。

8.3 制剂分析

8.3.1 浓度测定

制剂配好后立即取样并转运至公司内的分析化学实验室，用于浓度测定。

用验证了的分析方法测定供试品制剂取样浓度。

8.3.2 稳定性评估

高剂量和低剂量（高于“定标线”的最低浓度）的制剂样品，一套样品如 8.3.1 所述进行供试品浓度分析，分外的一套制剂样品将在室温下再放置 6 小时，然后进行供试品浓度分析，以评估供试品制剂 6 小时稳定性。以首套样品开始进行浓度分析为基础来评价供试品 6 小时的稳定性。

8.3.3 均一性确认

对于呈悬浊液的制剂，上层、中间层和下层部分的制剂样品都需要测定浓度以评价供试品制剂的均一性。

Reason:

澄清实验方案中文翻译的内容
APPROVED BY: 批准签名

I have read amendments to this protocol and I approve the test methods described.

我已阅读实验方案修正案，我同意上述描述的方法。

- 胡哲文

Zhewen Hu, MS 胡哲文，硕士
Genetic Toxicology 遗传毒理
(Study Director)（专题负责人）

Date 日期
2011-09-27

Bruce Bryan, MD
CEO Prolume/Nanolight
(Sponsor representative)（委托方代表）

Date 日期
2011-09-27

Ying Jiang, PhD 姜颖，博士
Executive Director, Laboratory Science
实验科学部执行主任
WuXi AppTec (Suzhou) Co., Ltd.
苏州药明康德新药开发有限公司
(Facility Management 机构管理层)

Date 日期
2011-09-27

Robert J Coldreck, BS, 学士 RQAP-GLP
Vice President, GLP QA 副总裁，GLP QA
QA Acknowledgement Signature /确认签名
(SFDA GLP Chapter 6 Article 29)
(SFDA GLP 第 6 章第 29 条)
hu_zhewen@wuxiapptec.com

From: Bruce Bryan [bryanmd@aol.com]
Sent: 2011年9月27日星期二 10:19
To: hu_zhewen@wuxiapptec.com
Cc: Bruce Bryan
Subject: Re: Protocol amendment

Dear Zhewen,

I have read the protocols and agree to proceed.

You hereby have my authorization.

Yours,

Bruce Bryan, MD

On Sep 26, 2011, at 6:48 PM, hu_zhewen@wuxiapptec.com wrote:

Dear Bruce

Enclosed please review the protocol amendments to chromosome aberration assay (139-0005) and in vivo micronucleus assay (139-0007). The dosing day for the chromosome aberration assay is this afternoon. Would you please approve the amendments by email if no further comments? Thank you.

Zhewen
PROTOCOL AMENDMENT NO. 02

STUDY TITLE: Coelenterazine: In Vitro Chromosomal Aberration Assay in Chinese Hamster Ovary Cells

实验名称： 肠腔素：中国仓鼠卵巢细胞外染色体畸变实验

STUDY NO.: 139-0005

实验编号：

SPONSOR STUDY NO.: NA

委托单位实验编号： 无

TESTING FACILITY: WuXi AppTec (Suzhou) Co., Ltd.
1318 Wuzhong Avenue, Wuzhong District
Suzhou 215104, China

研究机构： 苏州药明康德新药开发有限公司
中国江苏省苏州市吴中区吴中大道 1318 号，邮编：215104

SPONSORS: Prolume Ltd. POB 2746, Pinetop, AZ 85935
Office Tel: 1-928-367-1200
www.prolume.com

and 和

BioLume Inc. 6325 Old Mill Farm Drive, Wendell, NC 27591
Office Tel: 1-919-824-9299
www.biolume.net
1 SECTION TO BE AMENDED

9.8 Definitive Chromosome Aberration Assay

Add:

The definitive chromosome aberration assay will be repeated at dose levels of 20, 40, 60 and 70 µg/mL using 3-hour exposure with S9 group.

Reason:

An SOP deviation occurred in the definitive chromosome aberration assay that the incomplete McCoy’s 5A medium was placed on ice after mixing with the S9 mixture. And the incidence of polyploid and endoreduplicated cells (3.38% and 9.50%, respectively) in cultures with S9 was significantly higher when compared to the cultures without S9 (2.44% and 0.99%, respectively) in this study and historical control data (0-2.91% and 1.48-6.54%, respectively). It is not sure if this has impact on the outcome of the 3 hours with S9 series in the definitive chromosome aberration assay.

2 SECTION TO BE AMENDED

8.2 Dose Formulation Samples Collection

Add:

Only concentration will be verified for dose formulation analysis in the repeating assay.

Reason:

Dose levels in the repeating assay are in the range of dose levels used in the definitive chromosome aberration assay. Stability assessment and homogeneity verification are not necessary.

3 SECTION TO BE AMENDED

6 PROPOSED STUDY SCHEDULE

Original:

Proposed Experimental Initial Date: 2011-09-13
Proposed Experimental Completion Date: 2011-10-28
Proposed Audited Draft Report Date: 2011-11-11
Replaced With

Proposed Experimental Initial Date: 2011-09-13
Proposed Experimental Completion Date: 2011-11-04
Proposed Audited Draft Report Date: 2011-11-18

Reason:
Prolong the scheduling time due to repeating test.

4 SECTION TO BE AMENDED

16 APPENDIX: TRANSLATED PROTOCOL

9.8 染色体畸变实验主实验

Add:

染色体畸变实验主实验的 3 小时加 S9 系列组将在 20, 40, 60 和 70 μg/mL 剂量下重复。

8.2 制剂取样

Add:

在重复试验中，只需测定制剂的浓度。

6 预期实验计划

Original:

预期实验开始日期: 2011-09-13
预期实验结束日期: 2011-10-28
预期实验报告草案完成日期: 2011-11-11

Replaced with:

预期实验开始日期: 2011-09-13
预期实验结束日期: 2011-11-04
预期实验报告草案完成日期: 2011-11-18

Reason:
澄清实验方案中文翻译的内容
APPROVED BY:  批准签名

I have read amendments to this protocol and I approve the test methods described.

我已阅读实验方案修正案，同意上述描述的方法。

胡哲文
Zhewen Hu, MS
Genetic Toxicology遗传毒理
(Study Director)（专题负责人)

Bruce Bryan, MD
CEO Prolume/Nanolight
(Sponsor representative)（委托方代表）

Ying Jiang, PhD
Executive Director, Laboratory Science
实验科学部执行主任
WuXi AppTec (Suzhou) Co., Ltd.
苏州药明康德新药开发有限公司
(Facility Management)（机构管理层）

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Vice President, GLP QA 副总裁，GLP QA
QA Acknowledgement Signature 确认签名
(SFDA GLP Chapter 6 Article 29)
（SFDA GLP第6章第29条）

2011-10-26
Date

2011-10-26
Date
APPENDIX 4  QUALITY CONTROL & PRODUCTION CERTIFICATE OF S9

MOLTOX POST MITOCHONDRIAL SUPERNATANT (S-9) QUALITY CONTROL & PRODUCTION CERTIFICATE

<table>
<thead>
<tr>
<th>LOT NO.:</th>
<th>2753</th>
<th>SPECIES:</th>
<th>Rat</th>
<th>PREPARATION DATE:</th>
<th>April 12, 2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>VOLUME:</td>
<td>1 ml</td>
<td>SEX:</td>
<td>Male</td>
<td>BUFFER:</td>
<td>0.154 M KCl</td>
</tr>
<tr>
<td>TISSUE:</td>
<td>Liver</td>
<td>INDUCING AGENT(s):</td>
<td>Aroclor 1254 (Nemursato KI 615, 500 mg/kg i.p.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>REFERENCE:</td>
<td>Maron, D. &amp; Ames, B. Mutat Res 113:173, 1983</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

STORAGE: At or below -20°C

BIOCHEMISTRY:

- PROTEIN: 40 mg/ml Assayed according to the method of Lowry et al., JBC 193:265, 1951 using bovine serum albumin as the standard.
- ALKOXYRESORUFIN-O-DEALKYLASE ACTIVITIES

<table>
<thead>
<tr>
<th>Activity</th>
<th>P450</th>
<th>EROD</th>
<th>1A1, 1A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold - Induction</td>
<td>166.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BROD, &amp; methods</td>
<td>PROD</td>
<td>2B1, 2B2</td>
<td>28.1 MROD</td>
</tr>
<tr>
<td></td>
<td>PROD</td>
<td>2B1, 2B2</td>
<td>46.3</td>
</tr>
<tr>
<td>MROD</td>
<td>1A1, 1A2</td>
<td>101.5</td>
<td></td>
</tr>
</tbody>
</table>

Assays for ethoxyresorufin-O-deethylase (EROD), pentoxys-, benzyl-, and methoxyresorufin-O-dealkylases (PROD, MROD) were conducted using a modification of the methods of Burke, et al., Biochem Pharm 34:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SAs). Control SAs (pmoles/min/mg protein) were 50.7, 37.6, 94.1, & 20.4 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-ribose) media. Triplicate plates were read after 48-72 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. His+ revertants

<table>
<thead>
<tr>
<th>TA98</th>
<th>TA1535</th>
</tr>
</thead>
<tbody>
<tr>
<td>1552</td>
<td>560</td>
</tr>
</tbody>
</table>

The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., Mutat Res 129:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted as described by Maron & Ames, (Mutat Res 113:173, 1983).

µl S9 per plate/number His+ revertants per plate

<table>
<thead>
<tr>
<th>Promutagen</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP (5 µg)</td>
<td>146</td>
<td>386</td>
<td>694</td>
<td>835</td>
<td>1334</td>
<td>1341</td>
</tr>
<tr>
<td>2-AA (2.5 µg)</td>
<td>153</td>
<td>1114</td>
<td>1828</td>
<td>2161</td>
<td>1858</td>
<td>643</td>
</tr>
</tbody>
</table>

MOLTOX TOXICOLOGY, INC.
157 Industrial Park Dr.
Boone, NC 28607
(828) 264-9099
www.moltox.com

Approved: 04/14/11

Confidential
## APPENDIX 5  DEVIATION FORM

<table>
<thead>
<tr>
<th>Deviation Form</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment No.:</strong> 139-0005</td>
</tr>
<tr>
<td><strong>Study Day:</strong> D NA</td>
</tr>
<tr>
<td><strong>Date of Occurrence:</strong> 2011-09-27</td>
</tr>
<tr>
<td><strong>Deviation Type:</strong></td>
</tr>
<tr>
<td><strong>Deviation No.:</strong> SOP# SOP-GEN-0003-3.0</td>
</tr>
<tr>
<td><strong>Reason for Deviation:</strong> In section 9.5 of the SOP, it is requested that medium should be warmed (37±2°C) before adding to the culture. However the incomplete medium was placed on ice after mixing with the S9 mixer. Reason for deviation: the technician did not follow the SOP strictly.</td>
</tr>
<tr>
<td><strong>Corrective Action:</strong> The 3 hours with S9 series will be repeated.</td>
</tr>
</tbody>
</table>

**Impact**

<table>
<thead>
<tr>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>This deviation <strong>does</strong> not adversely affect the outcome of the study, because: The incidence of polyploid and endoreduplicated cells in cultures with S9 was significantly higher compared to the cultures without S9 and historical data.</td>
</tr>
<tr>
<td>This deviation <strong>should be</strong> not included in the final report.</td>
</tr>
<tr>
<td>This deviation <strong>should not be further investigated.</strong></td>
</tr>
</tbody>
</table>

**Signatures**

<table>
<thead>
<tr>
<th>Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>王松伟</td>
</tr>
<tr>
<td>日期/Date: 2011-10-24</td>
</tr>
</tbody>
</table>

**Copies to:**

| Facility Management: Ying Jiang |
| QA: Yingjie Yu |
| Study Director: Zhewen Hu |
| Study Coordinator: NA |
| Laboratory Supervisor: Ying Jiang |
| Lab Personnel: Jinjin Jiang |
| Deviation Tracking Personnel: Weilai Wang |
| Other: NA |

Effective date: 2011-09-19
APPENDIX 6  DOSE FORMULATION ANALYSIS REPORT
COELENTERAZINE: IN VITRO CHROMOSOMAL ABERRATION
ASSAY IN CHINESE HAMSTER OVARY CELLS
肠腔素：中国仓鼠卵巢细胞体外染色体畸变实验

Dose Formulation Analysis Report
制剂分析报告

WuXi AppTec Study Number: 139-0005
实验编号：139-0005
Sponsor Study Number: NA
委托单位实验编号：无

Author: Xiaosan Rong
制定者：荣小三
Document Status: Final
文件状态：最终版本
Release Date: 2012-01-11
释放日期：
SIGNATURES

Authored By/制定者：

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苏州药明康德新药开发有限公司

Formulation Analyst
Analytical Chemistry Laboratory
Laboratory Services

WuXi AppTec (Suzhou) Co., Ltd.
Suzhou, P.R. China

Reviewed By/审核者：

Ya Wang, BS/王亚，本科
主管
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苏州药明康德新药开发有限公司

Supervisor
Analytical Chemistry Laboratory
Laboratory Services

WuXi AppTec (Suzhou) Co., Ltd.
Suzhou, P.R. China

Approved By/批准者：

Millie Chen, PhD/陈秀芳，博士
高级主任
实验室服务部
苏州药明康德新药开发有限公司

Senior Director
Laboratory Services

WuXi AppTec (Suzhou) Co., Ltd.
Suzhou, P.R. China

Signature

Date

Signature

Date

Signature

Date
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1 KEY STUDY INFORMATION

Objective(s) of this report

This report contains the dose formulation assessment performed as part of the study (WuXi AppTec Study Number 139-0005).

<table>
<thead>
<tr>
<th>Test Item/Article</th>
<th>Coelenterazine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sample Collection Schedule for Dose Formulation Analysis

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Test Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coelenterazine (mg/mL)</td>
<td>Absence of test article</td>
</tr>
<tr>
<td>0 (Vehicle)</td>
<td>Day 1</td>
</tr>
<tr>
<td>0.5</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>1.5</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>NA</td>
</tr>
<tr>
<td>8</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>NA</td>
</tr>
</tbody>
</table>

The stability of Coelenterazine in 95% Ethanol at 0.5 mg/mL and 10 mg/mL was determined at room temperature conditions for 6 hours.
2 MATERIALS AND METHODS

2.1 Reference Standard(s) Used for Analytics

Reference Compound: Coelenterazine
CAS Number: 55779-48-1
Batch No.: TH03647-031
Purity: 93.57%
Molecular Weight: 423.46
Storage Conditions: Store at -20°C, protect from light
Supplier: WuXi AppTec (Tianjin) Co., Ltd
Description: Yellow powder
Expiration Date: June 2021

2.2 Sample Preparation

| Sample Solution Preparation | The entire content in the sample vial was accurately transferred into an appropriately sized volumetric flask; the vial was rinsed approximately 5-8 times with diluent; and the rinsate was transferred into the volumetric flask. An appropriate volume of diluent was added to the flask and mixed by manually shaking until a completely uniform solution was formed. The flask was then sonicated for about 2 minutes; allowed to equilibrate to room temperature; and then adjusted to final volume with the diluent and mixed well by inversion. The final concentration was approximately equal to the standard solution concentration, 0.005 mg/mL. (With the exception of control sample), as outlined in the following table.

Dilute each formulation sample only once.

<table>
<thead>
<tr>
<th>Nominal Formulation Sample Concentration (mg/mL)</th>
<th>Formulation Sample Volume (mL)</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Vehicle)</td>
<td>2.00</td>
<td>5</td>
</tr>
<tr>
<td>0.5</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>200</td>
</tr>
<tr>
<td>1.5</td>
<td>0.83</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>0.63</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>0.83</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>0.63</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>0.63</td>
<td>1000</td>
</tr>
<tr>
<td>7</td>
<td>0.90</td>
<td>1250</td>
</tr>
<tr>
<td>8</td>
<td>0.76</td>
<td>1250</td>
</tr>
<tr>
<td>10</td>
<td>1.00</td>
<td>2000</td>
</tr>
</tbody>
</table>

Standard Solution Preparation: Prepare 0.005 mg/mL of standard solution.
2.3 Method Parameters

Column: Agilent Eclipse XDB C18, 150 mm x 4.6 mm i.d., S-5 μm particle size
Wavelength: 215 nm
Column Temp.: 40°C
Flow Rate: 1.0 mL/min
Injection Volume: 10 μL
Wash Needle: Injection with needle wash; Flushport; Time: 2 sec
Auto-Sampler: Ambient
Mobile Phases:
- Mobile Phase A: 0.05% Trifluoroacetic Acid (TFA) in water
- Mobile Phase B: 0.05% Trifluoroacetic Acid (TFA) in ACN
Gradient:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>20</td>
</tr>
<tr>
<td>5.00</td>
<td>80</td>
</tr>
<tr>
<td>5.10</td>
<td>20</td>
</tr>
<tr>
<td>9.00</td>
<td>20</td>
</tr>
</tbody>
</table>
Run Time: 9.00 min
Retention Time (RT) of Coelenterazine: 4.48 min±10%
Response Factor (RF) of Coelenterazine: 29349.4±10%

2.4 Data Processing

Software for data acquisition and peak integration: Agilent ChemStation

3 RESULTS AND CONCLUSION

Dose formulation stability:

The 0.5 mg/mL and 10 mg/mL Coelenterazine Dose Formulations were all stable for 6 hours at Room Temperature.

Summaries of the Stability of Coelenterazine Dose Formulations at room temperature for 6 hours are presented in Table 3-1.

Concentration:
Day 1: The concentrations of dose formulations at 0.5 mg/mL to 6 mg/mL were within ±10% of nominal value, analysis results for 0.5 mg/mL to 6 mg/mL dose formulations were acceptable. The concentrations of dose formulations at 7 mg/mL to 10 mg/mL were within ±15% of nominal value, analysis results for 7 mg/mL to 10 mg/mL dose formulations were acceptable.

Day 1 (Repeat): The concentrations of dose formulations at 2 mg/mL, 4 mg/mL and 6 mg/mL were within ±10% of nominal value, analysis results for 2 mg/mL, 4 mg/mL and 6 mg/mL dose formulations were acceptable. The concentration of dose formulations at 7 mg/mL was within ±15% of nominal value, analysis results for 7 mg/mL dose formulations was acceptable.

The vehicle was tested and there was no detectable peak observed at the retention time of Coelenterazine on Day 1 and Day 1 (Repeat). The data were reported as <LOQ × DF (Dilution Factor).

Summaries of the Concentration of Coelenterazine Dose Formulations used during Day 1 are presented in Table 3-2. The Concentrations of Coelenterazine Repeat Dose Formulations are presented in Table 3-3.

**Homogeneity:** Homogeneities of 7 mg/mL, 8 mg/mL and 10 mg/mL dose formulations were confirmed (meeting the acceptance criteria).

Concentrations of the top, middle, and bottom portions were within 85% and 115% of nominal values for 7 mg/mL, 8 mg/mL and 10 mg/mL dose formulations on Day 1. The relative standard deviation (RSD) of top, middle, and bottom samples was ≤10%.

Summaries of the Homogeneity of Coelenterazine Dose Formulation used during Day 1 are presented in Table 3-2.

**Table 3-1 Stability of Coelenterazine Dose Formulation at Room Temperature for 6 Hours**

<table>
<thead>
<tr>
<th>Dose Level (mg/mL)</th>
<th>Time Point</th>
<th>Storage Condition</th>
<th>Conc. (mg/mL)</th>
<th>Accuracy* (%)</th>
<th>Difference* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Initial</td>
<td>Room Temperature</td>
<td>0.530</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>6 hours</td>
<td></td>
<td></td>
<td>0.499</td>
<td>100</td>
<td>-6</td>
</tr>
<tr>
<td>10</td>
<td>Initial</td>
<td>Room Temperature</td>
<td>9.853</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>6 hours</td>
<td></td>
<td></td>
<td>9.977</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

**Stability Conditions**

Room Temperature for 6 hours

**Acceptance Criteria**

For clear solution formulations, the results of the concentration verification are within the acceptance criteria: 90% to 110% nominal concentration. For suspension formulations, the results of the concentration verification are within the acceptance criteria: 85% to 115% nominal concentration.

The difference in %nominal concentration between stability time point and the baseline is within ±10%.
# Table 3-2  Concentration and Homogeneity of Coelenterazine in the Dose Formulations

<table>
<thead>
<tr>
<th>Occasion</th>
<th>Nominal Conc. (mg/mL)</th>
<th>Sampling Portion</th>
<th>Conc. (mg/mL)</th>
<th>Accuracy* (%)</th>
<th>RSD* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Mid</td>
<td>&lt;LOQ x DF (Dilution Factor)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>Mid</td>
<td>0.530</td>
<td>106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Mid</td>
<td>1.002</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>Mid</td>
<td>1.522</td>
<td>101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mid</td>
<td>1.998</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Mid</td>
<td>2.967</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Mid</td>
<td>3.915</td>
<td>98</td>
<td></td>
<td></td>
</tr>
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<td>5.008</td>
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<td></td>
</tr>
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<td>6</td>
<td>Mid</td>
<td>6.016</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Top</td>
<td>6.884</td>
<td>98</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Top</td>
<td>Mid</td>
<td>6.858</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottom</td>
<td></td>
<td>6.831</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Top</td>
<td>7.727</td>
<td>97</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Mid</td>
<td></td>
<td>7.771</td>
<td>97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottom</td>
<td></td>
<td>8.133</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Top</td>
<td>9.820</td>
<td>98</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Mid</td>
<td></td>
<td>9.853</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottom</td>
<td></td>
<td>9.908</td>
<td>99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Acceptance Criteria*

Accuracy: The concentration of the test item in the 0 mg/mL formulation must be lower than or equal to the Limit of Quantitation (LOQ) x Dilution Factor. For clear solution formulations, the concentration of test item in each dose formulation must fall within 90% to 110% of nominal. For suspension formulations, the concentration of test item in each dose formulation must fall within 85% to 115% of nominal.

Homogeneity: Concentrations from the top, middle, and bottom portions must fall within 85% to 115% of nominal, and with ≤10% difference in top, middle, and bottom samples.
Table 3-3  Concentration of Coelenterazine in the Repeat Dose Formulations

<table>
<thead>
<tr>
<th>Occasion</th>
<th>Nominal Conc. (mg/mL)</th>
<th>Sampling Portion</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Conc. (mg/mL)</td>
</tr>
<tr>
<td>Day1</td>
<td>0</td>
<td>Mid</td>
<td>&lt;LOQ × DF (Dilution Factor)</td>
</tr>
<tr>
<td>(Repeat)</td>
<td>2</td>
<td>Mid</td>
<td>1.891</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Mid</td>
<td>3.873</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Mid</td>
<td>6.086</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Mid</td>
<td>7.432</td>
</tr>
</tbody>
</table>

*Acceptance Criteria: Accuracy: For clear solution formulations, the concentration of test item in each dose formulation must fall within 90% to 110% of nominal. For suspension solution formulations, the concentration of test item in each dose formulation must fall within 85% to 115% of nominal.

4  ADMINISTRATIVE DETAILS

4.1 Facility and Study Personnel

Sponsor: Prolume Ltd. POB 2746, Pinetop, AZ 85935
Office Tel: 1-928-367-1200

and

BioLume Inc. 6325 Old Mill Farm Drive, Wendell, NC 27591
Office Tel: 1-919-824-9299

Test Facility: WuXi AppTec (Suzhou) Co., Ltd.
1318 Wuzhong Avenue, Wuzhong District
Suzhou, 215104, China

Study Director: Zhewen Hu, MS

WuXi AppTec (Suzhou) Co., Ltd.
1318 Wuzhong Avenue, Wuzhong District
Suzhou, 215104, China

Lab Management: Millie Chen, PhD

WuXi AppTec (Suzhou) Co., Ltd.
1318 Wuzhong Avenue, Wuzhong District
Suzhou, 215104, China

Formulation Analyst: Xiaosan Rong, BS

WuXi AppTec (Suzhou) Co., Ltd.
1318 Wuzhong Avenue, Wuzhong District
Suzhou, 215104, China
4.2 Analytical Details

Review of Analytical Data: All analytical data were reviewed for integrity and accuracy by the responsible laboratory personnel. Data transferred manually were crosschecked against the source data, which were part of the study raw data.

5 REFERENCES

Reference(s) to internal document(s)

Study Protocol: 139-0005 and Amendments
Analytical Method: AM-A110818-01-01

References to computer software

S1 Agilent Chemstation, version B, 04, 01.
S2 Microsoft Excel, version 2003 SP3, Microsoft Corporation, Redmond, WA, U.S.A.

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表 3-3 重复实验中的肠腔素制剂的分析浓度 ......................................................... 17
1 实验的主要信息

目的 该报告是实验 139-0005 的制剂评估部分

<table>
<thead>
<tr>
<th>测试项目/供试品</th>
<th>肠腔素</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

制剂分析计划表

<table>
<thead>
<tr>
<th>浓度 (mg/mL)</th>
<th>肠腔素</th>
<th>供试品的存在</th>
<th>均一性</th>
<th>浓度</th>
<th>稳定性</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (溶媒)</td>
<td>第一天</td>
<td>NA</td>
<td>NA</td>
<td>第一天和第一天(重复)</td>
<td>NA</td>
</tr>
<tr>
<td>0.5</td>
<td>NA</td>
<td>NA</td>
<td>第一天</td>
<td>第一天</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>第一天</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>NA</td>
<td>NA</td>
<td>第一天</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>NA</td>
<td>NA</td>
<td>第一天和第一天(重复)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>第一天</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>第一天和第一天(重复)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>NA</td>
<td>NA</td>
<td>第一天</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td>NA</td>
<td>第一天和第一天(重复)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>NA</td>
<td>第一天</td>
<td>第一天和第一天(重复)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>NA</td>
<td>第一天</td>
<td>第一天</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>NA</td>
<td>第一天</td>
<td>第一天</td>
<td>第一天</td>
<td></td>
</tr>
</tbody>
</table>

样品采集表

稳定性是指测试在 95%乙醇中的 0.5 mg/mL 和 10 mg/mL 肠腔素室温 6 小时的稳定性。
2 材料和方法

2.1 分析用的标准物质

<table>
<thead>
<tr>
<th>标准物</th>
<th>肾上腺素</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS 编号</td>
<td>55779-48-1</td>
</tr>
<tr>
<td>批号</td>
<td>TH 03647-031</td>
</tr>
<tr>
<td>纯度</td>
<td>93.57%</td>
</tr>
<tr>
<td>分子量</td>
<td>423.46</td>
</tr>
<tr>
<td>储存条件</td>
<td>避光，保存在-20℃</td>
</tr>
<tr>
<td>生产厂商</td>
<td>天津药明康德新药开发有限公司</td>
</tr>
<tr>
<td>性状</td>
<td>黄色粉末</td>
</tr>
<tr>
<td>失效日期</td>
<td>2021 年 6 月</td>
</tr>
</tbody>
</table>

2.2 样品配制

样品溶液的配制

将样品瓶中的制剂准确、完全地转移到相应的容量瓶中，然后用稀释液润洗样品瓶 5-8 次，同时需将润洗液转移到容量瓶中；加入适量的稀释液，手摇至样品混匀，超声 2 分钟，平衡到室温后，用稀释液定容，颠倒摇匀，最终浓度约等于标准溶液浓度，0.005 mg/mL（对照样品溶液除外），详见下表。

<table>
<thead>
<tr>
<th>制剂样品的标准浓度 (mg/mL)</th>
<th>取样体积 (mL)</th>
<th>稀释倍数</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (溶媒)</td>
<td>2.00</td>
<td>5</td>
</tr>
<tr>
<td>0.5</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>200</td>
</tr>
<tr>
<td>1.5</td>
<td>0.83</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>0.63</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>0.83</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>0.83</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>1.00</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>0.83</td>
<td>1000</td>
</tr>
<tr>
<td>7</td>
<td>0.90</td>
<td>1250</td>
</tr>
<tr>
<td>8</td>
<td>0.78</td>
<td>1250</td>
</tr>
<tr>
<td>10</td>
<td>1.00</td>
<td>2000</td>
</tr>
</tbody>
</table>

标准溶液的配制 配制 0.005 mg/mL 的标准溶液

定量限溶液的配制 配制 2%的标准溶液为定量限测试溶液，约等于 0.0001 mg/mL

稀释液 甲醇（HPLC 级）
2.3 方法的参数

色谱柱：Agilent Eclipse XDB C18, 150 mm × 4.6 mm i.d., S-5 μm particle size

吸光波长：215 nm

柱温：40℃

流速：1.0 mL/min

进样体积：10 μL

洗针：Injection with needle wash; Flushport; Time: 2 sec

自动进样器温度：室温

流动相：流动相 A: 0.05%三氟乙酸在纯水中
流动相 B: 0.05%三氟乙酸在乙腈中

梯度：

<table>
<thead>
<tr>
<th>时间 (min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>20</td>
</tr>
<tr>
<td>5.00</td>
<td>80</td>
</tr>
<tr>
<td>5.10</td>
<td>20</td>
</tr>
<tr>
<td>9.00</td>
<td>20</td>
</tr>
</tbody>
</table>

运行时间：9.00 min

肠腔素的保留时间：4.48 min ± 10%

肠腔素的响应因子：29349.4 ± 10%

2.4 数据处理

数据采集和峰积分所使用的软件：安捷伦化学工作站 (Chemstation)

3 结果和结论

制剂的稳定性：

0.5 mg/mL 和 10 mg/mL 的肠腔素制剂在室温 6 小时下是稳定的。

肠腔素制剂在室温 6 小时下的稳定性结果见表 3-1

制剂的测得浓度：

给药第一天：0.5 mg/mL 到 6 mg/mL 肠腔素制剂的浓度均在标识浓度的 90% 到 110% 之间，其结果均符合接受标准；7 mg/mL 和 10 mg/mL 肠腔素制剂的浓度均在标识浓度的 85% 到 115% 之间，其结果均符合接受标准。
给药第一天（重复）：2 mg/mL、4 mg/mL 和 6 mg/mL 肠腔素制剂的浓度均在标识浓度的 90% 到 110% 之间，其结果均符合接受标准。7 mg/mL 肠腔素制剂的浓度在标识浓度的 85% 到 115% 之间，其结果符合接受标准。

被分析的溶媒中没有发现肠腔素色素谱峰的出现，浓度被报告为小于定量限乘以稀释倍数（<LOQ × DF）。

肠腔素各个制剂浓度结果见表 3-2；重复的肠腔素各个制剂浓度结果见表 3-3

制剂的均一性：

7 mg/mL、8 mg/mL 和 10 mg/mL 肠腔素制剂的均一性被确认，均符合接受标准。

7 mg/mL、8 mg/mL 和 10 mg/mL 肠腔素制剂的上、中、下三层的浓度均在标识浓度的 85% 到 115% 之间，且相对标准偏差（RSD）均小于 10%。

7 mg/mL、8 mg/mL 和 10 mg/mL 肠腔素制剂均一性结果见表 3-2

### 表 3-1 肠腔素制剂在室温 6 小时的稳定性

<table>
<thead>
<tr>
<th>标识浓度 (mg/mL)</th>
<th>时间点</th>
<th>储存条件</th>
<th>测得浓度 (mg/mL)</th>
<th>准确度* (%)</th>
<th>差值* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>起始点</td>
<td></td>
<td>0.530</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>室温</td>
<td>0.499</td>
<td>100</td>
<td>-6</td>
</tr>
<tr>
<td>10</td>
<td>起始点</td>
<td></td>
<td>9.853</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>室温</td>
<td>9.977</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

- **稳定性条件**: 室温 6 小时

*接受标准

溶液样品的浓度应在标识浓度的 90% 到 110% 之间。溶液制剂样品的浓度应在标识浓度的 85% 到 115% 之间。

稳定性时间点的标识浓度百分比与起始点的标识浓度百分比的差值应在±10% 之内。
### 表 3-2 肠腔素制剂的分析浓度和均一性

<table>
<thead>
<tr>
<th>给药期间</th>
<th>标识浓度 (mg/mL)</th>
<th>取样位置</th>
<th>测得浓度 (mg/mL)</th>
<th>准确度* (％)</th>
<th>相对标准偏差* (％)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>中层</td>
<td>&lt;LOQ × DF (稀释倍数)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>中层</td>
<td>0.530</td>
<td>106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>中层</td>
<td>1.002</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>中层</td>
<td>1.522</td>
<td>101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>中层</td>
<td>1.998</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>中层</td>
<td>2.967</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>中层</td>
<td>3.915</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>中层</td>
<td>5.006</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>中层</td>
<td>6.016</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>上层</td>
<td>6.884</td>
<td>98</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>中层</td>
<td>6.858</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>下层</td>
<td>6.831</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>上层</td>
<td>7.727</td>
<td>97</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>中层</td>
<td>7.771</td>
<td>97</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>下层</td>
<td>8.133</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>上层</td>
<td>9.820</td>
<td>98</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>中层</td>
<td>9.853</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>下层</td>
<td>9.908</td>
<td>99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**接受标准**

准确度：0 mg/mL 的制剂浓度应不小于定量限乘以稀释倍数，溶剂样品的浓度应在标识浓度的 90％到 110％之间，溶剂样品的浓度应在标识浓度的 85％到 115％之间。

均一性：制剂的上、中、下三层的浓度应在标识浓度的 85％到 115％之间，且相对标准偏差 (RSD) 不得大于 10％。
表 3-3 重复实验中的肠腔素制剂的分析浓度

<table>
<thead>
<tr>
<th>给药期间</th>
<th>标识浓度 (mg/mL)</th>
<th>取样位置</th>
<th>结果</th>
<th>准确度* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>第一天 (重复)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>中层</td>
<td>&lt;LOQ × DF (稀释倍数)</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>中层</td>
<td>1.891</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>中层</td>
<td>3.873</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>中层</td>
<td>6.086</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>中层</td>
<td>7.432</td>
<td>106</td>
<td></td>
</tr>
</tbody>
</table>

*接受标准：准确度：0 mg/mL 的制剂浓度应不得大于定量限乘以稀释倍数，溶液样品的浓度应在标识浓度的 90% 到 110% 之间，悬浮液制剂样品的浓度应在标识浓度的 85% 到 115% 之间。

4 管理详细

4.1 设施和实验人员

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and 和

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实验室管理者：陈秀芳，博士
苏州药明康德新药开发有限公司
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制剂分析人员：荣小三，本科
苏州药明康德新药开发有限公司
中国苏州吴中区吴中大道 1318 号，邮编 215104
4.2 分析详细

分析数据的复核：所有分析数据都会被实验室相关人员完整、准确地复核。手动传输的数据和原始数据应交叉索引，同时手动传输的数据也作为实验原始数据的一部分。

5 参考文献

内部的参考文献
实验方案：139-0005 和其修正案
分析方法：AM-A110818-01-01

电脑软件参考文献
S1 安捷伦化学工作站，B.04.01 版本；
S2 微软 Excel，2003 SP3 版本；微软 Corporation, Redmond, WA, U.S.A.
## APPENDIX 7  HISTORICAL CONTROL VALUES

### Historical Negative and Positive Control Data of Structural Chromosome Aberration

<table>
<thead>
<tr>
<th></th>
<th>Negative Control (%)</th>
<th>Positive Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>3 hours without S9</td>
<td>1.9±0.6</td>
<td>1.0~2.5</td>
</tr>
<tr>
<td>20 hours without S9</td>
<td>2.1±1.0</td>
<td>1.0~3.0</td>
</tr>
<tr>
<td>3 hours with S9</td>
<td>2.1±0.8</td>
<td>1.5~3.0</td>
</tr>
</tbody>
</table>

Note:
The data were collected and calculated from 4 control (negative or positive) groups (duplicated cultures per group) in four studies: 100-0040, 100-0042, 100-0046, and 121-0004. The data shown was the frequency (%) of structural aberrant cells.
SD=Standard Deviation

### Historical Negative Control Data of Numerical Chromosome Aberration

<table>
<thead>
<tr>
<th></th>
<th>Cells with Polyploidy (%)</th>
<th>Cells with Endoreduplication (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>3 hours without S9</td>
<td>2.52±2.27</td>
<td>0~5.21</td>
</tr>
<tr>
<td>20 hours without S9</td>
<td>2.44±0.67</td>
<td>1.96~3.38</td>
</tr>
<tr>
<td>3 hours with S9</td>
<td>0.98±1.31</td>
<td>0~2.91</td>
</tr>
</tbody>
</table>

Note:
The data were collected and calculated from 4 negative control groups (duplicated cultures per group) in four studies: 100-0040, 100-0042, 100-0046, and 121-0004. The data shown was the frequency (%) of numerical aberrant cells.
SD=Standard Deviation