28-Day and 90-Day Repeated Dose Toxicity Test Using Mammals, Reverse Mutagenicity Test on Bacteria, Chromosomal Aberration Test Using Cultured Mammalian Cells and Mutagenicity Test by Mouse Lymphoma TK Assay

I.

This paper sets forth methods that should serve as standards for <28-Day Repeated Dose Toxicity Test Using Mammals, Reverse Mutagenicity Test on Bacteria, Chromosomal Aberration Test Using Cultured Mammalian Cells>.

II. 28-Day Repeated Dose Toxicity Test Using Mammals

Objective

The objective of this test is to demonstrate the toxicity of the test substance by observing the changes that emerge in bodily functions and forms when the test substance is repetitively administered on animals every day for a certain period of time.

1. **Test Animals**

   1-1. Animal types and sex

   In principle, male and female rats are used.

   1-2. Age

   Rats that are 5-6 weeks old[*1] with uniform weight are used. The allowable range of weight changes is within the respective average weight for males and females ± 20%.

   1-3. Number of animals

   Each group should have at least five male and female rats. If some rats are put down for examinations during the course of tests, the number of rats needed for examinations should be added from the beginning. For the observation of reversibility, persistence of changes as well as tardive toxicity by raising rats for at least 14 days upon completion of the administration of the test substance, satellite groups each consisting of at least five male and female rats are established for both control groups and high-dose groups.

2. **Test Substance**

2-1. Administration methods

In principle, the test substance is administered orally. When the test substance has features that do not allow oral administration, they are administered parenterally. In forced administration, the test substance is administered at regular hours every day.

2-2. Dosage

At least three levels of dosage should be set. The maximum dose is an amount that is proven to have the toxic impact of the test substance, while the minimum dose is an amount that is proven to have no toxic impact of the test substance throughout the test period. Furthermore, one or more dosage levels should be established between the maximum and minimum doses, with corresponding control groups.

The intake of the test substance when administered by mixing in feed or drinking water is calculated on the basis of the intake of feed or water and the concentration level of the test substance.

In case of forced oral administration, the maximum allowable dose of the test substance is set at 1,000mg/kg, and when the test substance are administered by adding it to feed or drinking water, the maximum allowable dose is at a level equivalent to the intake of 1,000mg/kg[*2] of test substance consumed via feed or drinking water. When no toxicity is observed at this level of dosage, tests using three levels of dosage are not necessary.

2-3. Period of administration

The test substance is administered for 28 days.

3. **Observation and examination**

At least once every day, the general condition of all animals should be observed and recorded, at the same time of day as much as possible and taking into account the peak time of expected effects after the administration of the test substance. In order to prevent the decline of the number of test animals due to cannibalism or self-destruction, animals should be observed at least twice a day for signs of illness or death. Furthermore, all animals should be observed in detail and observation results recorded once before the first administration of the test substance and at least once a week thereafter[*3, *4].

Functional examinations are conducted on all animals in the fourth week of the administration, and when some anomaly is observed, functional examinations should be conducted on all animals in satellite groups as well[*5].

Furthermore, examinations are conducted on the following items.

3-1. Fatality rate

3-2. Weight, feed intake and intake of water (when the test substance are added to drinking water)[*6]

3-3. Blood tests

3-3-1. Hematological test[*7]

3-3-2. Blood biochemical test[*8]

3-4. Urine test[*9]

3-5. Pathological test

3-5-1. Observation with the naked eye and internal organ weight[*10]

3-5-2. Histopathological test[*11]

3-6. Other necessary items

The cause of deaths of animals that died during the course of tests should be investigated as much as possible.

Furthermore, animals whose general condition deteriorated extremely and are about to die should be put down promptly for autopsy.

4. **Summary of test results**

The results of tests should be summarized using Form 4, with a final report attached. Furthermore, items, where applicable, should be evaluated with the use of appropriate statistical methods.

*[*1]: When animals 5-6 weeks old are not available, animals less than nine weeks old may be used.*

*[*2]: In the case of rats, the concentration level of the test substance in feed or drinking water that is equivalent to the dose of 1,000mg/kg may vary depending on the length of administration periods, but the tentative measure is set at 2%.*

*[*3]: Observations should be made in breeding rooms or standard places of observation outside cages with equivalent conditions. It is desirable that observation results are recorded using scoring systems clearly defined by testing organizations. Care should be taken to minimize the variation of test results. It is desirable that observations are*

conducted by observers who are not informed of the administration of the test substance.

*[*4]: Observations should check at least the condition of skin, fur, eyes, mucosa, secretions and excrement, the emergence of activities of the autonomic nerve system (salivation, dacryorrhea, piloerection, pupil widening, abnormal breathing, etc.), walking, posture and responses to handling, clonic and tonic convulsion, stereotypical behaviors (excessive grooming, repetitive circling movements, etc.), and abnormal behaviors (self-injurious behaviors, shying, etc.).*

*[*5]: In the observation in the fourth week of the administration of the test substance, measuring equipment should be used as necessary to measure kinetic responses to the stimulation of different kinds of senses (acoustic sense, sense of sight, proprioceptive sensation, etc.), grip strength and motor activity.*

*[*6]: They should be measured at least once a week.*

*[*7]: Examinations should be conducted on all animals (except for dying animals and animals that died during the course of tests). Hematological examinations should cover the following items: red blood cell count, hemoglobin concentration, hematocrit value, white blood cell count, white blood cell percentage, blood platelet count and other indicators of the blood's ability to clot. Indicators of the blood's ability to clot include blood-clotting time, prothrombin time and thromboplastin time.*
On top of these, examinations should be conducted on other items that may indicate the toxic impact given chemical structures of the test substance, such as reticulocyte count and methemoglobin.

*[*8]: Examinations should be conducted on all animals (except for dying animals and animals that died during the course of tests). One-night abrosia is desirable before blood samples are taken.*
Blood biochemistry tests should cover the following items: total protein, albumin, blood sugar, total cholesterol, urea nitrogen, creatinine, Na, K, Cl, at least two kinds of enzymes that may indicate effects on hepatic cells (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, sorbitol dehydrogenase, etc.).
On top of these, examinations should be conducted on other items that may indicate the

toxic impact given chemical structures of the test substance, such as cholinesterase, triglyceride, hormones, Ca, P, and total bilirubin.

*[*9]: When toxic effects are expected, or when the observation of the appearance of urine samples indicates, urine examinations should be conducted in the final week of tests using fresh urine or previously collected urine on the description, quantity, osmotic pressure or specific gravity, pH, albuminoid, sugar, occult blood and sediment.*

*[*10]: All animals used in the tests should be fully examined macroscopically, including the observation of the surface of the body, clear apertures, cranial cavity, chest cavity, abdominal cavity and its contents. The following organs and tissues should be stored in appropriate preservation liquids for histopathological tests: all grossly-affected areas, brain* (the cerebra, cerebella, and other typical regions, including pontile), pituitary gland, spinal cord, eyeballs, thyroid gland (including the parathyroid gland), heart*, trachea and lungs (maceration after the injection of fixing solution), liver*, kidney*, thymus gland*, spleen*, adrenal gland*, stomach, intestines and colon (including Peyer's Patch), reproductive glands (testicles* or ovaries), accessory reproductive glands (uterus or prostate gland, epididymis*), bladder, lymph nodes (those related to administration routes of the test substance and those apart from administration ruotes), peripheral nerves close to muscles (ischiadic nerves or tibial nerves), bone marrow (thigh bones), and other organs and tissues suspected as target organs on the basis of observations with the naked eye and other information and examinations.*
Organs marked with an asterisk should be weighed.

*[*11]: These examinations should be conducted on all preserved organs and tissues of all animals in the maximum-dose groups and control groups. As for organs and tissues which show changes likely to have been caused by the test substance in maximum-dose groups in particular, animals in groups of all other levels of dose should be examined with focus on the observations concerned.*
On animals in satellite groups, histopathological tests examinations should be made on organs and tissues that show signs of effects after the 28-day administration of the test substance. In addition, all grossly-affected areas should be examined.

III. 90-Days Repeated Dose Toxicity Test

The test is conducted pursuant to the OECD guidelines 408.

IV. Mutagenicity Test

Objective

The objective of this test is to detect genetic toxicity and carcinogenicity of the test substance with relatively simple short-term tests.

Test methods

In this test, reverse mutagenicity test on bacteria is conducted as a test that gives an indication of genetic mutagenicity and chromosome aberration test using. Cultured mammalian cells is conducted as a test that gives an indication of chromosomal aberration inducibility.
When the above-described tests cannot be conducted for solid scientific reasons, they can be substituted with tests with similar genetic indicators.

1. **Reverse Mutagenicity Test on Bacteria**

   1-1. Objective

   The objective of this test is to detect the presence of genetic mutagenicity inducibility of the test substance using bacteria.

   1-2. Strains of bacteria to be used

   Tests are conducted using the following five strains.
   (1) *Salmonella typhimurium* TA98
   (2) *Salmonella typhimurium* TA100
   (3) *Salmonella typhimurium* TA1535
   (4) *Salmonella typhimurium* TA1537, TA97 or TA97a
   (5) *Escherichia coli* WP2 *uvrA*, *Escherichia coli* WP2 *uvrA*/pKM101 or *Salmonella typhimurium* TA102.

   In detecting chemical compounds cross-linked to DNA, in the case of *Salmonella typhimurium*, TA102 should be included, or in the case of *Escherichia coli*, the WP2 or WP2/pKM101 with the wild-type strain of excision repair capability should be added. Other strains may be added as needed.

   1-3. Test method

   Implement the test using either the pre-incubation method or the plate method. If there is a scientifically valid reason, it is acceptable to use another method. Whichever method is used, conduct tests on the case due to metabolic activation and the case not due to metabolic activation. In the case due to metabolic activation, use S9 mix with coenzymes, etc. added to rodent (normally rat) liver homogenate 9,000×g supernatant fraction (S9) treated with an appropriate drug-metabolizing enzyme inducing agent (for example,

combined use of Phenobarbital and 5,6-Benzoflavone, etc.). Make the final concentration of the S9 within the 5% to 30% range (normally 10%).

1-4. Dosage stages

Use a dosage that can be analyzed in five stages or more at appropriate intervals. Conduct a dose-range finding test in advance and establish the maximum dosage, taking into account growth inhibition and solubility. As a rule, make the maximum dosage the dosage for which growth inhibition appears. If growth inhibition does not appear, make the maximum dosage 5 mg/plate. If it is a very insoluble substance and no growth inhibition can be seen at all, the dosage which forms a precipitation can be made the maximum dosage.

1-5. Control

Establish a group treated with a solvent as a negative control and a group treated with an appropriate known mutagen as a positive control.

1-6. Number of plates

As a rule use two or more plates for each dosage of the test substance and the negative and positive control.

1-7. Observation of the reverse mutagenicity colony

After cultivating all of the plates, as a rule at 37°C for 48-72 hours, measure the number of reverse mutagenicity colonies on each plate and record the number. At the same time, observe growth inhibition, and if growth inhibition is discovered, record the dosage. Also record the dosage if precipitation of the test substance is discovered.

1-8. Reproducibility

As a rule, the test results must be reproducible. However, if all strains of bacteria are being used and the dose-range finding study is being conducted, including negative control and positive control, using two or more plates for each dosage, it is possible to use it to verify reproducibility.

1-9. Judgment of test results

If the number of reverse mutagenicity colonies clearly increases relative to the negative control, and dosage dependence or reproducibility are discovered in its chemical action, determine that it is positive. If reproducibility is not discovered in the results of the dose-range finding test and this test, implement a test to verify the reproducibility. When

it is impossible to give a clear positive or negative conclusion, a confirmation test must be carried out under appropriate experimental conditions.

1-10. Display of results

Show the number of reverse mutagenicity colonies for each plate and display their average value for each dosage.

1-11. Summary of test results

The test results should be summarized in Form 5.

2. **Chromosome Aberration Test Using Cultured Mammalian Cells**

2-1. Objective

The objective of this test is to use cultivated mammalian cells to search for whether the test substance has chromosomal structural aberration inducibility. When polyploids appear, record this fact.

2-2. Cells used

Use Chinese hamster fibroblast cell lines (for example, CHL/IU, CHO), human peripheral blood lymphocytes, or other primary, secondary, or established cells. Study the modal number, whether there is mycoplasma contamination, the cell cycle, etc. of the cells being used in the test.

2-3. Test method

Use cells in the growth phase and firstly, as a short-term treatment process, in the case due to metabolic activation and the case not due to metabolic activation, treat with the test substance for 3-6 hours and make the chromosome sample approximately 1.5 cell cycles after the commencement of treatment. If the result of the short-term treatment process is negative, next implement a test using a 1.5-cell cycle continuous treatment process in the case not due to metabolic activation. Some test substances will cause a marked delay in the cell cycle. Sometimes continuous treatment longer than 1.5 cell cycles is necessary in the case not due to metabolic activation, and a sample making time longer than 1.5 cell cycles is necessary in the case due to metabolic activation. For this reason, conduct a confirmation test as necessary.

In the case due to metabolic activation, use S9 mix with coenzymes, etc. added to rodent (normally rat) liver homogenate 9,000×g supernatant fraction (S9) treated with an

appropriate drug-metabolizing enzyme inducing agent (for example, combined use of Phenobarbital and 5,6-Benzoflavone, etc.). Make the final concentration of the S9 within the 1% to 10% range (normally 5%).

2-4. Preparation of the test substance

Dissolve the test substance in an appropriate solvent or suspend it in an appropriate medium. If the test substance is a liquid, it is acceptable to directly add it to the test system. If the test substance is soluble in water, use a normal saline solution, etc. to dissolve it. If the test substance is not soluble in water, use dimethylsulfoxide (DMSO), etc. to dissolve it.
As necessary, use carboxymethylcellulose (CMC) sodium, etc. to prepare a homogenous suspension.

2-5. Dosage stages

Use a dosage for which it is possible to do a chromosome analysis of three stages or more at appropriate intervals (as a rule, geometric ratio 2). It is desirable to establish the maximum dosage by conducting in advance a cell growth inhibition test using a maximum dosage of 5mg/mL or 10mM (the lowest of the two). As a rule, regardless of the solubility of the test substance in the culture solution, make the maximum dosage the dosage that clearly suppresses cell growth 50% or more. In tests using short-term treatment processes and the tests using continuous treatment processes, measure cell growth at the time the chromosome sample is made. If cell growth suppression of 50% or more is not confirmed, make the maximum dosage 5mg/mL or 10mM (the lowest of the two). If cell toxicity is not confirmed and precipitation of the test substance is confirmed at the time of completion of treatment, the dosage which forms a precipitation can be made the maximum dosage.

2-6. Control

As a negative control, establish a solvent treatment group; as a positive control, establish a treatment group using an appropriate known chromosome aberrations inducing substance.

2-7. Number of plates

As a rule, use two plates for each dosage group of the test substance and the negative and positive control groups.

2-8. Observation of chromosome aberrations

Slide samples must be coded and observed under the condition that treatment conditions cannot be identified. With respect to chromosome structural aberration, observe at least 200 well-spread metaphase cells (for which the number of chromosomes is equal to mode ± 2) for each dosage, and record the number of cells with chromosome structural aberration and the number of cells for each kind of aberration. If two plates are used, as a rule observe at least 100 metaphase cells per plate. Classify gaps separately from other aberrations and record them, but do not include them in structural aberrations. Gaps are defined as achromatic regions that are narrower than chromatids. With respect to the chromosome numerical aberration, observe 200 or more metaphase cells for each dosage and record the incidence of polyploids.

2-9. Judgment of test results

Positive determination is given when the incidence of cells with chromosome aberrations clearly increases as compared to the negative control, and dose dependency or reproducibility is observed in its action. When it is impossible to give a clear positive or negative conclusion, a confirmation test must be carried out under appropriate experimental conditions.

2-10. Display of results

Display the number of cells having chromosome structural aberrations, their incidence (%), and the number of cells for each type of structural aberration for all of the plates in the test by short-term treatment process and the test by continuous treatment process. Also, display the average value of the plate for each group. Display the number and incidence (%) of polyploids. Display data on cell growth in each dosage group and the negative control group for the cell growth inhibition test, the test by short-term treatment process and the test by continuous treatment process. If precipitation of the test substance is seen, record that dosage.

2-11. Summary of test results

The test results should be summarized in Form 6.