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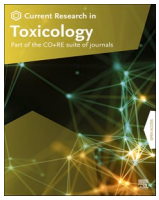

A step-by-step approach for assessing acute oral toxicity without animal testing for additives of quasi-drugs and cosmetic ingredients

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ABSTRACT

Animal testing of cosmetic ingredients and products has been banned in the European Union since 2013. However, in Japan, the application of new quasi-drugs requires the generation of data on acute oral toxicity through animal testing. A weight of evidence approach for assessing oral toxicity was challenged. This approach used a combination of safety data, including a neutral red uptake cytotoxicity assay using BALB/c3T3 cells (3T3-NRU cytotoxicity assay), which can assess the acute oral toxicity of quasi-drugs or cosmetic ingredients. We conclude that the step-by-step approach can be used to assess test substances that cause low acute oral toxicity, such as the median lethal dose (LD 50) > 2000 mg/kg, thereby avoiding animal testing.

Introduction

Single-dose toxicity is a response that is guided by changes in the symptoms of acute toxic reactions, including lethality, resulting from a single dose of a test substance exposed by oral, dermal, or inhalation (ICH, 2009). Single-dose toxicity study is conducted to assess the quantitative and qualitative toxicity of a chemical substance and predict the toxic concentration and symptoms associated with acute toxicity of the substance (Japan Cosmetic Industry Association, 2015; Anon, 2017).

In the past, acute oral toxicity studies in rats and mice have been conducted using gavage applications for marketing approval of quasi-drugs. Moreover, these studies have been used to revise the cosmetic standards to predict the acute toxic reactions caused by accidental ingestion of the product. Acute oral toxicity studies in animals should be conducted at a dose level of 2000 mg/kg for less toxic substances. For more toxic substances that produce mortality at one dose level, signs of acute toxicity and the associated doses should be identified (Anon, 2017). To reduce the number of animals required for testing and avoiding animal stress, the Organization for Economic Cooperation and Development (OECD) has issued test guidelines, that identify alternative methods, such as acute oral toxicity–fixed dose procedure (TG420), acute oral toxicity–acute toxic class method (TG 423), and acute oral toxicity: up-and-down procedure (TG425) (OECD, 2002a; 2002b; 2008). However, it is desirable to establish alternative methods to replace animal testing to ensure animal welfare.

The Neutral Red Uptake cytotoxicity assay using BALB/c3T3 cells (3T3-NRU cytotoxicity assay), a cell line derived from mouse fibroblasts, is widely used for assessing cytotoxicity, and its capacity to predict acute oral toxicity has been reported in a previous study (Halle, 2003). National Toxicology Program Interagency Center for the Validation of Alternative Toxicological Methods (NICEATM) and EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) jointly conducted a validation study of the 3T3-NRU and reported that this method can be used to assess the starting dose for acute oral toxicity (NICEATM,

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2006, ICCVAM, 2006). Based on these results, the OECD Guidance Document No. 129, titled "Guidance document on using cytotoxicity assays to estimate starting doses for acute oral systematic toxicity tests" was published in 2010 (OECD, 2010). The document determines the starting dose for acute oral toxicity studies by predicting LD 50 from the regression analysis based on the correlation between the 50 % inhibitory concentration (IC 50: half maximum interference concentration) assessed for the 3T3-NRU cytotoxicity study and the median lethal dose (LD 50) obtained from the database. Prediction of the starting dose using the 3T3-NRU cytotoxicity assay reduced the number of animals.

EURL ECVAM subsequently conducted a follow-up validation study demonstrating the usefulness of the 3T3-NRU cytotoxicity assay for the determination of substances with LD 50 > 2000 mg/kg (ECVAM, 2011, Prieto et al., 2013), and published the "EURL ECVAM Recommendations on the 3T3 Neutral Red Uptake Cytotoxicity Assay for Acute Oral Toxicity Testing" in 2013 (EURL ECVAM, 2013). Although the 3T3-NRU cytotoxicity assay can be used to identify substances with an LD50 >2000 mg/kg, it was recommended that the 3T3-NRU cytotoxicity assay should always be used in conjunction with other information, rather than alone, because substances with neuro-or cardiac-specific toxicities or those with metabolic activation cannot be evaluated in this cytotoxicity assay. The Japanese Center for the Validation of Alternative Methods (JaCVAM) also evaluated the 3T3-NRU cytotoxicity assay, which predicts LD 50 > 2000 mg/kg based on the ECVAM outcome, and recommended a weight of evidence (WoE) assessment in combination with other reliable information (JaCVAM, 2018, JaCVAM, 2019). The WoE assessment refers to a method of using the strengths and weaknesses of collected information as a basis for obtaining conclusions that are not clear from a single data (OECD, 2010).

Based on the JaCVAM recommendations, we developed a step-by-step approach, a flow for the WoE assessment of acute oral toxicity using a combination of safety data, including 3T3-NRU cytotoxicity studies.

Theory

Basic consideration

Acute oral toxicity of a chemical substance can be assessed using sufficient data from *in vivo* studies that use this substance. The definition of"safety data" is a safety concern, regarding additives of quasi-drug and cosmetic ingredients based on the combination with the existing information such as the *in vivo* data of acute oral toxicity studies of the test substance and similar ones, or a history of dietary consumption and no cytotoxicity *in vitro* study. However, safety data of a substance cannot be assessed on its own using *in vivo* studies. In such cases, safety data should be assessed using the WoE assessment in combination with acute oral toxicity data of the test substance and/or its analogs. However, when toxicity data from analogous substances are used, it is necessary to explain the selection process of this substance and show that the selection is appropriate.

In vivo data

When evaluating acute oral toxicity based on *in vivo* data, it is necessary to check the detailed test conditions and results, including conditions of animal husbandry, detailed treatment conditions, body weight, general state, and autopsy results (ICH guideline M3(R2), 2009). If such data are available, acute oral toxicity can be assessed using the test substance alone. However, when acute oral toxicity is assessed using data retrieved from other sources, such as data from original research papers, toxicity database, and the Cosmetic Ingredient Review (CIR) (Cosmetic Ingredient Review, 2022), it is often difficult to obtain detailed data as these sources describe summarized test conditions and results. In this context, the *in vivo* information was corrected by a team at the Japan Cosmetic Industry Association (JCIA) in the present study, *in vivo* data was assessed for (1) species of rat or mouse used for testing, (2) oral route of administration, (3) toxicity results when LD 50 > 2000 mg/kg, and (4) source information, if available. This summary was one of the key data for the WoE assessment. In the absence of a summary of *in vivo* data for the test substance itself, the *in vivo* data for substances with similar chemical structures and functions should be considered useful data for such an WoE assessment, if the substance belongs to the predefined range of ingredients with probably low acute oral toxicity (see Table 1).

Cytotoxicity assay data

In vitro 3T3-NRU cytotoxicity assay is considered as one of the most reliable data for WoE assessment. However, this method is generally used when the test data results can be easily obtained and detailed test records and results are available. Therefore, the JCIA team collected data to predict acute oral toxicity in conjunction with 3T3-NRU cytotoxicity assays. These data were obtained from multiple sources that are listed below: (1) a validation study of 3T3-NRU was conducted to determine the starting dose for acute toxicity studies (NICEATM, 2006, ICCVAM, 2006). Based on these results, the OECD Guidance Document No. 129, titled "Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests (OECD, 2010), (2) a follow-up validation study based on the data obtained was subsequently conducted by EURL ECVAM, which demonstrated the effectiveness of using 3T3-NRU cytotoxicity assay for identifying substances with LD 50 > 2000 mg/kg (ECVAM 2011, Prieto et al., 2013), and (3) in-house data on the 3T3 NRU cytotoxicity assay (JaCVAM, 2018).

Other data

The 3T3-NRU cytotoxicity study for acute oral toxicity cannot be assessed alone and should be combined with safety data related to the acute oral toxicity of a test substance and/or its analogs. Additionally, even *in vivo* studies may not be assessed independently if the data are inadequate. However, the 3T3-NRU cytotoxicity study alone could not comprehensively evaluate acute oral toxicity. Hence, data regarding dietary experience, human use, and the threshold of toxicological concern (TTC) (SCCS/1602/18, 2018, Mitsubishi Chemical Research Corporation, 2015) was collected by the JCIA team for the WoE assessment.

Results

"Acute oral Toxicity Assessment of additives for Quasi-Drug and Cosmetic Ingredients Combining 3T3-NRU Cytotoxicity Studies and Other Safety Data" is performed using the following procedure (Fig. 1):

Step 1: The test substance is assessed for acute oral toxicity as an additive to a quasi-drug or cosmetic ingredient. Otherwise, the approach is not applicable.

This approach does not apply to the active ingredients of quasi-drugs and ingredients that require revision of cosmetic standards.

Step 2: The *in vivo* data of acute oral toxicity studies of the test substance are summarized from existing data, such as original articles and reliable databases. Proceed to step 3, if this step is applicable. If not, proceed to step 4.

1. During literature review of the existing data (steps 2, 3, and 4), the "test substance" includes "all components of the test substance". Therefore, data from tests performed on the test substance itself as well as on the same ingredients as the test substance can be used. If a test substance consists of more than one component, the data for each component can be used. It is necessary to note that even if a component is indicated by a single component name, some compounds may have different molecular weights (for example, hexadecanol) and others may contain impurities (for example, sodium

Table 1
Range of test substances with probably low acute oral toxicity.

No.	Categories	Range of test substances with low acute oral toxicity in this evaluation[36-38)]	Examples
1	Sugars[39)]	Compounds with one carbonyl group or aldehyde group and two or more hydroxy groups with 3 to 6 carbon atoms (monosaccharides) and compounds in which two or more of monosaccharides are bonded by glycosidic bonds (disaccharides, oligosaccharides, polysaccharides). In addition, compounds (sugar alcohols) in which the carbonyl group of a monosaccharide is reduced to a hydroxy group.	Glyceraldehyde, Dihydroxyacetone (Triose), D-Ribose (Pentose), D-Glucose (Hexose), D-Sucrose (Disaccharide), Glucomannan (Polysaccharide), Xylitol (Sugar alcohol)
2	Amino acids, Peptides	Compounds (amino acids) that have both amino group and carboxy group. However, L-Cysteine is excluded. In addition, compounds (peptides) in which amino acids are used as monomers and linked in a chain by peptide bonds.	19 kinds of amino acids excluding L-cysteine out of 20 kinds of amino acids that make up proteins (Amino acid), *Nori* oligopeptide (Oligopeptide)
3	Polyols	Compounds having a structure in which two or more hydrogen atoms of a hydrocarbon are replaced with a hydroxy group. However, 1,4-butanediol is excluded.	1,2-Ethanediol, 1,3-Butanediol, 1,6-Hexanediol (Dihydric alcohol), Glycerol (Trihydric alcohol)
4	Macromolecules (Molecular weight 1000 or more)	Compounds with a molecular weight of 1000 or more.[40, 41)]	Polyethylene glycol of various molecular weights, PEG-32, PEG-75, PEG-150, PEG-20 M
5	Fatty acids (Carbon number 9 or more)	Compounds having a structure in which the hydrogen atom of a hydrocarbon is replaced with a carboxyl acid and having 9 or more carbon atoms.	Nonanoic acid (C9), Decanoic acid (C10), Dodecanoic acid (C12), Hexadecanoic acid (C16)
6	Alcohols (Carbon number 3 or less or 8 or more)	Compounds having a structure in which one hydrogen atom of a hydrocarbon is replaced with a hydroxy group and having 3 or less or 8 or more carbon atoms.	Methanol (C1), Ethanol (C2), Isopropanol (C3), Octanol (C8), Dodecanol (C12), Hexadecanol (C16)
7	Esters	Compounds obtained by the dehydration reaction of acid and alcohol. The acid is a fatty acid, and the alcohol is a monohydric or polyhydric alcohol.	Isopropyl myristate, Glyceryl laurate, Ethyl acetate
8	Wax esters	Compounds in which a higher fatty acid (usually 12 or more carbon atoms) and a higher alcohol (usually 6 or more carbon atoms) are ester-bonded. In addition to these, free fatty acids, higher alcohols, hydrocarbons, etc. are contained.	Candelilla wax, Jojoba oil, Lanolin
9	Triglycerides	Compounds in which three molecules of fatty acids are ester-bonded to one molecule of glycerol	Triheptanoin, Triisostearin
10	Hydrocarbons (Carbon number 6 or more)	Compounds consisting of carbon and hydrogen with 6 or more carbon atoms	Hexane (C6), Isododecane (C12), Isohexadecane (C16), Squalane (C30), Polyisobutene
11	Silicones	Polymers with silicon oxide as the basic skeleton	Polydimethylsiloxane, Dodecamethylcyclohexasiloxane

Note 1) Data of examples of test substances belonging to 11 categories with low scute oral toxicity and data of some test substances excluded from the range of test substances with low acute oral toxicity within these 11 categories are shown in Appendix 1.
Note 2) Even if the test substance belongs to the classification 1 to 11, it does not mean that the acute oral toxicity is probably low. The acute toxicity of the test substance should be evaluated based on the data of similar substances.

polyacrylate). Consider the actual chemical substances that constitute the ingredients, regardless of their name.
2. During the investigation of existing data, efforts should be made to obtain as much detailed information as possible.
3. The reliable database refers to public database (a database that is included after data evaluation by experts), including existing chemical toxicity databases such as (Japan Existing Chemical Database (NIHS, 2022a), integrated platform for hazard assessment support systems (Hazard Evaluation Support System Integrated Platform) (National Institute of Technology and Evaluation, 2022), OECD QSAR Toolbox (2022), Registry of Toxic Effects of Chemical Substances (2022), European Chemicals Agency. Information on Chemicals (2022), ChemIDplus (U.S. National Library of Medicine, 2022), and Cosmetic Ingredient Review (2022). The database used to collect and assess hazard information for toxicological designation in Japan is considered reliable and informative (NIHS, 2022b).
4. "Summary of *in vivo* data" refers, at minimum, to (1) rat or mouse species, (2) oral route of administration, (3) LD 50 $>$ 2000 mg/kg results, and (4) the source

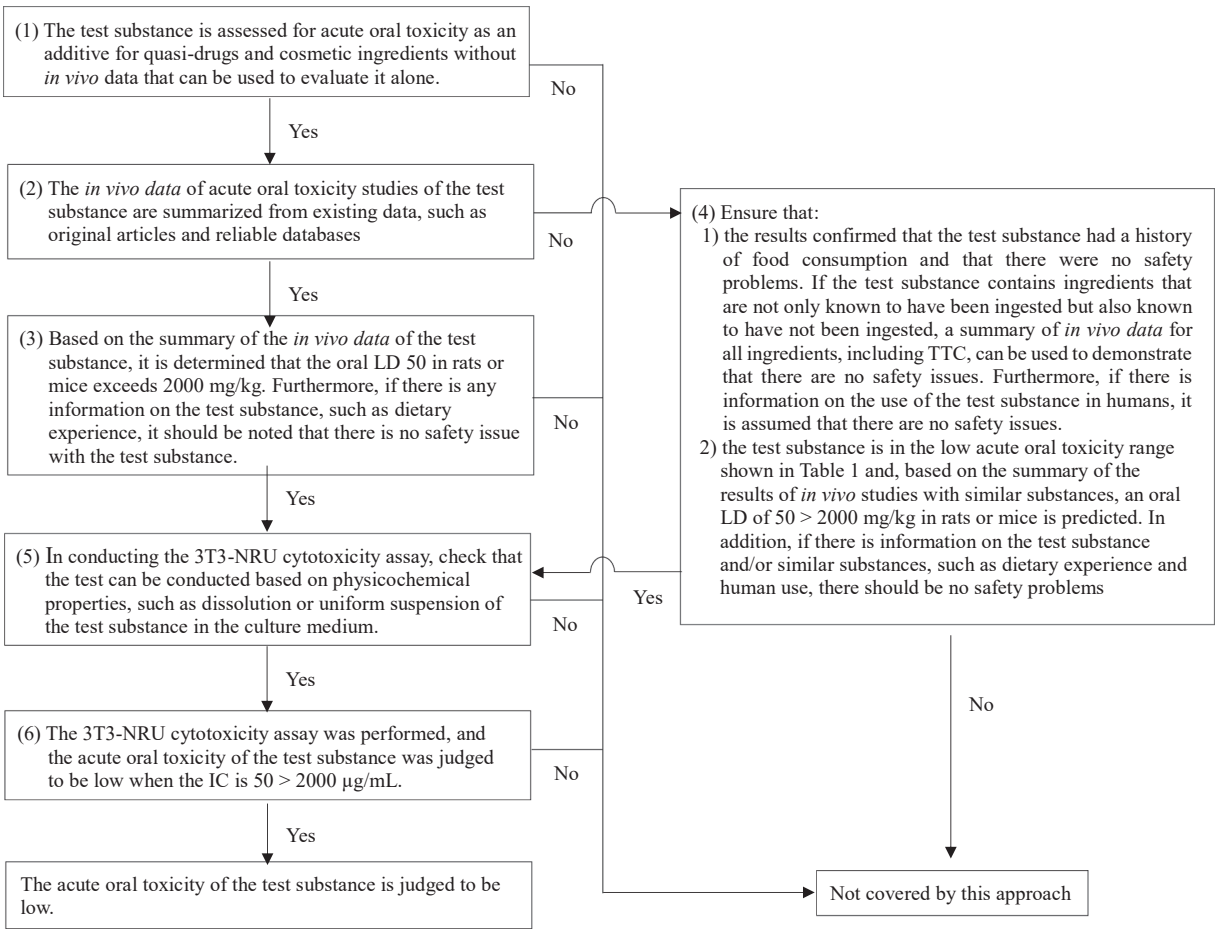

Fig. 1. Acute oral toxicity assessment system for quasi-drug additives and cosmetic ingredients that combines 3T3-NRU cytotoxicity test and other safety data.

5. If data such as *in vivo* repeated-dose toxicity studies are found to be useful in the evaluation of the acute oral toxicity of the test substance, these data will be used as the data in the summary of *in vivo test* results.

Step3: In summary, the *in vivo* data of the test substance determines that an oral LD 50 in rats or mice exceeds 2000 mg/kg. Furthermore, if there is any information on the test substance, such as food experience, it should be noted that there is no safety issue with the test substance. Proceed to step 5, if this step is applicable. Otherwise, the approach is not applicable.

1. It is important to check that no specific neuro- or cardiac-specific toxicity has been reported in the data, and that toxicity due to metabolic activation has not been mentioned. One of these methods is to confirm that the oral LD 50 in rats or mice exceeds 2000 mg/kg *in vivo*. If the test substance is an acute oral toxic substance as a result of a specific mechanism or metabolic activation, then the toxicity is reflected in the LD 50 values in the rat or mouse that is tested. Therefore, an LD 50 of >2000 mg/kg for the test substance can be considered to indicate that the test substance is not an acute oral toxic substance via a specific mechanism or metabolic activation.
2. For the summary of *in vivo* data, data for predicting acute oral toxicity from *in vivo* repeated-dose toxicity studies and other data can be used. Bulgheroni et al. (2009) reported that a NOAEL (no observed adverse effect level) of 200 mg/kg or higher in a 28 day oral toxicity study in rats predicted oral LD50s > 2000 mg/kg in rats, which would be helpful in checking that there are no problems with acute oral toxicity and that the substance does not exhibit acute oral toxicity, particularly due to specific mechanisms or metabolic activation (JaCVAM, 2018, JaCVAM, 2019).
3. Data to be assessed when information is available includes the diet of the animal and the human-use condition. The term “dietary experience” refers to a case in which it is generally accepted that the same product (or related ingredients) has been eaten as part of a diet for a long period of time without changing the conditions of the food product such as its ingredients or processing methods, and no safety problem has been associated with the diet. If these conditions are met, the food product can be considered safe (FSCJ, 2004). Therefore, dietary conditions may provide valuable information for assessing the acute oral toxicity of the test substance. The quality and quantity of information (including the duration of the dietary experience) should be assessed to ensure that the information obtained based on the food experience is sufficient and can be used to determine that acute oral intake of the test substance is not toxic to the body, does not specifically cause neuro- or cardiac-specific toxicity, and does not cause toxicity due to metabolic activation.

Even though cosmetics are used through the transdermal or transmucosal route, they do not provide sufficient oral safety information and are generally considered less important than dietary experience for the evaluation of acute oral toxicity. Considerations based on TTC can also be used to assess the safety of ingredients.

4. Dietary experience can be used to assess acute oral toxicity. To date, the use of test substance-related foods in Japan or other countries or regions, including cooking and processing methods, composition of ingredients, forms of consumption, methods of consumption,

amounts of consumption, areas of consumption, populations of consumption, duration of consumption, and hazard information, should be investigated to determine whether the data can be used to assess the acute oral toxicity of the targeted quasi-drug excipients in this approach (JHNFA, 2008).

5. Information on human use of the test substance is useful if there is detailed safety information, especially data on accidental ingestion as well as data that shows the amount of oral exposure to humans.
6. The TTC is the threshold for human exposure to all chemicals, below which no apparent adverse effects are expected (SCCS/1602/18, 2018). Therefore, it can be used to assess the acute oral toxicity of an ingredient. The European Commission's Scientific Committee on Consumer Safety (SCCS) has assessed TTC criteria (SCCS/1602/18, 2018). In addition, International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) has established the permissible intake based on the TTC in its "Guidelines for the Evaluation and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Reduce Potential Carcinogenic Risks" (MHLW, 2015, ICH, 2014). In the safety assessment of quasi-drug excipients, 90 μg/person/day, which is equivalent to 1.5 μg/kg body weight per day for the substances without genotoxicity (warning substructure), serves as a reference standard for TTC. A reference value of 1.5 μg/person/day, equivalent to 0.025 μg/kg body weight per day, was used as a potential DNA-reactive carcinogen (SCCS/1602/18, 2018). When assessing the safety of an ingredient based on TTC, it is necessary to check whether the subject is within the scope of TTC. The substances not covered by TTC are mixtures of substances including aflatoxin-like compounds, azoxy compounds, *N*-nitroso compounds, benzidine, hydrazine, inorganic compounds, metals and organometals, proteins, steroids, and substances with known or expected bioaccumulation potential (polyhalogenated dioxin/dibenzofuran and dioxin-like polyhalogenated biphenyl), nanomaterials, radioactive materials, and unknown chemical structures. However, based on the guidelines of the European Food Safety Authority (EFSA), TTC can be applied to the sum of the components of a mixture if sufficient information or analysis is available to indicate that TTC does not include the substance in a category that is not applicable (EFSA, 2019). As a specific application of TTC, there is an example in which the test substance was analyzed using a general analytical instrument and no safety problem was found at a concentration below the TTC standard in a product, which is acceptable (SCCS/1610/19, 2019, Koster et al., 2011).

Step 4: Ensure that the test article: The results confirmed that the test substance had a history of food consumption and that there were no safety problems. If the test substance contains ingredients that are not only known to have been ingested but also known to have not been ingested, a summary of *in vivo* data for all ingredients, including TTC, can be used to demonstrate that there are no safety issues. Furthermore, if there is information on the use of the test substance in humans, it is assumed that there are no safety issues.

The test substance is in probably low acute oral toxicity range shown in Table 1, and based on the summary of the results of *in vivo* studies with similar substances, an oral LD of 50 > 2000 mg/kg in rats or mice is predicted (Annex1 is a supporting document). In addition, if there is information on the test substance and/or similar substances, such as dietary experience and human use, there should be no safety problems. Proceed to the next step if this step is applicable. Otherwise, the approach is not applicable.

1. In the evaluation of dietary experience in step3-1), it is possible to show that there is no toxicity based on dietary experience with respect to the acute oral toxicity in humans after ingestion of 2000 mg/kg of the test substance. The 3T3-NRU cytotoxicity assay requires that the substance does not show acute oral toxicity by a specific mechanism or metabolic activation at a relatively high dose.
2. The evaluator indicated the history of selection of similar substances. When certain components of the test substance were to be assessed, "similar components" were selected.
3. The analogous substances referred to here are those that have similar chemical structures, physical and chemical properties, and functions and are found to have LD50 > 2000 mg/kg. The similarity explanation for the slight difference in the chemical structure indicates that the physical and chemical properties and functions are similar. The data of structure similarity in OECD QSAR Toolbox is available (OECD QSAR Toolbox). For example, if the test substance at C 16 is different only in the carbon chain, and C 14 and C 18 are assessed as similar substances, it is considered that the substance can be selected as similar if various characteristic values are shown to fall between C 14 and C 18.
4. For at least one of the analogous substances, select the substance for which a summary of the *in vivo* data exists.
5. It should be noted that the range of substances with probably low acute oral toxicity shown in Table 1 is generally excluded when special functional groups or chemical structures are added. For example, L-cysteine is a substance of low acute oral toxicity, whereas acetylated *N*-acetyl L-cysteine differs in chemical structure, physical and chemical properties, and function, and is excluded from components of low acute oral toxicity.
6. Data to be assessed when information is available includes dietary experience and human use experience. Considerations based on TTC can also be used to assess the safety of ingredients and product.
7. If a test substance for which no summary of *in vivo* data is available has been used in humans as a cosmetic, the rationale for the evaluation that acute oral toxicity is not a problem should be provided. It should be noted that if the safety evaluation performed for human use lacks validity, the use results obtained will not be accepted as valid data.

Step 5: In conducting the 3T3-NRU cytotoxicity assay, check that the test can be conducted based on physicochemical properties, such as dissolution or uniform suspension of the test substance in the culture medium (JaCVAM, 2018). Proceed to be step 6 if this step is applicable. Otherwise, the approach is not applicable.

1. The substance to be tested should be subjected to a solubility test to check whether the test is feasible, but ultimately, only well data that have been dissolved or uniformly suspended until the end of application of the test substance should be used in the cytotoxicity assay.
2. Test facilities conducting new 3T3-NRU cytotoxicity tests should use the data described in Annex 8 of OECD Guidance Document No. 129 (OECD, 2010) to improve accuracy that the same substance will give the equivalent results.
3. This test is not applicable to substances with the following physicochemical properties.

1) Substances insoluble in cell cultures
2) Evaluation is possible when the substance is dissolved in the cell culture medium or uniformly suspended; however, evaluation is not possible when a precipitate is produced and the substance is exempted from application.
3) Substances that react with cell cultures
4) Highly volatile substance
5) Substances with saturated vapor pressures above 4 kPa at 25 °C may be exempted. When evaluating such substances, it is necessary to explain that a reasonable evaluation was made.
6) Colored substances

Red, or colored substance that inhibits the absorbance measurement of NR, with properties that persist in cells.

4. Substances that can be falsely negative.

This test method should be used with caution because false negatives may occur when toxicity mechanisms and effects on cell function are present.

1) Substances that are metabolically activated to cause toxicity
2) Substances that are toxic by organ-specific mechanisms of action, such as neurotoxicity and cardiotoxicity
3) These substances can be identified by comparing the viability measured in the 3T3-NRU cytotoxicity test to viability measured using image analyses. Cell viability of 3T3-NRU measurement will be higher (JaCVAM, 2018).

Step 6. The 3T3-NRU cytotoxicity assay was performed, and the acute oral toxicity of the test substance was judged to be low when the IC is 50 > 2000 μg/mL. IC 50 ≤ 2000 μg/mL was not evaluable and was not included in this approach.

In addition, three case studies are addressed.

Case study 1: Butoxydiglycol

In case of case study1, the *in vivo* data of acute oral toxicity studies of the test substance are summarized from existing data.

INCI Name: Butoxydiglycol.

INCI Monograph ID: 333.

Synonym(s): 2-(2-Butoxyethoxy)ethanol, Diethylene glycol butyl ether.

CAS No.: 112-34-5.

Molecular weight: 162.23.

Chemical formula: $CH_3(CH_2)_3OCH_2CH_2OCH_2CH_2OH$.

Step 1: The test substance is assessed for acute oral toxicity as an additive to a quasi-drug or cosmetic ingredient.

Butoxydiglycol is not listed in the List of Quasi-Drug Additives in Japan (MHLW, 2008), but it is listed in the International Nomenclature for Cosmetic Ingredients (INCI). Therefore, it is one of the substances that may be evaluated as an additive for a quasi-drug or cosmetic ingredient. (1) in Fig. 1, which shows the acute oral toxicity evaluation system, becomes Yes and proceeds to (2).

Step 2: The *in vivo* data of acute oral toxicity studies of the test substance are summarized from existing data, such as original articles and reliable databases.

Data on acute oral toxicity in rats or mice were investigated for Butoxydiglycol. ChemIDplus (U.S. National Library of Medicine, 2022), ECHA Information on Chemicals (ECHA, 2022), and SCCP "Opinion on diethylene glycol monobutyl ether (DEGBE)" (SCCP/1043/06) and are shown in Table 2. Detailed test conditions and results, i.e., animal rearing conditions, detailed administration conditions, body weight, general condition, autopsy results, etc., were not available in complete data, but summary data of acute oral toxicity were obtained. (2) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes Yes and proceeds to (3).

Step 3: Based on the summary of the *in vivo* data of the test substance, it is determined that the oral LD 50 in rats or mice exceeds 2000 mg/kg. Furthermore, if there is any information on the test substance, such as food experience, it should be noted that there is no safety issue with the test substance.

Summary data of acute oral toxicity of Butoxydiglycol all showed LD 50 > 2000 mg/kg. In addition, a search on Cosmetic-Info.jp (2022), a website that allows users to search for all ingredients labeling, revealed that Butoxydiglycol was incorporated into one product (a sheet mask product released on August 5, 2016). Although no serious adverse reactions have been reported with this product, Butoxydiglycol is not permitted as an additive in foods.

Based on the above, (3) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes Yes and proceeds to (5).

Step 5: In conducting the 3T3-NRU cytotoxicity assay, check that the test can be conducted based on physicochemical properties, such as

Table 2
Summary data of acute oral toxicity for Butoxydiglycol.

Species	Route	LD50 or not	Results	Detailed data or summary	First searched source	Original Source
Rat	Oral	LD50	5660 mg/kg	Summary	ChemIDplus	Dow Chemical Company Reports. Vol. MSD-41,
Rat	Unreported#	LD50	4500 mg/kg	Summary	ChemIDplus	Gigiena i Sanitariya. For English translation, see HYSAAV. Vol. 46(2), Pg. 14, 1981.
Mouse	Oral	LD50	2400 mg/kg	Summary	ChemIDplus	Journal of the American College of Toxicology. Vol. 12, Pg. 139, 1993.
Mouse	Unreported#	LD50	6050 mg/kg	Summary	ChemIDplus	Gigiena i Sanitariya. For English translation, see HYSAAV. Vol. 46(2), Pg. 14, 1981.
Mouse	Oral	LD50	2410 mg/kg (fasted animal)	Detailed data, but no environmental conditions for animals	ECHA Information on Chemicals	Unnamed study report, 1981.
Mouse	Oral	LD50	5530 mg/kg (fed animal)	The same as above	ECHA Information on Chemicals	Unnamed study report, 1981.
Rat	Oral	LD50	5660 mg/kg	Summary	SCCP Opinion	ChemID Lite.
Rat	Oral	LD50	6560 mg/kg	Summary	SCCP Opinion	Budavari, S. (ed.). The Merck Index - Encyclopedia of Chemicals, Drugs and Biologicals. Rahway, NJ: Merck and Co., Inc., p. 239, 1989
Rat	Oral	LD50	9623 mg/kg	Summary	SCCP Opinion	Eastman Kodak Co. Toxicity studies with diethyl glycol monobutyl ether. Acute oral LD50. Submitted to EPA, Washington, April 1984
Rat	Oral	LD50	7292 mg/kg	Summary	SCCP Opinion	The same as above
Mouse	Oral	LD50	2400 mg/kg	Summary	SCCP Opinion	ChemID Lite.
Mouse	Oral	LD50	5526 mg/kg	Summary	SCCP Opinion	Eastman Kodak Co. Toxicity studies with diethyl glycol monobutyl ether. Acute oral LD50. Submitted to EPA, Washington, April 1984
Mouse	Oral	LD50	2406 mg/kg	Summary	SCCP Opinion	The same as above

#: Unreported route remained in this Table.

dissolution or uniform suspension of the test substance in the culture medium.

Butoxydiglycol is water soluble and can be tested for 3T3-NRU cytotoxicity without any solubility issues. (5) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes Yes and proceeds to (6).

Step 6: The 3T3-NRU cytotoxicity assay was performed.

The results of the 3T3-NRU cytotoxicity test of Butoxydiglycol were IC 50 > 2000 μg/mL. Specifically, data from the 3 sites that performed the study on Butoxydiglycol, the Health and Safety Laboratory (UK), the Institute for Health and Consumer Protection (European Commission Joint Research Centre in Italy), and the Institute for In Vitro Sciences, Inc. (USA), were 2017.7, 2596.8, and 2670.0 μg/mL, respectively, all of which exceeded 2000 μg/mL (EURL ECVAM). (6) in Fig. 1 of the Acute Oral Toxicity Evaluation System was set to Yes, and in the end, the acute oral toxicity of Butoxydiglycol was evaluated to be low.

Case study 2: Plum extract

In case of case study 2, test substance had a history of food consumption.

Step 1: The test substance is assessed for acute oral toxicity as an additive to a quasi-drug or cosmetic ingredient. Plum extracts are listed on the list of quasi-drug additives in Japan (MHLW, 2008), but the types and concentrations that can be formulated are limited (3.0 % for hair removers such as medicated soap, shampoo, and rinse). The product may be evaluated as a quasi-drug or cosmetic ingredient when it is formulated in a new type of quasi-drug and cosmetic formulation or when the specifications of Plum extract are different. As a case study, a dilution of Plum extract from commercial foods (diluted to 40 % and then added ethanol to achieve a final concentration of 20 %) was used as the test substance for acute toxicity evaluation as a quasi-drug or cosmetic ingredient. Plum extract, which accounts for 40 % of the test substance, has the same specifications as Plum extract of commercial foods. In addition, the test substance has different specifications from Plum extract, a quasi-drug or cosmetic ingredient (MHLW, 2021). (1) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes Yes and proceeds to (2).

Step 2: The *in vivo* data of acute oral toxicity studies of the test substance are summarized from existing data, such as original articles and reliable databases.

There are no summary data for acute oral toxicity of Plum extracts of commercially available foods in rats or mice, and (2) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes No and proceeds to (4).

Step 4: Ensure that the results confirmed that the test substance had a history of dietary consumption and that there were no safety problems. If the test substance contains ingredients that are known to have been ingested as well as ingredients not known to have been ingested, a summary of *in vivo* data for all ingredients, including TTC, can be used to demonstrate that there are no safety issues. Furthermore, if there is information on the use of the test substance in humans, it is assumed that there are no safety issues.

The test substance is a 40 % diluted liquid of commercial Plum extract, which has been sold as food in Japan for >50 years and has been used safely. Since the specifications of Plum extract and commercial Plum extract contained in the test substance are identical, the dietary experience of commercial Plum extract is a reference for evaluation. In addition, 20 % ethanol was incorporated into the test substance, but the literature value for rat oral LD 50 of ethanol is over 7000 mg/kg, indicating low acute oral toxicity (Registry of Toxic Effects of Chemical Substances). The standard amount of Plum extract used as food is 55 g/day, which may be used in cooking or taken as is at one time. A person weighing 60 kg ingesting 2 g/kg of the test substance is within the range of the standard usage because it is equivalent to ingesting 48 g of Plum extract of food (formula: 2 g/kg × 60 kg × 0.4 = 48 g). In addition, a search on Cosmetic-Info.jp (2022), a website that allows users to search for all ingredient labels, showed that Plum extract was incorporated into 25 products (the oldest product being a lip balm released on November 2, 2007). No serious adverse reactions have been reported with these products. Therefore, (4) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes Yes and proceeds to (5).

Step 5: In conducting the 3T3-NRU cytotoxicity assay, check that the test can be conducted based on physicochemical properties, such as dissolution or uniform suspension of the test substance in the culture medium.

The test substance is water soluble and can be tested for 3T3-NRU cytotoxicity without problems from solubility. (5) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes Yes and proceeds to (6).

Step 6: The 3T3-NRU cytotoxicity assay was performed.

The results of the 3T3-NRU cytotoxicity test of Plum extract were IC 50 > 2000 μg/mL (in-house data), indicating low acute oral toxicity.

Case study 3: Polyethylene glycol **60 (PEG-60)**.

In case of case study 3, the summary of the results of *in vivo* studies with similar substances.

INCI Name: PEG-60.

INCI Monograph ID: 5425.

Synonym(s): PEG-3000.

CAS No.: 25322–68-3.

Calculated average molecular weight: 2658 (Cosmetic Ingredient Review, 2011).

Chemical formula: $H(0CH_2CH_{2)n}OH$ where n has an average value of 60.

Step 1: The test substance is assessed for acute oral toxicity as an additive to a quasi-drug or cosmetic ingredient. Although PEG-60 is not listed in the Quasi-Drug Additives List in Japan (MHLW, 2008), it is listed in the INCI. Therefore, it is one of the substances that may be evaluated as a quasi-drug or cosmetic ingredient. A case study was conducted to evaluate the acute oral toxicity of PEG-60 as a quasi-drug additive. (1) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes Yes and proceeds to (2).

Step 2: The *in vivo* data of acute oral toxicity studies of the test substance are summarized from existing data, such as original articles and reliable databases.

When the single-dose toxicity of PEG-60 was ascertained for the specified molecular weight, no data on acute oral toxicity *in vivo* were available. (2) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes No and proceeds to (4).

Step 4: Ensure that the test article: The test substance is in probably low acute oral toxicity range shown in Table 1 and, based on the summary of the results of *in vivo* studies with similar substances, an oral LD of 50 > 2000 mg/kg in rats or mice is predicted. In addition, if there is information on the test substance and/or similar substances, such as dietary experience and human use, there should be no safety problems.

PEG-60 is a macromolecule with a molecular weight of >1000 kDa and is within the range of substances with low acute oral toxicity. Furthermore, the Cosmetic Ingredient Review (CIR) had data of PEG-32 (concentration 50 %) rat oral LD 50 > 16 g/kg and PEG-75 (concentration 50 %) rat oral LD 50 > 50 g/kg (Cosmetic Ingredient Review, 2010), and the rat oral LD 50 of PEG-60 was predicted to exceed 2000 mg/kg. There was no finding of acute oral toxicity in any of the PEG analogues. On the contrary, a search on Cosmetic-Info.jp (2022), a site that allows users to search for all ingredients labeling, revealed that PEG-60 was incorporated into 2 products (2017–04-05 gel products, August 27, 2007 sheet mask pack products). No serious adverse reactions have been reported with these products. In addition, PEG-60 is not permitted to be added to foods. Based on the above, (4) in Fig. 1 of the Acute Oral Toxicity Assessment System becomes Yes and proceeds to (5).

Step 5: In conducting the 3T3-NRU cytotoxicity assay, check that the test can be conducted based on physicochemical properties, such as dissolution or uniform suspension of the test substance in the culture medium.

PEG-60 is water soluble and can be tested for cytotoxicity without any solubility problems. (5) in Fig. 1 of the Acute Oral Toxicity

Assessment System becomes Yes and proceeds to (6).

Step 6: The 3T3-NRU cytotoxicity assay was performed.

The results of the 3T3-NRU cytotoxicity study of PEG-60 were IC 50 $>$ 2000 μg/mL (in-house data), indicating low acute oral toxicity.

Discussion

This step-by-step approach is based on the idea that acute oral toxicity can be evaluated by combining the data above. The scope of application of this approach is limited to additives for quasi-drugs and cosmetic ingredients. For the active ingredients of quasi-drugs and ingredients for which a request for revision of the cosmetic standards is required, further careful consideration is necessary for the completion of the WoE assessment, and they are excluded at present.

One of endpoints on *in vitro* cytotoxicity study results in cell death. Ekwall proposed that cell death is the result of a non-specific effect on basic cell function (basal cytotoxicity) and that a mechanism similar to cell death works *in vivo* in the blood concentration range where chemical cell death is induced (Ekwall B, et al., 1998). In other words, the normal structure and function of the cell membrane and cytoskeleton, which are common to all cells, are impaired; metabolic and synthetic abilities are inhibited; degradation and dysfunction of cellular components, regulation of intracellular and extracellular ion concentrations, and cell division are impaired, resulting in cell death. Widespread cell death can cause serious tissue damage, which, in turn, can affect unexposed organs and lead to death. Therefore, this assay is useful as a first step in the application of alternative test methods in acute oral toxicity tests.

However, substances that cause neurotoxicity or cardiac-specific toxicity or that cause toxicity by metabolic activation cannot be assessed in the 3T3-NRU cytotoxicity assay (ECVAM 2011, Prieto et al., 2013, JaCVAM, 2018, JaCVAM, 2019). Substances that act specifically on nerve receptors and ion channels can cause death without causing cytotoxicity. For example, tetrodotoxin (TTX), a pufferfish poison, and aconitine, which are found in aconite, act on TTX-sensitive sodium channels and cause death via respiratory depression. The mechanisms of cell death and individual death are different for these substances, causing individual death at concentrations much lower than those at which basal cytotoxicity is observed.

Hence, when the acute oral toxicity of an unknown substance is evaluated, it is necessary to consider the potential toxicity based on specific mechanisms and toxicity based on metabolic activation (JaCVAM, 2018, JaCVAM, 2019). However, if the cut-off for LD 50 is predicted to be 2000 mg/kg, *in vitro* cytotoxicity studies, regardless of the specific mechanism or metabolic activation, view any toxic effects as cytotoxic, and it has been found that *in vitro* cytotoxicity assays are unlikely to miss toxicity at LD 50 $\leq$ 2000 mg/kg (JaCVAM, 2018). In the acute oral toxicity assessment of unknown substances, the WoE assessment can be completed by combining predictions from cytotoxicity with other reliable information.

The approach discussed here, namely the "3T3-NRU Cytotoxicity Study and Acute oral Toxicity Evaluation System for additives of Quasi-Drug and cosmetic ingredients Combining Other Safety Data" is an example of the WoE assessment that can be used to assess the acute oral toxicity. For the active ingredients of quasi-drugs and ingredients for which a request for revision of cosmetic standards is required, other types of approaches and combinations that can be assessed using them should be considered in the future.

Conclusions

In the present study, we developed the step-by-step approach for assessing the applicability of acute oral toxicity for additives for quasi-drugs and cosmetic ingredients without animal testing. This approach was challenged in the WoE assessments of acute oral toxicity using a combination of safety data, including 3T3-NRU cytotoxicity studies that can assess the acute oral toxicity for additives of quasi-drugs or cosmetic ingredients. However, our approach is out of scope for major ingredients of quasi-drugs and a request for revision of cosmetic standards. We conclude that the step-by-step approach can be used to assess test substances that cause low acute oral toxicity within this applicability domain, such as the median lethal dose (LD 50) $>$ 2000 mg/kg based on our proposal, thereby avoiding animal testing.

CRediT authorship contribution statement

Hajime Kojima: Conceptualization, Writing – review & editing. **Tokio Nakada:** Writing – original draft. **Akiko Yagami:** Writing – original draft. **Hiroaki Todo:** Writing – original draft. **Jihei Nishimura:** Writing – original draft. **Mio Yagi:** Writing – original draft. **Keiko Yamamoto:** Writing – original draft. **Mariko Sugiyama:** Writing – original draft. **Yoshiaki Ikarashi:** Writing – original draft. **Hitoshi Sakaguchi:** Writing – original draft. **Masahiko Yamaguchi:** Writing – original draft. **Morihiko Hirota:** Conceptualization, Writing – original draft. **Sakiko Aizawa:** Conceptualization, Methodology, Data curation. **Shota Nakagawa:** Conceptualization, Methodology, Data curation. **Shigenobu Hagino:** Conceptualization, Methodology, Data curation, Writing – original draft. **Masato Hatao:** Conceptualization, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Author Contribution Statement

No.

Author Disclosure Statement

No competing financial interests exist.

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Appendix A. Supplementary data

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References

Anon, 2017. Guidebook for Cosmetics/Quasi-drug Manufacturing and Sales [in Japanese]. Yakuji Nippo, Limited, Tokyo, Japan.

Bulgheroni, A., Kinsner-Ovaskainen, A., Hoffmann, S., et al., 2009. Estimation of acute oral toxicity using the No Observed Adverse Effect Level (NOAEL) from the 28 days repeated dose toxicity studies in rats. Regul. Toxicol. Pharmacol. 53, 16–19.

Cosmetic Ingredient Review (2010) Final Report of the Cosmetic Ingredient Review Expert Panel. Amended Safety Assessment of Triethylene Glycol and Polyethylene Glycols (PEGs)-4, -6, -7, -8, -9, -10, -12, -14, -16, -18, -20, -32, -33, -40, -45, -55, -60, -75, -80, -90, -100, -135, -150, -180, -200, -220, -240, -350,-400, -450, -500, -800, -2M, -5M, -7M, -9M, -14M, -20M, -23M, -25M,-45M, -65M, -90M, -115M, -160M and -180M and any PEGs $\geq$ 4 as used in Cosmetics.

Cosmetic Ingredient Review. https://www.cir-safety.org/ingredients (Last accessed Mar. 2, 2022).

Cosmetic-Info.jp. https://www.cosmetic-info.jp/ (Last accessed Sep. 12, 2022).

ECHA, Information of chemicals. https://echa.europa.eu/registration-dossier/-/registered-dossier/15952/7/3/2 (Last accessed Sep. 12. 2022).

ECVAM. Follow-up study on the predictive capacity of the 3T3 Neutral Red Uptake cytotoxicity assay to correctly identify substances not classified for acute oral toxicity under the EU CLP system (LD50>2000mg/kg), Final study report. 2011. https://www.jacvam.jp/list.html (Last accessed Mar. 2, 2022).

EURL ECVAM. EURL ECVAM recommendation on the 3T3 Neutral Red Uptake cytotoxicity assay for acute oral toxicity. 2013. https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/eurl-ecvam-recommendation-3t3-neutral-red-uptake-cytotoxicity-assay-acute-oral-toxicity (Last accessed Mar. 2, 2022).

EFSA. EFSA Scientific Committee, European Food Safety Authority. Guidance on the use of the threshold of toxicological concern approach in food safety assessment. 2019. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2019.5708 (Last accessed Mar. 2, 2022).

Ekwall, B., Clemedson, C., Crafoord, B., et al., 1998. MEIC evaluation of Acute Systemic Toxicity: Part V. Rodent and human toxicity data for the 50 reference chemicals. Altern. Lab. Anim. 26 (Suppl. 2), 571–616.

European Chemicals Agency. Information on Chemicals. https://echa.europa.eu/information-on-chemicals (Last accessed Mar. 2, 2022).

FSCJ. Expert Committee on Novel Foods, Food Safety Commission of Japan. Basic concept regarding safety evaluation of foods for specified health uses [in Japanese]. 2004. https://www.fsc.go.jp/senmon/sinkaihatu/tokuho_kangaekata.pdf (Last accessed Mar. 2, 2022).

Halle, W., 2003. The registry of cytotoxicity: toxicity testing in cell cultures to predict acute toxicity (LD50) and to reduce testing in animals. Altern. Lab. Anim. 31, 89–198.

ICCVAM, NICEATM. ICCVAM test method evaluation report: *In vitro* cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity testing. 2006. https://ntp.niehs.nih.gov/iccvam/docs/acutetox_docs/brd_tmer/at-tmer-complete.pdf (Last accessed Sep. 1, 2021).

ICH. European Medicines Agency. ICH guideline M3 (R2) on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. ICH M3 (R2). pp 1–26; 2009.

ICH. Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk M7. 2014. https://www.pmda.go.jp/files/000208234.pdf (Last accessed Mar. 2, 2022).

JaCVAM acute toxicity editorial committee. Peer review report, *In vitro* cytotoxicity test for predicting acute oral toxicity [in Japanese], Dec. 11, 2018. https://www.jacvam.jp/files/list/07/07_02_C1.pdf (Last accessed Mar. 2, 2022).

JaCVAM regulatory acceptance board. Report of JaCVAM regulatory acceptance board. *In vitro* cytotoxicity test for predicting acute oral toxicity [in Japanese]. Apr. 24, 2019. https://www.jacvam.jp/files/list/07/07_02_B1.pdf (Last accessed Mar. 2, 2022).

Japan Cosmetic Industry Association, 2015. Guidance for the Safety Evaluation of Cosmetics [in Japanese]. Yakuji Nippo, Limited, Tokyo, Japan.

JHNFA. Review Committee on Health Food Safety Assessment, Japan Health and Nutrition Food Association. Guideline for the safety evaluation of Health foods [in Japanese]. 2008. http://www.jhnfa.org/topic76.pdf (Last accessed Mar. 2, 2022).

Koster, S., Boobis, A.R., Cubberley, R., et al., 2011. Application of the TTC concept to unknown substances found in analysis of foods. Food Chem. Toxicol. 49, 1643–1660.

MHLW. Additives list of quasi-drugs. 2008, https://www.mhlw.go.jp/topics/2008/04/dl/tp0407-2a.pdf (Last accessed Sep. 12, 2022).

MHLW. Chief of pharmaceuticals examination and management division, pharmaceutical and living hygiene bureau, Ministry of Health, Labor and Welfare. Assessment and control guideline of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk [in Japanese]. Nov. 10, 2015. https://www.pmda.go.jp/files/000208287.pdf (Last accessed Mar. 2, 2022).

MHLW. Standards for quasi-drug ingredients 2021. https://www.mhlw.go.jp/content/000843321.pdf (Last accessed Sep. 15, 2022).

Mitsubishi Chemical Research Corporation. Research report on risk assessment methods using threshold of toxicological concern (TTC) [in Japanese]. 2015. http://www.fsc.go.jp/fsciis/survey/show/cho20150030001 (Last accessed Mar. 2, 2022).

National Institute of Technology and Evaluation. Hazard Evaluation Support System Integrated Platform [in Japanese]. https://www.nite.go.jp/chem/qsar/hess.html (Last accessed Mar. 2, 2022).

NICEATM, NIEHS. Background review document: *In vitro* cytotoxicity test methods for estimating acute oral systemic toxicity. 2006. https://ntp.niehs.nih.gov/pubhealth/evalatm/test-method-evaluations/acute-systemic-tox/in-vitro-validation/index.html (Last accessed Sep. 1, 2021).

NIHS. Division of Risk Assessment, National Institutes of Health Sciences. Collection and evaluation of hazard information for designation of poisonous and deleterious substances [in Japanese]. http://www.nihs.go.jp/law/dokugeki/hyoukaindex.html (Last accessed Mar. 2, 2022a).

NIHS. Division of Risk Assessment, National Institutes of Health Sciences. Japan Existing Chemical Database [in Japanese]. http://dra4.nihs.go.jp/mhlw_data/jsp/SearchPage.jsp (Last accessed Mar. 2, 2022b).

OECD QSAR Toolbox. http://www.qsartoolbox.org (Last accessed Mar. 2, 2022).

OECD. Test Guideline 420. OECD guideline for testing of chemicals. Acute oral toxicity - Fixed dose procedure. 2002a. https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl420.pdf (Last accessed Sep. 1, 2021).

OECD. Test Guideline 423. OECD guideline for testing of chemicals. Acute oral toxicity - Acute toxic class method. 2002b. https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl423.pdf (Last accessed Sep. 1, 2021).

OECD. Test Guideline 425. OECD guideline for testing of chemicals. Acute oral toxicity: Up-and-down procedure. 2008. https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecdtg425.pdf (Last accessed Sep. 1, 2021).

OECD. Series on testing and assessment No. 129. Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests. 2010. https://ntp.niehs.nih.gov/pubhealth/evalatm/test-method-evaluations/acute-systemic-tox/in-vitro-validation/index.html (Last accessed Sep. 1, 2021).

Prieto, P., Cole, T., Curren, R., et al., 2013. Assessment of the predictive capacity of the 3T3 neutral red uptake cytotoxicity test method to identify substances not classified for acute oral toxicity (LD50>2000 mg/kg): results of an ECVAM validation study. Regul. Toxicol. Pharmacol. 65 (3), 344–365.

Registry of Toxic Effects of Chemical Substances. https://www.cdc.gov/niosh/rtecs/RTECSaccess.html (Last accessed Mar. 2, 2022).

SCCP/1043/06. https://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_081.pdf (Last accessed Sep. 12, 2022).

SCCS/1602/18. The SCCS notes of guidance for the testing of cosmetic ingredients and their safety evaluation 10th revision. 2018. https://ec.europa.eu/health/sites/health/files/scientific_committees/consumer_safety/docs/sccs_o_224.pdf (Last accessed Mar. 2, 2022).

SCCS/1610/19. Opinion on tetrabromophenol blue, 4,4'-(4,5,6,7-tetrabromo-1,1-dioxido-3H-2,1-benzoxathiol-3-yliden)bis-2,6-dibromophenol (C183) - Submission IV –. 2019. https://ec.europa.eu/health/sites/health/files/scientific_committees/consumer_safety/docs/sccs_o_232.pdf (Last accessed Mar. 2, 2022).

U.S. National Library of Medicine. ChemIDplus. https://chem.nlm.nih.gov/chemidplus/ (Last accessed Mar. 2, 2022).