The following reagents will be used and stored according to the instructions of manufacturers.

- $NaH_2PO_4 \cdot 2H_2O$ (CAS No. 13472-35-0)
- $Na_2HPO_4 \cdot 12H_2O$ (CAS No. 10039-32-4)
- p-Nitrosodimethylaniline (RNO, CAS No. 138-89-6)
- Imidazole (CAS No. 288-32-4)
- Nitroblue tetrazolium chloride (NBT, CAS No. 298-83-9)
- Purified water

Preparation of reagents

All reagents should be sonicated and used within 1 month after preparation. Representative preparation methods are shown as follows;

- 20 mM sodium phosphate buffer (NaPB), pH 7.4

  Weigh 593 mg of $NaH_2PO_4 \cdot 2H_2O$ and 5.8 g of $Na_2HPO_4 \cdot 12H_2O$, add 900 mL of purified water, adjust with HCl to a pH of 7.4, dilute with purified water up to 1 L, and mix.

  Stored in a refrigerator or at room temperature.
- 0.2 mM p-Nitrosodimethylaniline (RNO)

  Dissolve 3 mg of RNO in 100 mL of 20 mM NaPB.

  Stored in a refrigerator and protect from light.
- 20 mM imidazole

  Dissolve 13.6 mg of imidazole in 10 mL of 20 mM NaPB.

  Dilute the 2×10^{-2} M imidazole solution 100 times with 20 mM NaPB.

  Stored in a refrigerator and protect from light.
- 0.4 mM nitroblue tetrazolium chloride (NBT)

  Dissolve 32.7 mg of NBT in 100 mL of 20 mM NaPB.

  Stored in a refrigerator and protect from light.

Test chemicals

The test chemical should be stored appropriately until termination of the study and its stability during the test period should be confirmed. Two concentration levels, 20 and 200 μM (final concentration), should be used.

Preparation of test chemicals

The test chemicals will be prepared using DMSO just before use. Each test chemical will be weighed in a tube, and added DMSO at the concentration 10 mM at first. The tube will be mixed with vortex mixer and sonicated for 5 to 10 min under UV-cut illumination or shade. All

preparations should be kept to protect from light. The final concentration in a reaction mixture will be set at 200 μM. When precipitation is observed at 20 μM in the reaction mixture under a microscope, 1 mM of the test chemical solution should be prepared using DMSO. In the case of DMSO-insoluble chemical, the final concentration in the reaction mixture including 20 μL of DMSO will be used at the maximum concentration without precipitation (20 or 200 μM).

Positive and negative control chemicals

Qunine HCl (CAS No. 6119-47-7) should be used at 200 μM (final concentration) as a positive control. Sulisobenzone (CAS No. 4065-45-6) should be used at 200 μM (final concentration) as a negative control.

Preparation of positive and negative control chemicals

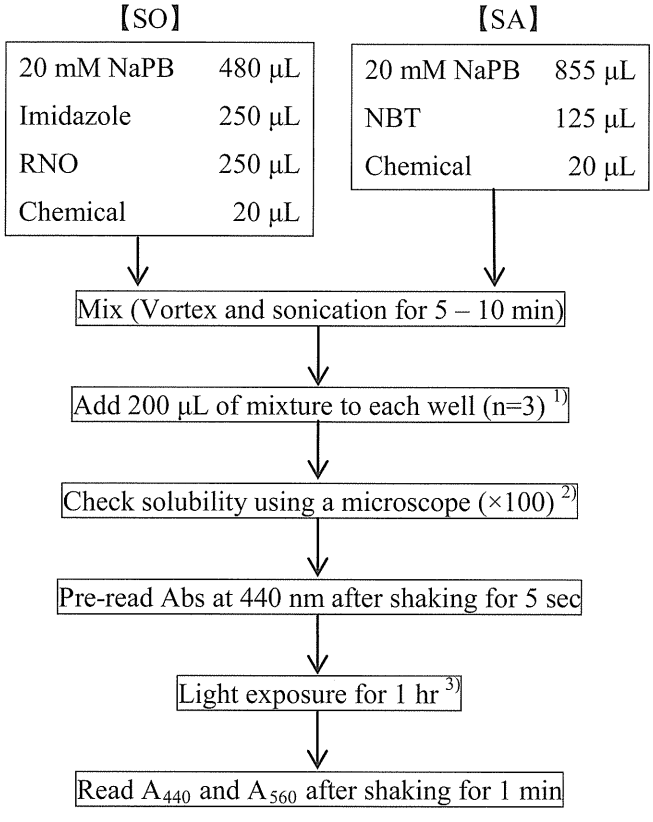
Stock solutions of qunine and sulisobenzone will be prepared at 10 mM each in DMSO (the final concentration of 200 μM) according to the above procedure, divided into some tubes, and stored in a freezer (generally below -20°C) for up to 1 month. The stock solution will be thawed just before the experiment and used within the day.

Solvent

DMSO (analytical grade) should be used at first. In the case of DMSO-insoluble chemical, 20 mM NaPB should be used. When a test chemical is insoluble in either DMSO or 20 mM NaPB, bovine serum albumin (BSA) or other solvent might be helpful [7-8]. Prior to use of BSA or other solvent, a feasibility study (see section 6) should be conducted using the reference chemicals to determine appropriate test conditions. However, classification for regulatory purposes should not be based on a ROS assay using BSA or other solvent until these solvents have been properly evaluated.

Test procedure

A tube (e.g. 1.5 mL of micro tube) and a plastic clear flat bottomed 96-well microplate will be used. The reaction mixture should be prepared by vortex mixing and/or sonication under UV-cut illumination or shade. The same volume of DMSO, 20μL, will be added in a blank instead of test chemical solution.



Notes

1) It should be avoided using peripheral wells. More than one test chemical could be tested on a plate. An example of 96-well plate configuration is shown as follows;

	1	2	3	4	5	6	7	8	9	10	11	12
A					Singlet oxygen							
B		B	P	N	T1 L	T1 H	T2 L	T2 H	T3 L	T3 H		
C		B	P	N	T1 L	T1 H	T2 L	T2 H	T3 L	T3 H		
D		B	P	N	T1 L	T1 H	T2 L	T2 H	T3 L	T3 H		
E		B	P	N	T1 L	T1 H	T2 L	T2 H	T3 L	T3 H		
F		B	P	N	T1 L	T1 H	T2 L	T2 H	T3 L	T3 H		
G		B	P	N	T1 L	T1 H	T2 L	T2 H	T3 L	T3 H		
H					Superoxide anion							

B: Blank (DMSO)
P: Quinine, 200 μM
N: Sulisobenzone, 200 μM
T1-T3: Test chemical No. 1-3
L: 20 μM
H: 200 μM

2) Some chemicals may precipitate in the reaction mixture. Therefore it is important to check the solubility before the irradiation. The solubility of each reaction mixture in a well should be observed by using a microscope before irradiation. Test chemicals concentrations should be selected so as to avoid precipitation or cloudy solutions.

3) The 96-well plate will be placed in to the Quartz reaction container. A quarts cover will be set on the plate and fasten with bolts. The solar simulator and the temperature control unit (or its equivalent) will be used under a stable condition. Before and after irradiation, UVA intensity and temperature at the plate position will be measured using a UVA detector and thermometer.

5. DATA AND REPORTING

Data analysis

Data from three wells for each chemical concentration will be used to calculate mean and standard deviation.

- SO

  Decrease of $A_{440} \times 1000 = [A_{440}(-) - A_{440}(+) - (a - b)] \times 1000$

  $A_{440}(-)$: Absorbance before light exposure at 440 nm

  $A_{440}(+)$: Absorbance after light exposure at 440 nm

  a: Blank before light exposure (mean)

  b: Blank after exposure (mean)

- SA

  Increase of $A_{440} \times 1000 = [A_{560}(+) - A_{560}(-) - (b - a)] \times 1000$

  $A_{560}(-)$: Absorbance before light exposure at 560 nm

  $A_{560}(+)$: Absorbance after light exposure at 560 nm

  a: Blank before light exposure (mean)

  b: Blank after exposure (mean)

Criteria for data acceptance

The following criteria should be satisfied in each experiment.

- No precipitation of test chemical in the reaction mixture before light exposure.
- No technical problems when collecting data set (including temperature within prescribed range).
- Raw OD value: 0.02 to 1.5
- Historical positive and negative control values should be developed by each laboratory based

on a mean +/-2 SD. The following range was defined based on the 95% confidence interval (mean +/- 1.96SD) obtained from the validation data. When other solar simulators are used, establish modified criteria based on 95% confidence interval.

Positive control value at 200 μM (mean of 3 wells)

- SO: 319 to 583
- SA: 193 to 385

Negative control value at 200 μM (mean of 3 wells)

- SO: -9 to 11
- SA: -20 to 2

Judgment criteria

Each test chemical will be judged as follows;

Judgment[1)]	Concentration judged	SO (mean of 3 wells)		SA (mean of 3 wells)
Photoreactive	20 and/or 200 μM[2)]	≥25	and	≥70
		<25 and/or P[3)]	and	≥70
		≥25	and	<70 and/or P
Weakly photoreactive	20 and 200 μM[2)]	<25	and	≥20, <70
Non-photoreactive	20 and 200 μM[2)]	<25	and	<70
Inconclusive	The results do not meet the above-mentioned criterion.[4)]			

Notes

1) It can be judged based on results of one experiment because the ROS assay shows good reproducibility in the validation studies.
2) It would be judged at 20 μM only when precipitation is observed at 200 μM.
3) Precipitation before irradiation.
4) When precipitation is observed at 20 and 200 μM before irradiation, the compound is regarded incompatible with the ROS assay.

Data quality

For a regulatory purpose, the study should be conducted under high-quality standards with data collection records readily available, preferably in compliance with GLP/GMP regulations and all documents should be checked by the Quality Assurance Unit of the laboratory.

Test report

The test report must include the following information:

- Test chemical

  - Name and lot No.
  - Physical nature and purity
  - Storage condition
  - Stability during the test period
  - UV/vis absorption spectrum, maximum molar extinction coefficient at 290 to 700 nm, and/or photostability, if known
  - Preparation of test chemical solution
  - Final concentrations tested
- Control chemicals
  - Name, manufacturer, and lot No.
  - Physical nature and purity
  - Storage condition
  - Preparation of control chemical solutions
  - Final concentrations tested
- Solvent
  - Name, manufacturer, and lot No.
  - Justification for choice of solvent
- Irradiation condition
  - Manufacturer and type of the solar simulator used
  - Rationale for selection of the solar simulator used
  - UVA detector used
  - UVA irradiance, expressed in mW/cm^2
  - UVA dose, expressed in J/cm^2
  - Temperature before and after irradiation
- ROS assay procedure
- Acceptance and decision criteria
- Results
- Discussion
- Conclusions

Archives

The study report and all raw data will be retained according to the SOP in the testing facility.

6. REFERENCE CHEMICALS FOR THE FEASIBILITY STUDY

For establishing ROS assay, irradiation condition to satisfy the recommended criteria should be determined using the positive and negative controls at 200 μM. The reference chemicals should be tested at 200 μM in a feasibility study prior to test. The reference chemicals should be selected from the following 21 chemicals judged at 200 μM in the validation studies. Recommended reference chemical set and acceptable range are shown in Table 1 and Table 2. Values of SO and SA should be close to the values.

- Photoreactive chemicals

  Acridine, acridine hydrochloride, chlorpromazine hydrochloride, diclofenac, doxycycline hydrochloride, furosemide, ketoprofen, 6-methylcoumarine, 8-methoxy psoralen (8-MOP), nalidixic acid, nalidixic sodium salt, norfloxacin, ofloxacin, omeprazole, promethazine hydrochloride, and tetracycline.

- Non-photoreactive chemicals

  Aspirin, benzocaine, camphor sulfonic acid, erythromycin, and p-aminobenzoic acid (PABA).

Table 1 Recommended chemical set for solar simulators used in the validation studies and the acceptable range at 200 μM: 3 photoreactive chemicals showing strong (No. 11), moderate (No. 12), and weak (No. 13) responses and 3 non-photoreactive chemicals (No. 14-16)

No.	Chemical	CAS No.	SO	SA
Photoreactive chemicals				
11	Doxycycline hydrochloride	10592-13-9	≥115 (115 to 429)	≥230 (230 to 468)
12	Norfloxacin	70458-96-7	≥131 (131 to 271)	≥57 (57 to 161)
13	8-MOP	298-81-7	≥31 (31 to 137)	≥20 (0 to 126)
Non-photoreactive chemicals				
14	Benzocaine	94-09-7	<25 (7 to 9)	<20 (7 to 17)
15	Erythromycin	114-07-8	<25 (-15 to 11)	<20 (9 to 19)
16	PABA	150-13-0	<25 (-8 to 12)	<20 (-11 to 7)

DMSO should be used for preparation of the chemicals.

The values in parenthesis were calculated as mean +/- 1.96 SD from the validation data.

Table 2 Recommended chemical set for the other solar simulators and the acceptable range at 200 μM: 11 photoreactive chemicals (No. 21-31) and 3 non-photoreactive chemicals (No. 32-34)

No.	Chemical	CAS No.	SO	SA
Photoreactive chemicals				
21	Acridine	260-94-6	≥182 (182 to 328)	≥121 (121 to 243)
22	Chlorpromazine hydrochloride	69-09-0	Usually <25	≥66 (66 to 106)
23	Diclofenac	15307-79-6	≥34 (34 to 416)	≥47 (47 to 437)
24	Doxycycline hydrochloride	10592-13-9	≥115 (115 to 429)	≥230 (230 to 468)
25	Furosemide	54-31-9	≥31 (31 to 225)	≥20 (-7 to 109)
26	Ketoprofen	22071-15-4	≥120 (120 to 346)	≥77 (77 to 151)
27	8-MOP	298-81-7	≥31 (31 to 137)	>20 (0 to 126)
28	Nalidixic acid	389-08-2	≥54 (54 to 246)	≥88 (88 to 470)
29	Norfloxacin	70458-96-7	≥131 (131 to 271)	≥57 (57 to 161)
30	Omeprazole	73590-58-6	Usually <25	≥30 (30 to 216)
31	Promethazine hydrochloride	58-33-3	≥25 (20 to 168)	≥20 (-3 to 77)
Non-photoreactive chemicals				
32	Benzocaine	94-09-7	<25 (-7 to 9)	<20 (-7 to 17)
33	Erythromycin	114-07-8	<25 (-15 to 11)	<20 (-9 to 19)
34	PABA	150-13-0	<25 (-8 to 12)	<20 (-11 to 7)

DMSO should be used for preparation of the chemicals.

The values in parenthesis were calculated as mean +/- 1.96 SD from the validation data.

7. GLOSSARY

ROS: Reactive Oxygen Species, including superoxide anion (SA) and singlet oxygen (SO).

3T3 NRU-PT: *In vitro* 3T3 neutral red uptake phototoxicity test.

Irradiance: The intensity of UV or visible light incident on a surface, measured in W/m^2 or mW/cm^2.

Dose of light: The quantity [= intensity × time (seconds)] of UV or visible light incident on a surface, expressed in J/m^2 or J/cm^2.

MEC: Molar Extinction Coefficient (also called molar absorptivity) is a constant for any given molecule under a specific set of conditions (e.g., solvent, temperature, and wavelength) and reflects the efficiency with which a molecule can absorb a photon (typically expressed as $L\ mol^{-1}\ cm^{-1}$).

Photoreactivity: The property of chemicals that react with another molecule as a consequence of absorption of photons.

Phototoxicity: An acute light-induced tissue response to a photoreactive chemical.

UVA: Ultraviolet light A (wavelengths between 320 and 400 nm).

UVB: Ultraviolet light B (wavelengths between 290 and 320 nm).

UVC: Ultraviolet light B (wavelengths between 190 and 290 nm).

8. REFFERENCES

[1] ROS assay validation management team. Validation report for the international validation study on ROS (Reactive Oxygen Species) assay as a test evaluating phototoxic potential of chemicals (Atlas Suntest version), 2013.

[2] ROS assay validation management team. Validation report for the international validation study on ROS (Reactive Oxygen Species) assay as a test evaluating phototoxic potential of chemicals (Seric version), 2013.

[3] Onoue S, Hosoi K, Wakuri S, Iwase Y, Yamamoto T, Matsuoka N, Nakamura K, Toda T, Takagi H, Osaki N, Matsumoto Y, Kawakami S, Seto Y, Kato M, Yamada S, Ohno Y, Kojima H. Establishment and intra-/inter-laboratory validation of a standard protocol of reactive oxygen species assay for chemical photosafety evaluation. J Appl Toxicol, Article first published online, 13 June, 2012 (DOI: 10.1002/jat.2776).

[4] I. Kraljić, S. El Mohsni. A new method for the detection of singlet oxygen in aqueous solutions. Photochem Photobiol, 28, 577-81, 1978.

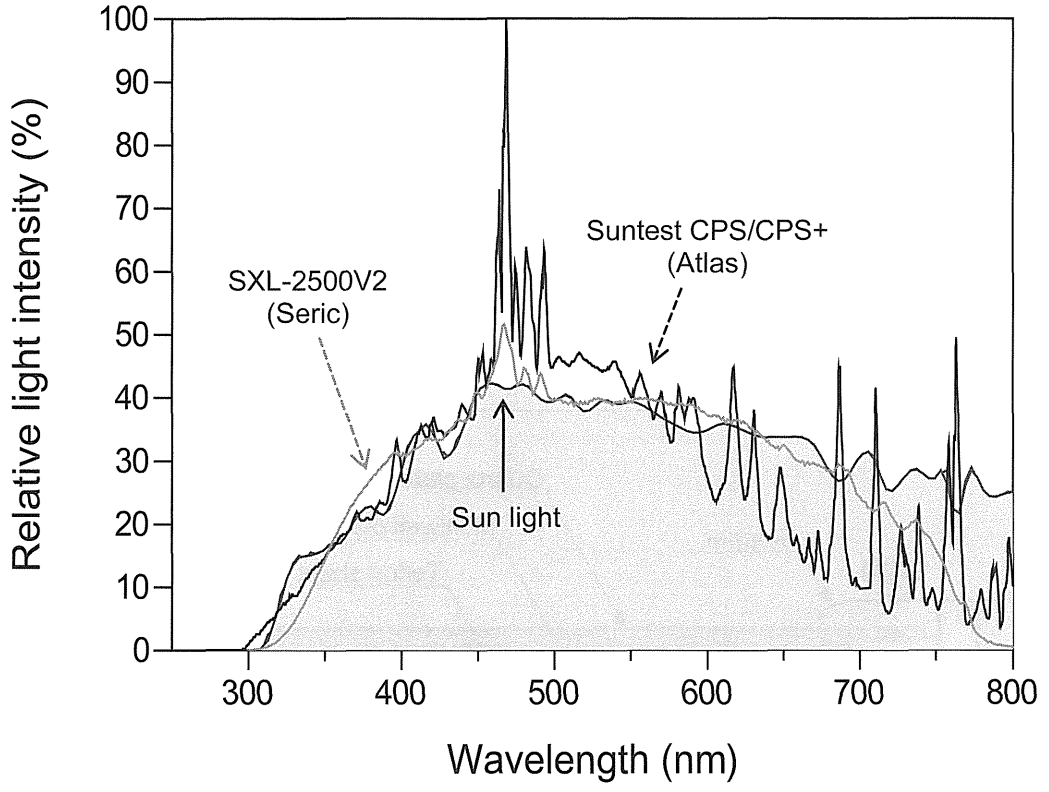
[5] Pathak MA, Joshi PC. Production of active oxygen species ($^1O_2$ and $O_2^{\cdot-}$) by psoralens and ultraviolet radiation (320-400 nm). Biochim Biophys Acta, 798, 115-26, 1984.

[6] Onoue S, Igarashi N, Yamada S, Tsuda Y. High-throughput reactive oxygen species (ROS) assay: an enabling technology for screening the phototoxic potential of pharmaceutical substances. J Pharm Biomed Anal, 46, 187-93, 2008.

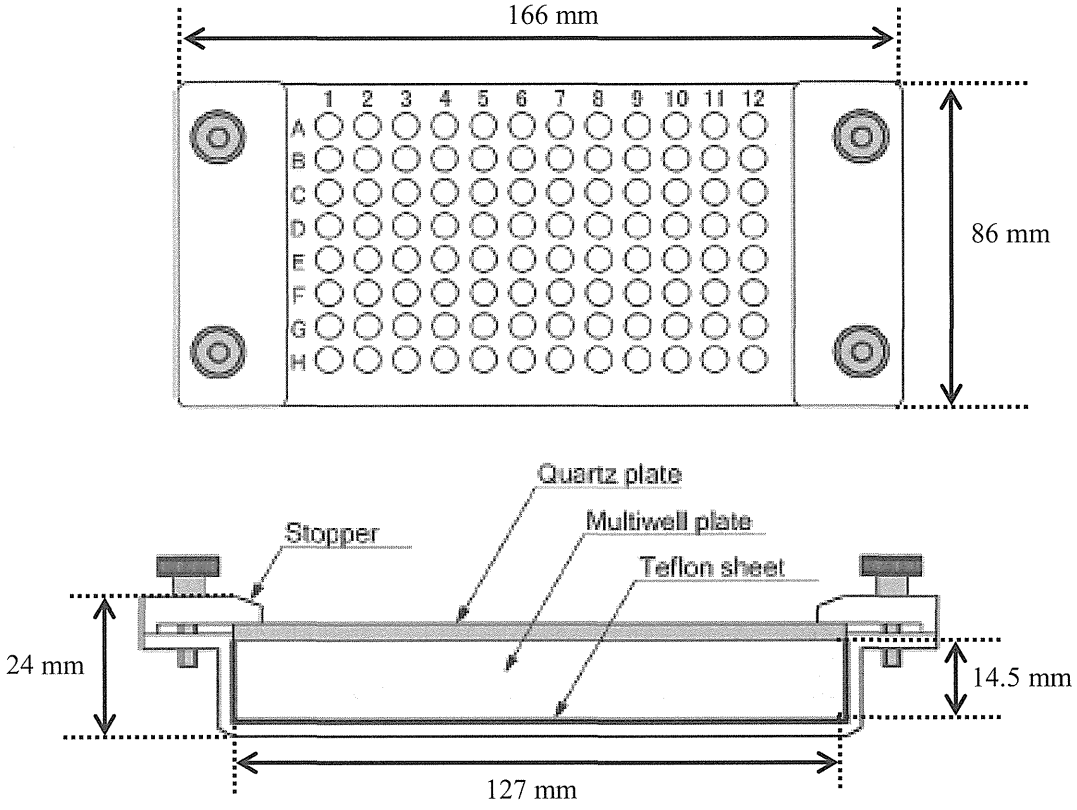
[7] Onoue S, Yamauchi Y, Kojima T, Igarashi N, Tsuda Y. Analytical studies on photochemical behavior of phototoxic substances; effect of detergent additives on singlet oxygen generation. Pharm Res, 25, 861-868, 2008.

[8] Onoue S, Kato M, Yamada S. Development of an albuminous reactive oxygen species assay for photosafety evaluation under experimental biomimetic conditions. J Appl Toxicol, Article first published online, 28 Jan, 2013 (DOI: 10.1002/jat.2846).

Appendix 1 Spectrum of solar simulators used in the validation studies



Appendix 2 Quarts reaction container used in the validation studies



Peer Review Panel Evaluation of the Reactive Oxygen Species (ROS) Photosafety Assay

Japanese Center for the Validation of Alternative Methods
National Institute of Health Sciences
Tokyo, Japan

16 October 2013

Table of Contents

Executive Summary

The Japanese Center for the Validation of Alternative Methods (JaCVAM) convened an independent scientific peer review panel to evaluate the validation status of the Reactive Oxygen Species (ROS) assay in accordance with established international criteria (OECD, 2005). The ROS assay is a test method proposed as a component of an integrated photosafety testing strategy to evaluate whether test substances such as pharmaceuticals have the potential to cause phototoxicity.

The panel met initially in February and again in August 2013 in Tokyo, Japan. The panel considered the reports of two international validation studies and a proposed ROS Assay protocol at their initial meeting. The panel subsequently reviewed updated versions of the ROS Assay protocol and the validation study reports as revised by the Validation Management Team (VMT). This report summarizes the panel's final evaluation and conclusions.

Overall conclusion: The panel concluded that the reproducibility and predictivity of the ROS assay is sufficient to support its use in an integrated photosafety testing and decision strategy for drug research and development. In this strategy, negative results in the ROS assay would not require further testing in animals or other tests, while positive, weakly positive, and inconclusive results would proceed to the next level of testing in an *in vitro* test system such as the 3T3 Phototoxicity Assay (OECD Test Guideline 432). The panel also concluded that use of the ROS assay could potentially provide significant savings in time, cost and reduced animal use for photosafety assessments. Furthermore, incorporating the ROS assay into a photosafety testing strategy is expected to significantly reduce the overall number of substances that would require additional testing in the in vitro 3T3 Phototoxicity Assay and subsequent testing in animals.

Regulatory rationale: The panel concluded that the ROS Assay is applicable for use within the ICH regulatory testing framework for photosafety evaluation of pharmaceutical products. Regulatory authorities (e.g. PMDA/MHLW, U.S. FDA, EMEA) require non-clinical photosafety testing prior to approving First-in-Human Phase I studies so that appropriate precautions and observations can be taken during initial human studies. Such non-clinical photosafety testing typically includes an assessment of the potential for a drug to cause phototoxic reactions, which are characterized clinically by dermal redness, swelling, irritation, and inflammation. The panel also agreed that the ROS assay is applicable to in-house drug research and development.

Scientific rationale: The panel recognized that ROS production is the most important mechanism for chemically-induced phototoxicity, and is therefore a critical pathway initiating event leading to phototoxicity. The ROS assay quantitatively measures two common reactive oxygen species generated by photoreactive chemicals after exposure to simulated sunlight. In this validation study, chemicals that did not produce sufficient ROS to meet the photoreactivity threshold classification criteria for the ROS assay are uniformly non-phototoxic, while chemicals that met or exceeded the photoreactivity classification criteria include all known phototoxicants. Therefore if a chemical is not photoreactive in the ROS assay, it is unlikely that phototoxicity will occur in living systems.

Limitations: The panel noted that the ROS assay assesses chemical photoreactivity in a non-biological system, and therefore may overpredict phototoxicity potential since it does not assess the direct interaction of chemicals with biological tissues. The assay may also overestimate the

potential for phototoxicity because some chemicals may not achieve sufficient concentration in skin for phototoxic reactions to occur, or photodegradation may occur. Accordingly, positive results in the ROS assay are generally recommended for further evaluation in a photosafety testing strategy.

Validation study reference chemicals: The panel agreed that the reference chemicals selected for the validation studies were appropriate and sufficiently representative of the chemicals likely to be evaluated in the assay. The 42 reference chemicals incorporated most known human phototoxicants and included 23 known positives and 19 negatives. The chemicals were backed by data from human patch testing and in vitro 3T3 phototoxicity assay results. All data from the validation studies were made available in the validation study reports.

Assay Reproducibility: The panel concluded that the assay had excellent reproducibility both within and between laboratories for the 42 reference chemicals evaluated in the validation studies. Additionally, the positive and negative control chemicals had 100% reproducibility within and between laboratories based on classification outcome, which further supports the reproducibility of the ROS assay.

Test method predictivity: After reviewing analyses provided in the validation study reports, the panel agreed that conducting a single assay per chemical provided optimal predictivity. The panel concluded that the classification criteria for test outcomes have been appropriately optimized to avoid false negatives while minimizing false positives. The panel also noted that chemicals positive for both reactive oxygen species were uniformly phototoxic.

Data quality: The panel agreed that the high level of within and between laboratory reproducibility suggested a consistently high level of quality of the validation studies. While the studies were not conducted in strict accordance with GLPs, most of the labs were GLP certified. The validation management team also confirmed that quality control audits found that validation report data accurately reflected the raw data results.

Test method protocols: The panel considered the test method protocols used for the two validation studies and key aspects of a proposed standardized ROS assay protocol. The panel recommended that the solar simulator should be equipped with an appropriate temperature control unit or fan since ROS production can be influenced by temperature. The panel concluded that the list of proficiency chemicals provided in the test method protocol for laboratories to use to demonstrate ability to perform the assay was appropriate. The panel recommended that each lab should develop historical positive and negative control value acceptance ranges that can be used to determine the acceptability of an individual test. The panel also agreed with the appropriateness of the reference chemicals identified for qualification of solar simulators other than the two used in the validation studies.

Applicability domain: The applicability domain of the ROS assay is currently restricted to only those chemicals that meet the solubility criteria outlined in the protocol. The panel recommended that as experience is gained from use of the ROS assay, the applicability domain could be more fully described in terms of physicochemical properties and/or chemical classes. This will contribute to increased efficiency by providing criteria that can be used to identify whether a chemical may be satisfactorily tested in the ROS assay, or whether an alternate assay should be used initially.

Peer Review Panel Evaluation of the ROS Assay

Introduction

The Japanese Center for the Validation of Alternative Methods (JaCVAM) convened an independent scientific peer review panel to evaluate the validation status of the Reactive Oxygen Species (ROS) Assay in accordance with established international validation and acceptance criteria (OECD, 2005). The ROS Assay is a test method proposed to evaluate whether test substances such as pharmaceuticals may have the potential to cause phototoxicity.

The panel met initially in February and again in August 2013 in Tokyo. The panel considered the reports of two international validation studies and a proposed outline for a ROS assay protocol at their initial meeting. Following provision of a complete ROS assay protocol by the Validation Management Team (VMT) and updating of the validation study reports, the panel met a second time to complete its evaluation. In conducting its evaluation, the panel addressed each of the evaluation criteria that correspond to internationally harmonized validation and acceptance criteria. This report summarizes the panel's final evaluation and conclusions.

Evaluation Criterion 1: A rationale for the test method should be available, including description of toxicological mechanisms, a clear statement of scientific need, and regulatory application.

The panel concluded that the ROS assay is applicable for use within the ICH regulatory testing framework for photosafety evaluation of pharmaceutical products. Regulatory authorities (e.g. PMDA/MHLW, U.S. FDA, EMEA, KFDA) require non-clinical photosafety testing prior to approving First-in-Human Phase I studies so that appropriate precautions and observations can be taken during initial human studies. Such non-clinical photosafety testing typically includes an assessment of the potential for a drug to cause phototoxic reactions, which are characterized by dermal redness, swelling, irritation, and inflammation. The panel also recognized that the ROS assay is applicable to in-house drug research and development. A proposed integrated photosafety testing strategy incorporating the ROS assay is provided below as Figure 1.

Chemicals that exhibit the potential for phototoxicity should be identified and if appropriate, eliminated in the early stages of drug discovery and development. Ideally, drugs should not be phototoxic. However, some beneficial drugs that have phototoxicity potential may be unavoidable, in which case it is important to ensure that there are appropriate precautions on drug labels so that patients can avoid exposures to sunlight that could lead to adverse reactions.

ROS production is the most important mechanism for inducing chemical phototoxicity. Physicochemical tests such as the ROS Assay enable the identification of ROS production by chemicals after exposure to UV and/or visible light.

.

Figure 1
Proposed integrated photosafety testing strategy incorporating the ROS Photosafety Assay (courtesy of Dr. Hosoi)

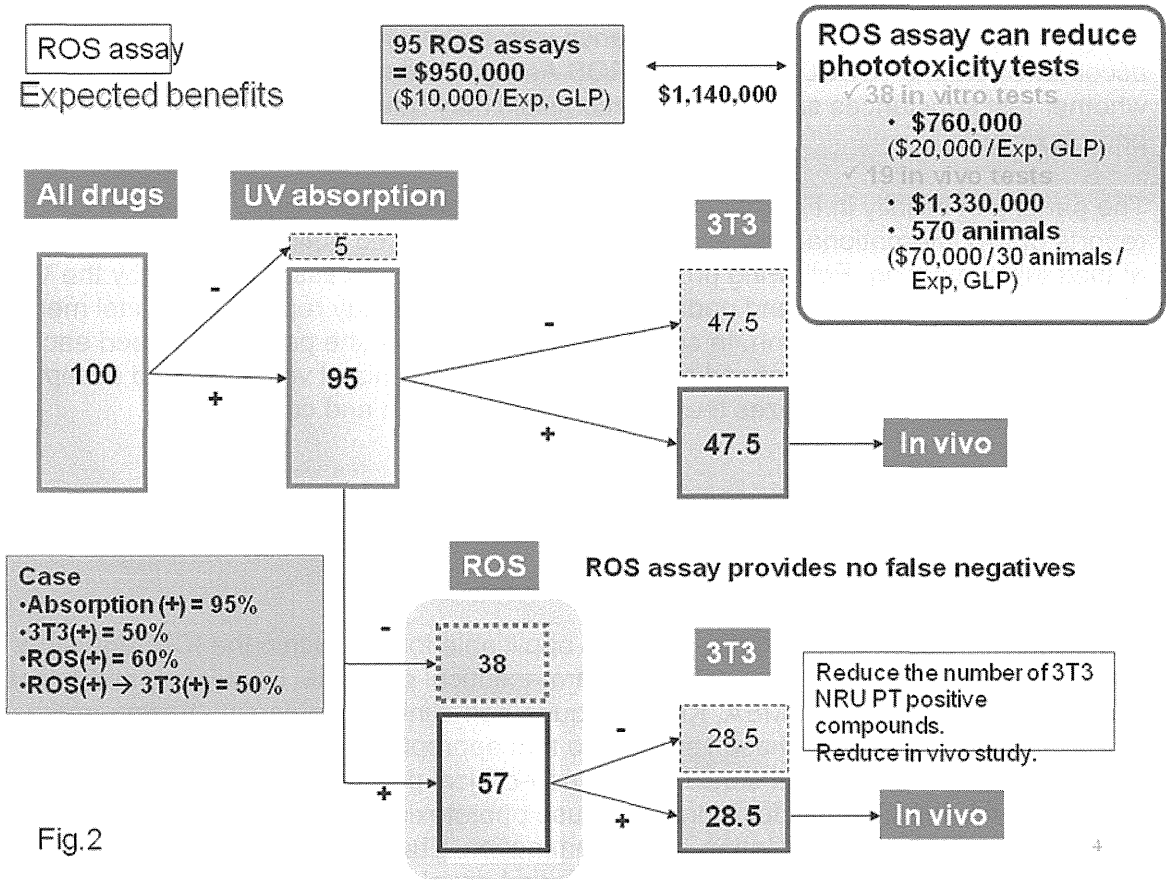


Fig.2

Evaluation Criterion 2: The relationship between the test method endpoint(s) and the biological effect and to the toxicity of interest should be addressed, describing limitations of the test methods.

Scientific rationale The ROS assay is based on identifying reactive oxygen species produced by photoreactive chemicals after exposure to UV and/or visible light. This mechanism is the basis for phototoxic reactions in the skin of humans, e.g., redness, swelling, irritation, and inflammation. The panel recognized that ROS production is the most important mechanism for chemically-induced phototoxicity, and is therefore a critical pathway initiating event leading to phototoxicity. The ROS assay quantitatively measures two common reactive oxygen species generated by photoreactive chemicals after exposure to simulated sunlight. In this validation study, chemicals that did not produce sufficient ROS to meet the positive photoreactivity threshold classification criteria for this assay are uniformly non-phototoxic, while chemicals that met or exceeded the positive classification criteria include all known phototoxicants. Therefore if a chemical is negative in the ROS assay it is unlikely that phototoxicity will occur in living systems.

Limitations: The panel noted that the ROS assay assesses chemical photoreactivity in a non-biological system, and therefore may overpredict phototoxicity potential since it does not assess the direct interaction of chemicals with biological tissues. Additionally, the initiation of phototoxic reaction in humans depends on pharmacokinetics and sufficient concentration in the target tissue, which cannot be assessed in this assay. The assay may also overestimate the potential for phototoxicity because some chemicals may not achieve sufficient concentration in skin for phototoxic reactions to occur, or photodegradation may occur. Accordingly, positive results in the ROS assay are generally recommended for further evaluation in a photosafety testing strategy.

Evaluation Criterion 3: A detailed test method protocol should be available.

The panel considered the test method protocols used for the two validation studies and key aspects of a proposed standardized ROS assay protocol. The panel concluded that the proposed ROS assay protocol was sufficiently detailed to allow for users to successfully perform the procedure. The panel also concluded that the protocol included adequate and appropriate analysis and classification criteria. The panel recommended that the solar simulator should be equipped with an appropriate temperature control unit or fan since ROS production can be influenced by temperature. The panel concluded that the list of proficiency chemicals provided in the test method protocol for laboratories to use to demonstrate ability to perform the assay was appropriate. The panel recommended that each lab should develop historical positive and negative control value acceptance ranges that can be used to determine the acceptability of an individual test.

Evaluation Criterion 4: Within- and between-laboratory reproducibility of the test method should be demonstrated.

The panel concluded that the assay demonstrated excellent reproducibility both within and between laboratories for the 42 reference chemicals evaluated in the validation studies. Additionally, the positive and negative control chemicals had 100% reproducibility within and between laboratories based on classification outcome, which further supports the reproducibility of the ROS assay.

Evaluation Criterion 5: Demonstration of the test method's performance should be based on testing of representative, preferably coded reference chemicals.

The panel agreed that the reference chemicals selected for the validation studies were appropriate, and sufficiently representative of the chemicals likely to be evaluated in the assay. The 42 reference chemicals incorporated most known human phototoxicants and included 23 known positives and 19 negatives. The chemicals were backed by data from human patch testing and in vitro 3T3 phototoxicity assay results. The validation reference chemicals were appropriately coded to minimize bias by performing labs. All data from the validation studies were made available in the validation study reports.

The panel noted the potential importance of chemical structure, and acknowledged the VMT for incorporating chemical structures for all chemicals in the validation report. In addition, the panel noted that the VMT also assessed and described whether the current drug label information for Japan and U.S. included precautionary language for phototoxicity.

Evaluation Criterion 6: Accuracy or predictive capacity should be demonstrated using representative chemicals. The performance of test methods should have been evaluated in relation to existing relevant toxicity data as well as information from the relevant target species.

After reviewing analyses provided in the study reports, the panel agreed with the VMT that a single assay per chemical provided optimal predictivity. The panel concluded that the classification criteria for test outcomes had been appropriately optimized to avoid false negatives while minimizing false positives (see ROS assay protocol judgment criteria). Appropriate criteria are provided for photoreactive, weakly photoreactive, non-photoreactive, and inconclusive classifications. In the first validation study (Atlas solar simulator), two phototoxic and one non-phototoxic reference chemicals were classified as inconclusive due to solubility issues, and were not included in the integrated accuracy calculations. In the second validation study (Seric solar simulator), three phototoxic and four non-phototoxic reference chemicals were classified as inconclusive due to solubility issues, and were not included in the integrated accuracy calculations.

All of the phototoxic reference chemicals that produced conclusive results were identified as photoreactive in both validation studies, resulting in a sensitivity of 100% and a false negative rate of 0%. In the first validation study, of the 18 non-phototoxic reference chemicals that provided conclusive results, 15 were identified as non-photoreactive and three were classified as weakly photoreactive, resulting in a specificity of 83.3 %(15/18), and a false positive rate of 16.7% (3/18). In the second study, of the 15 non-phototoxic chemicals for which there were conclusive results, 12 were identified as non-phototoxic, two were classified as weakly