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

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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.

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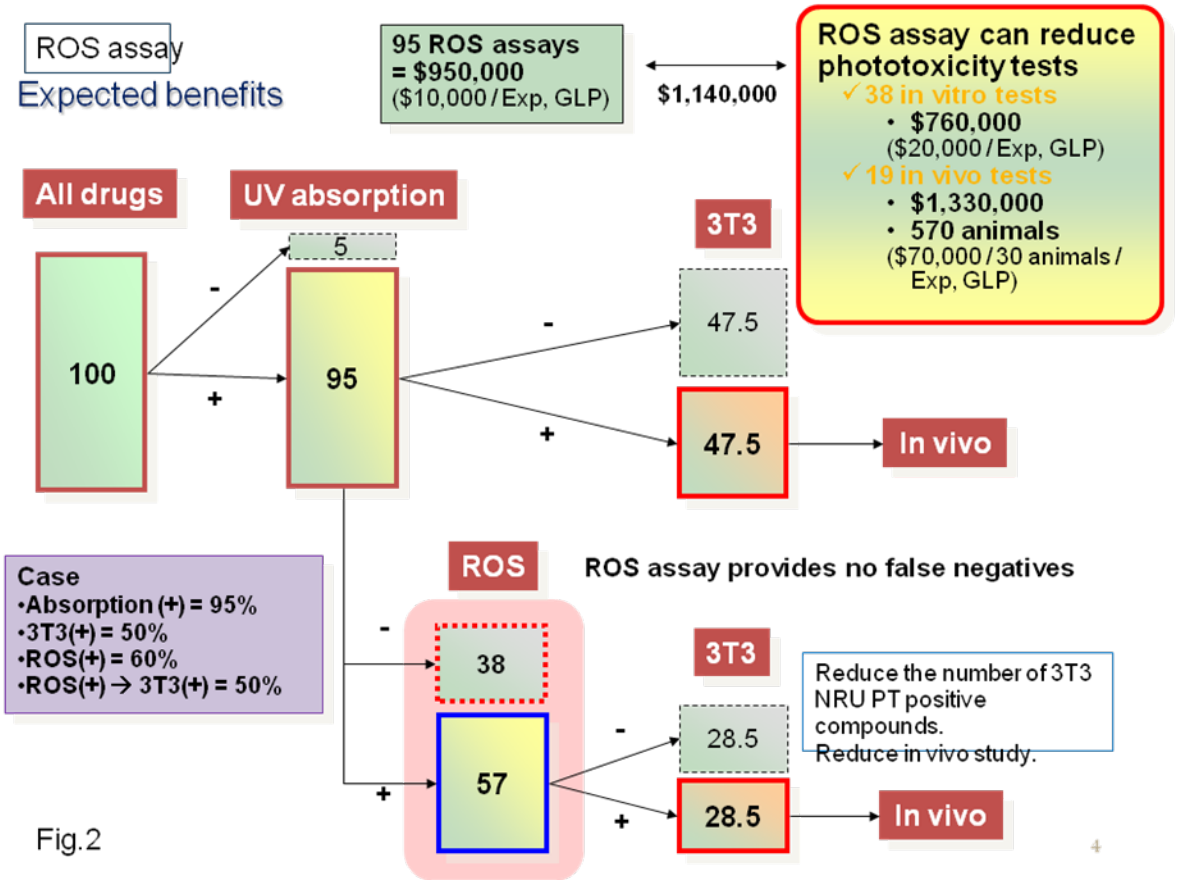
Figure 1
Proposed integrated photosafety testing strategy incorporating the ROS Photosafety Assay (courtesy of Dr. Hosoi)



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

photoreactive, and one was classified as phototoxic, resulting in a specificity of 80% (12/15), and a false positive rate of 20% (3/15). However, it is important to note that of the non-phototoxic chemicals producing photoreactive results, all three responses were categorized as weakly photoreactive in the first study, and 2 of the 3 responses were categorized as weakly photoreactive in the second study.

Evaluation Criterion 7: All data supporting the assessment of the validity of the test method should be available for expert review.

All raw data for the two validation studies was provided in the validation study reports, which are readily available electronically from the Japanese Center for the Validation of Alternative Methods at the National Institute of Health Sciences, Tokyo, Japan.

Evaluation Criterion 8: Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of Good Laboratory Practice (GLP).

The panel concluded that there was a high level of within and between laboratory reproducibility, which suggested a consistently high level of quality of the validation studies. While the studies were not conducted in strict accordance with GLPs, six of the seven laboratories participating in the validation studies were GLP certified. This included two of three of the labs in Study #1 (Atlas), and all four of the labs participating in Study #2 (Seric). There was no significant variability between laboratories, which suggested a consistent level of quality. The validation management team also confirmed that quality control audits found that validation report data accurately reflected the raw data results.

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Evaluation Criterion 9: The applicability domain of the validity of the test method should be defined for expert review.

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 would 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.

Chemicals that are insoluble in the recommended vehicles and therefore are not suitable for testing with this assay may be able to be tested in other vehicles, such as BSA, alcohol, and acetone. However, further characterization and standardization of procedures using these alternative vehicles should be performed before incorporation into routine use.

Evaluation Criterion 10: Proficiency chemicals should be provided in the proposed protocol.

The panel concluded that the list of 9 proficiency chemicals provided in the test method protocol for laboratories to use to demonstrate ability to perform the assay was appropriate. These 9 chemicals were selected from the validation study reference chemicals and represent a wide range of responses in the assay as well as a wide range of solubilities.

Evaluation Criterion 11: Performance standards should be developed for the proposed protocol.

The panel agreed with the appropriateness of the 17 reference chemicals identified for qualification of proposed solar simulators other than the two solar simulators used in the validation studies. The reference chemicals were appropriately selected from the reference chemicals used for the validation studies. While performance standards were not specifically proposed, the panel considered that these reference chemicals would be appropriate for incorporation in future performance standards for the ROS assay.

Evaluation Criterion 12: Are there advantages in terms of time, cost and animal welfare?

The ROS assay can potentially provide significant savings in time, cost and reduced animal use when used in an integrated photosafety testing strategy by allowing decisions to be made earlier and with fewer overall tests for many chemicals. These advantages are illustrated in Figure 1, which shows that chemicals that are non-photoreactive in the ROS assay need not be tested in animals or other tests. The ROS assay also reduces the number of chemicals which progress to testing in the 3T3 Phototoxicity Assay, with a subsequent reduction in the number of positive results in the 3T3 assay that may progress to in vivo tests for confirmation.

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 integrated 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 will provide significant potential savings in time, cost and reduced animal use for photosafety assessments. Furthermore, incorporating the ROS assay into a photosafety testing strategy will significantly reduce the overall number of substances that require additional testing in the in vitro 3T3 Phototoxicity Assay, and substantially reduce the number of substances that require subsequent testing in animals.

Appendix 1

Independent Peer Review Panel Members

Horst Spielmann, M.D., Panel Chairman
Dr. Spielmann is a Professor for Regulatory Toxicology in the Institute for Pharmacy, Faculty of Biology, Chemistry, and Pharmacy at the Freie Universität Berlin in Berlin, Germany. He is the former and first head of the National German Center for the Validation of Alternative Methods (ZEBET) at the Federal Institute for Risk Assessment (BfR) in Berlin, Germany, where he directed the standardization and international validation of several in vitro methods and also served as the head of the Department of Scientific Services. He served as Germany's first national representative on the Scientific Advisory Committee for the European Centre for the Validation of Alternative Methods at the European Commission's Joint Research Centre in Ispra, Italy, and has served as an expert for many years for the European Commission's Framework Programs on alternatives to animal testing and in vitro toxicology. In December 2012, he was appointed as the Animal Welfare Officer for the State of Berlin.

William S. Stokes, D.V.M., Rapporteur
Dr. Stokes is an Adjunct Professor in the Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, U.S.A. He recently retired from the United States Public Health Service where he was an Assistant U.S. Surgeon General. He served as the first Director of the U.S. National Toxicology Program's Interagency Center for the Evaluation of Alternative Toxicological Methods at the National Institute of Environmental Health Sciences from 1997-2012, where he directed the international validation and scientific peer review of numerous new test methods for regulatory safety assessments. He also served as the first Executive Director of the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods from 1997-2012. He is a Fellow of the Academy of Toxicological Sciences and a board certified environmental scientist, and is also board certified in laboratory animal medicine and animal welfare.

Ikuo Horii, Ph.D.
Dr. Horii is a global consultant for Pfizer and former Executive Director of Pfizer Drug Safety Research and Development in Nagoya, Japan. He is a Certified Toxicologist in the Japanese Society of Toxicology with a background in biochemistry, pharmacology, pathology, and molecular toxicology. He is currently a Visiting Professor at several institutions, including the School of Pharmacy at Showa University in Japan, Dalian Medical University in China, and Cambridge University in the United Kingdom. He is a Visiting Research Fellow at the National Institute of Health Sciences in Tokyo, Japan and Lecturer at Kyoto University, Tokyo University, and Chiba Institute of Science. He is President of Horii Science Associates and a board member of the Japanese Society of Toxicology.

Bae-Hwan Kim, D.V.M., Ph.D.
Dr. Kim is a Professor in the Department of Public Health at Keimyung University in Daegu, Republic of Korea, where he leads a biomedical research program and lectures in the College of Natural Sciences. He previously worked in the pharmaceutical and cosmetic industry for 15 years as a Team Leader in the Preclinical Department. His research includes investigation of the oxidative stress and oxidative photodamage induced by UV radiation and interventional strategies for avoidance of UV irradiation damage. His focus is on the safety evaluation of substances applied to the skin and the development of alternative methods to animal

experiments. Dr. Kim serves on the Editorial Board of the Journal of Biomedical Research and is a Council Member of the Korean Association for Laboratory Animal Science and the Korean Society for Alternatives to Animal Experiments.

Appendix 2

Acknowledgements

The Peer Review Panel members gratefully acknowledge Steven Venti from BHK Limited for his invaluable assistance with translation and editing during the peer review process. The Panel also acknowledges the members of the Validation Management Team for the completeness of the validation study reports, their cooperation and responsiveness to requests for additional information and analyses, and their responsive consideration of suggestions and updating of the validation study reports and test method protocol. Finally, the Panel expresses its appreciation to Dr. Hajime Kojima and his staff at the National Institute of Health Sciences for their excellent support and arrangements for the peer review panel meetings.

Appendix 3

Glossary[1]

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

Dose of light: The quantity [= intensity Å~ time (seconds)] of UV or visible light incident on a surface, expressed in J/m2 or J/cm2.

Irradiance: The intensity of UV or visible light incident on a surface, measured in W/m2 or mW/cm2.

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 a chemical to react with another molecule as a consequence of photon absorption. Excitation of molecules by light can lead to generation of reactive oxygen species (ROS) such as superoxide anion (SA) and singlet oxygen (SO) through energy transfer mechanisms.

Phototoxicity: acute toxic response that is elicited after the first exposure of skin to certain chemicals and subsequent exposure to light, or that is induced similarly by skin irradiation after systemic administration of a chemical.

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

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

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

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

[1] Note: definitions derived from OECD TG 432 and the ROS assay protocol

Appendix 4

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