*Title of Research:*16_S02-02-2

Prediction method (Amino acid Derivative Reactivity Assay: ADRA) for skin sensitization using novel lysine and cysteine derivatives

Principal Investigator:

Toshihiko Kasahara, PhD (Engineering Manager, FUJIFILM Corporation, Safety Evaluation Center, Ecology & Quality Management Division, CSR Division)
210, Nakanuma, Minamiashigara-shi, Kanagawa 250-0193, Japan
(tel)+81-465-73-7440 (e-mail)ktoshihiko.kasahara@fujifilm.com

Collaborators:

Masaharu Fujita, PhD（FUJIFILM Corporation,Safety Evaluation Center）
210, Nakanuma, Minamiashigara-shi, Kanagawa 250-0193, Japan
(tel)+81-465-73-7440 (e-mail)masaharu.fujita@fujifilm.com
Yusuke Yamamoto (FUJIFILM Corporation,Safety Evaluation Center）
210, Nakanuma, Minamiashigara-shi, Kanagawa 250-0193, Japan
(tel)+81-465-73-7440 (e-mail)yusuke.yamamoto@fujifilm.com
Atsuishi Ono, PhD (Professor, Okayama University, Graduate school of Medicine, Dentistry and Pharmaceutical sciences, Division of Pharmaceutical Sciences)
1-1-1, Tsushimanaka, Kita-ku, Okayama-shi, Okanaya 700-8530, Japan
(tel)086-251-7942 (e-mail) atsushiono3@okayama-u.ac.jp
Hajime Kojima, PhD (Director, National Institute of Health Sciences, Biological safety Research Center, Division of Risk Assessment)
1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
(tel) 03-3700-9874 (e-mail)h-kojima@nihs.go.jp
Shinichi Wantanabe (LION CORPORATION, HUMAN & ENVIRONMENTAL SAFETY EVALUATION CENTER)
100, Tajima, Odawara-shi, Kanagawa 256-0811
(tel)0465-49-4475 (e-mail)shin-w@lion.co.jp
Tsunetsugu Sugawara (LION CORPORATION, HUMAN & ENVIRONMENTAL SAFETY EVALUATION CENTER)
100, Tajima, Odawara-shi, Kanagawa 256-0811
(tel)0465-49-4475 (e-mail) tsune-s@lion.co.jp
Koji Wakabayashi (Manager, MITSUI CHEMICALS, INC., Chemical Safety Department
1144, Togo, Mobara-shi, Chiba 297-0017, Japan
(tel)0475-23-8251 (e-mail)Koji.Wakabayashi@mitsuichemicals.com
Yu Tahara (MITSUI CHEMICALS, INC., Chemical Safety Department
1144, Togo, Mobara-shi, Chiba 297-0017, Japan
(tel)0475-23-8251 (e-mail)Yu.Tahara@mitsuichemicals.com
Nobuyuki Horie (Senior Research Scientist, SUMITOMO Chemical Co., Ltd., Environmental Health Science Laboratory)
3-1-98, Kasugadenaka, Konohana-ku, Osaka-shi, Osaka 554-8558, Japan
(tel)06-6466-5107 (e-mail)horien@sc.sumitomo-chem.co.jp
Keiichi Fujimoto (SUMITOMO CHEMICAL Co., Ltd., Environmental Health Science Laboratory)
3-1-98, Kasugadenaka, Konohana-ku, Osaka-shi, Osaka 554-8558, Japan
(tel)06-6439-9753 (e-mail) fujimotok2@sc.sumitomo-chem.co.jp
Kei Kusakari (NISSAN CHEMICAL INDUSTRIES, LTD. Biological Research Laboratories)
1470, Shiraoka, Shiraoka-shi, Saitama 349-0294, Japan
(tel)0480-92-2513 (e-mail)kusakarik@nissanchem.co.jp
Yoshihiko Kurokawa (NISSAN CHEMICAL INDUSTRIES, LTD. Biological Research Laboratories)
1470, Shiraoka, Shiraoka-shi, Saitama 349-0294, Japan
(tel)0480-92-2513 (e-mail)kurokawayo@nissanchem.co.jp
Takashi Sozu, PhD (Assistant Professor, Department of Information and Computer Technology, Faculty of Engineering, Tokyo University of Science)
6-3-1, Shinjuku, Katsushika-ku, Tokyo 125-8585, Japan
(tel)03-5876-1688 (e-mail)sozu@rs.tus.ac.jp
Takuto Nakayama (Department of Management Science, Graduate School of Engineering, Tokyo

University of Science)
6-3-1, Shinjuku, Katsushika-ku, Tokyo 125-8585, Japan
(tel)03-5876-1688 (e-mail)4416624@ed.tus.ac.jp
Tsuyoshi Kawakami, PhD (Director, National Institute of Health Sciences, Biological safety Research Center, Division of Risk Assessment)
1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
(tel) 03-3700-1141 (e-mail)tkawa@nihs.go.jp
Kohichi Kojima、PhD （Director-General, Food and Drug Safety Center, Hatano Research Institute）
729-5, Ochiai, Hadano-shi, Kanagawa 257-8523, Japan
(tel)0463-82-4751 (e-mail)kojima.k@fdsc.or.jp
Nicol C. Kleinstreuer, PhD（NTP Interagency Center for the Evaluation of Alternative Toxicological methods）
(tel) (919) 541-7997 (e-mail) nicole.kleinstreuer@nih.gov
Jon Richmind, PhD（Ethical Biomedical Research and Testing Advice & Consultancy）
Doubledykes Carslogie Road, Cupar, Fife KY154HY, United Kingdom
(tel)077-86-381-757 (e-mail)dr.richmond@gmail.com
Bae-Hwan Kim, PhD（Professor, Keimyung University, Department of Public Health, College of Natural Science）
1095, Dalgubeol-daero, Dalseo-Gu, Daegu 42601, Republic of Korea
(tel)+82-53-580-5933 (e-mail)kim9399@kmu.ac.kr

Summary of Research:

In this study, we performed the validation study so that the alternative method for skin sensitization (ADRA) is adopted as OECD test guideline.

Part1)

For the four participating laboratories, the two training sessions were held, and the two confirmation tests (Pre-training test and Training test) were performed because the lead laboratory confirmed that the assay technique was correctly transferred for the participating laboratories.

Compared with cell-based *in vitro* test methods, DPRA is an easy-to-use test method and exhibits excellent predictive capacity. Nevertheless, the DPRA test method has significant limitations:

- One of the nucleophilic reagents, cysteine peptide, is easily oxidized rendering some test results invalid, unreliable or difficult to interpret.
- It requires relatively high concentrations of the test chemical, making it unsuitable for evaluating poorly soluble chemicals.
- It requires a relatively large quantity of both test chemicals and nucleophilic reagents to perform.
- Hydrophobic test chemicals tend to precipitate easily in the reaction solution.

ADRA, an *in chemico* test which is also based on protein reactivity, does not have these limitations thanks to the use of highly sensitive nucleophilic reagents, which allows reagent solutions to be prepared at test chemical concentrations just 1% of those required in DPRA.

Following a modular approach applying the ECVAM principles on test validity (OECD Series on Testing and Assessment, Number 34, 2005, Hartung *et al.* 2004), the Validation Management Team (VMT) empirically evaluated modules one through four (test definition, within-laboratory reproducibility, transferability, and between-laboratory reproducibility), and used these results also to evaluate modules five and six (predictive capacity and applicability domain).

During a preliminary training phase of the ADRA validation study using ten test chemicals, the results obtained by the four participating laboratories were 100% concordant with the lead laboratory. During the subsequent transferability phase of the study, three of the four participating laboratories were 100% (10/10) concordant and the fourth laboratory was 90% (9/10) concordant with the lead laboratory. The ADRA VMT considers these results to have confirmed that the ADRA test method is easily transferred to naïve laboratories.

During Phase I of the Study Plan, each of the four participating laboratories performed three test runs of identical sets of 10 coded test chemicals to evaluate within-laboratory reproducibility. The results, based on concordance, were 100% (10/10), 100% (10/10), 100% (7/7), and 90% (9/10). When the results from the 10 test chemicals of Phase I were combined with those of Phase II, in which each participating laboratory performed one test run of identical sets of an additional 30 test chemicals, between-laboratory reproducibility for 40 test chemicals was 92%. The VMT considers

this to satisfy standards for both within- and between-laboratory reproducibility for this class of test method.
Moreover, a review of predictive capacity relative to LLNA based on the results of Phases I and II yielded a sensitivity of 81%, specificity of 98%, and accuracy of 86%. The VMT therefore considers ADRA to be an *in chemico* method with sufficient specificity, sensitivity, and predictive capacity for regulatory acceptance.

Part 2)

During a recent validation study conducted at multiple laboratories as part of the process to include ADRA in an existing OECD test guideline, one of the nucleophilic reagents used in ADRA—*N*-(2-(1-naphthyl)acetyl)-*L*-cysteine (NAC)—was found to be susceptible to oxidation in much the same manner that the cysteine peptide used in DPRA was. Due to this, we undertook a study to clarify the cause of the promotion of NAC oxidation.

In general, cysteine and other chemicals that have thiol groups are known to be oxidized in the presence of minute quantities of metal ions. When Fe and Cu ions were added to the ADRA reaction solution, the Cu ions facilitated NAC oxidation significantly. When 0.25 µM of EDTA was added in the presence of Cu ions, NAC oxidation was suppressed. Based on this, we predicted that the addition of EDTA to the NAC stock solution would suppress NAC oxidation. Next, we tested 82 chemicals used in developing ADRA to determine whether or not EDTA affects ADRA's ability to predict sensitization. The results showed that the addition of EDTA has virtually no effect on the reactivity of NAC with a test chemical and yielded an accuracy of 87% for predictions of skin sensitization, which was roughly the same as ADRA.

Timeline: March 1, 2017- (it continues from March 1, 2016)
Topics: none
Publications:

1. Presented at the 44th The Japanese Society of Toxicology (2017.7, Yokohama) 「Multicenter Validation Study of Novel *in chemico* Skin Sensitization Assay (ADRA): 1st Report」
   Atsushi Ono, Shin-ichi Watanabe, Tsunetsugu Sugawara, Koji Wakabayashi, Yu Tahara, Nobuyuki Horie, Keiichi Fujimoto, Kei Kusakari, Yoshihiko Kurokawa, Takashi Sozu, Takuto Nakayama, Takeru Kusao, Tsuyoshi Kawakami, Kohichi Kojima, Hajime Kojima, Jon Richmond, Nicole Kleinstreuer, Bae-Hwa Kim, Yusuke Yamamoto, Masaharu Fujita, and Toshihiko Kasahara
2. Presented at the 10th World Congress on Alternatives and Animals in the Life Sciences (WC10) (2017.8) (Seattle, WA, USA) 「Phase-1 of the validation study of Amino acid Derivative Reactivity Assay (ADRA) : a novel *in chemico* alternative test method of skin sensitization.」
   Atsushi Ono, Shin-ichi Watanabe, Tsunetsugu Sugawara, Koji Wakabayashi, Yu Tahara, Nobuyuki Horie, Keiichi Fujimoto, Kei Kusakari, Yoshihiko Kurokawa, Takashi Sozu, Takuto Nakayama, Takeru Kusao, Tsuyoshi Kawakami, Kohichi Kojima, Hajime Kojima, Jon Richmond, Nicole Kleinstreuer, Bae-Hwa Kim, Yusuke Yamamoto, Masaharu Fujita, and Toshihiko Kasahara
3. Presented at the 30th Annual Meeting of the Japanese Society for Alternatives to Animal Experiments」 (2017.11, Tokyo) 「Multi-laboratory validation study of ADRA as novel in chemico alternative test method for skin sensitization: 2nd report」 Atsushi Ono, Shin-ichi Watanabe, Tsunetsugu Sugawara, Koji Wakabayashi, Yu Tahara, Nobuyuki Horie, Keiichi Fujimoto, Kei Kusakari, Yoshihiko Kurokawa, Takashi Sozu, Takuto Nakayama, Takeru Kusao, Tsuyoshi Kawakami, Kohichi Kojima, Hajime Kojima, Jon Richmond, Nicole Kleinstreuer, Bae-Hwa Kim, Yusuke Yamamoto, Masaharu Fujita, and Toshihiko Kasahara
4. The Cause of and Countermeasures for Oxidation of the Cysteine-Derived Reagent Used in the Amino acid Derivative Reactivity Assay. (in submission to Journal of Applied Toxicology) Masaharu Fujita, Yusuke Yamamoto, Shinichi Watanabe, Tsunetsugu Sugawara, Koji Wakabayashi, Yu Tahara, Nobuyuki Horie, Keiichi Fujimoto, Kei Kusakari, Yoshihiko Kurokawa, Tsuyoshi Kawakami, Kohichi Kojima, Hajime Kojima, Atsushi Ono, Yasuhiro Katsuoka, Hideto Tanabe, Hiroshi Yokoyama, and Toshihiko Kasahara.