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Evaluation of radioisotopic and non-radioisotopic versions of local lymph node assays for subcategorization of skin sensitizers compliant to UN GHS rev 4

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ABSTRACT

Recently UN GHS has introduced the sub-categorization of skin sensitizers for which ECt (concentration estimated to induce stimulation index above threshold) of the murine local lymph node assay (LLNA) is used as criteria. Non-radioisotopic variants of LLNA, LLNA: DA, LLNA: BrdU-ELISA, LNCC and LLNA: BrdU-FCM were developed yet their utilities for potency sub-categorization are not established. Here we assessed the agreement of LLNA variants with LLNA or human data in potency sub-categorization for 22 reference substances of OECD TG429. Concordance of sub-categorization with LLNA was highest for LLNA: BrdU-FCM(91%, $\kappa = 0.833$, weighted kappa) followed by LLNA: BrdU-ELISA (82%, $\kappa = 0.744$) and LLNA: DA (73%, $\kappa = 0.656$) whereas LNCC only showed a modest association (64%, $\kappa = 0.441$). With human data, LLNA agreed best (77%) followed by LLNA: DA and LLNA: BrdU-FCM(73%), LLNA: BrdU-ELISA (68%) and LNCC(55%). Bland-Altman plot revealed that ECt's of LLNA variants largely agreed with LLNA where most values fell within 95% limit of agreement. Correlation between ECt's of LLNA and LLNA variants were high except for LNCC(pair-wise with LLNA, LLNA: DA, $r = 0.848$, LLNA: BrdU-ELISA, $r = 0.744$, LLNA: BrdU-FCM, r=0.786, and LNCC, $r = 0.561$ by Pearson). Collectively, these results demonstrated that LLNA variants exhibit performance comparable to LLNA in the potency sub-categorization although additional substances shall be analyzed in the future.

1. Introduction

Local lymph node assay (LLNA), OECD TG 429 (OECD, 2010a), and its non-radioisotopic versions, LLNA:DA (TG 442A) (OECD, 2010a), LLNA: BrdU-ELISA (TG 442B) (OECD, 2010b), LNCC (Basketter et al., 2012) and LLNA: BrdU-FCM (Jung et al., 2010, 2012; Kim et al., 2016; Yang et al., 2015), have been developed as a stand-alone method to replace *in vivo* skin sensitization tests employing guinea pigs, OECD TG 406 (OECD, 1992; Ryu et al., 2016), with an aim to reflect '3R' principles and to improve animal welfare. Indeed, the employment of LLNAs has considerably reduced the sacrifice of animals (from 45 to less than 25), improved animal welfare by avoiding the use of painful adjuvant, and substantially reduced time and cost compared with conventional guinea pig tests. However, LLNAs are still "*in vivo*" and with the recent advent of true *in vitro* and *in chemico* assays that include KertinoSens™ (OECD, 2015b), hCLAT and Direct Peptide Reactivity Assay (DPRA) (OECD, 2015a), there is a strong voice to phase out LLNAs.

However, *in vitro* and *in chemico* alternatives described above have not fully overcome their intrinsic limitations yet, namely, their applicability has not exceeded the realm of hazard identification. Ultimately, skin sensitization tests must be able to provide potency information that can contribute to the exposure-based quantitative risk assessment/management and subsequent prioritization or

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classification for the regulation of chemicals. LLNAs can give information concerning the potency of skin sensitizers as ECt values (mathematically estimated concentration of chemical required to induce a threshold stimulation index (SI)). In this regard, LLNAs would be "currently in use" for substantial length of time as a gold standard for the assessment of sensitization.

United Nation Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) employs a classification system to designate categories to substances by types or level of hazard (United_Nations, 2003). UN GHS has been revised to introduce subcategory for the skin sensitizers (United_Nations, 2009). New system recommends the sub-categorization of skin sensitizers into Cat 1A and 1B depending on their potency. Criteria for the sub-categorization into Cat 1A is established as the substance with the EC3 of LLNA $\leq$2%, or $\geq$30% responding at $\leq$ 0.1% intradermal induction dose in guinea pig maximization test (or positive at $\leq$ 500 $\mu g/cm^2$ in Human Repeated Insult Patch Test (HRIPT)). Indeed, in the risk assessment of cosmetics, "No Expected Sensitization Induction Level (NESIL)" concept has been recently taken up, which is based on ECt produced by LLNA (Api et al., 2008), supporting the utility of LLNA for quantitative risk assessment.

Yet, it is to be demonstrated whether the non-radioisotopic variants of LLNA, (i.e., LLNA: DA, LLNA: BrdU-ELISA, LNCC and LLNA: BrdU-FCM) are compatible to the original LLNA for quantitative risk assessment. Since LLNA has a critical drawback, namely, *in vivo* use of radioisotope, ^{3}H-labeled thymidine during the experimental procedure, which seriously deters its use in some countries, proof of the utility of non-radioisotopic variants in the potency subcategorization may be helpful in replacing further conventional guinea pig tests. The main purpose of this study was to examine agreement between LLNA and its non-radioisotopic variants, LLNA: DA, LLNA: BrdU-ELISA, LNCC, and LLNA: BrdU-FCM, for classification of 22 reference substances enlisted in the performance standards of OECD TG429. As the secondary purpose, we compared the ECt values of LLNA and its variants.

2. Materials & methods

2.1. ECthreshold data for radioisotopic and non-radioisotopic LLNAs

Currently, one radioisotopic LLNA and two non-radioisotopic LLNAs, (LLNA: BrdU-ELISA and LLNA: DA) have been officially approved by OECD as test guidelines for the test of skin sensitization. In case of radioisotopic LLNA (TG 429), stimulation index (SI), which represents the measured extent of proliferation of lymph node cells (LNCs), and ECt, a hypothetical intra-polated concentration of a test chemical that induces SI beyond the threshold, or cutoff to determine as a sensitizer were published for 22 reference substances in OECD TG 429 performance standards (OECD, 2010a). These data are the basis for the sub-categorization of sensitizers into Category 1A and 1B compliant to UN GHS 4th revision. For LLNA:DA and LLNA: BrdU-ELISA, different threshold values (1.8 for DA and 1.6 for BrdU-ELISA) are adopted and ECt for reference substances was published in the ICCVAM 2010 review report (ICCVAM, 2010a; ICCVAM, 2010b). Data for some substances unavailable in the report, were provided by JaCVAM (xylene for LLNA: DA and methyl methacrylate, chlorobenzene, xylene and nickel chloride for LLNA: BrdU-ELISA). In addition, data for LLNA: BrdU-FCM and LNCC were published with which ECt values were derived (Ahn et al., 2016; Basketter et al., 2012; Kim et al., 2016) and for LLNA: BrdU-FCM, optional 4 substances (Sodium lauryl sulfate (Sigma-Aldrich, St. Louis, MO), ethylene glycol dimethacrylate, xylene, Nickel chloride) were tested again to conform to the current protocol version 1.3. LLNA: BrdU-FCM test was done in Biotoxtech (Ochang, Korea) in a coded fashion and the experimental procedure was well-described in the recent paper (Ahn et al., 2016). When multiple values were known, the lowest values among them were used to sub-categorize the potency.

2.2. United nation global harmonization system (UN GHS) classification

Based on the collected ECt values, the potency of sensitizers were subcategorized into 3 levels, namely, No category, Category 1B and 1A based on ECt. UN GHS classification system was revised to incorporate the potency concept into labeling recently (rev 4.). And this is based on EC3 value of radioisotopic LLNA. When the EC3 of the substance is measured to be $\leq$ 2% in LLNA, it is categorized to Category 1A, if EC3 > 2% then, Category 1B and when EC3 could not be determined (when maximum SI values did not exceed threshold SI value for respective LLNA variant), then No category. In a same vein, the categorization with other LLNAs were done according to respective ECt value.

2.3. Statistical analysis

2.3.1. UN GHS classification

The proportion of concordance of LLNA variants with LLNA or human data for binary decision (sensitizer vs non-sensitizer) and for the UN GHS subcategorization was assessed. The concordance in the UN GHS subcategorization (Cat 1A, Cat 1B, or No Category) was further assessed with weighted kappa since the UN GHS is an ordinal variable. Concordance is generally interpreted as "almost perfect agreement" when Kappa value is 0.81–0.99, "substantial agreement" at 0.61–0.8, "moderate agreement" at 0.41–0.6, "fair agreement" at 0.21–0.40 and "slight agreement" at 0.01–0.20 (Hunt, 1986; Landis and Koch, 1977).

2.3.2. ECt values

ECt value is a continuous data ranging between 0 and 100%. First, Bland-Altman analysis was employed to describe the agreement of LLNA variants with LLNA through drawing plot and 95% limit of agreement lines (Altman and Bland, 1983; Bland and Altman, 1986). In addition, Pearson correlation analysis (Jang et al., 2015) was done to quantify the pairwise correlation of ECt values between two LLNAs. Correlation coefficient is between -1 and 1, and the closer to 1 the absolute value is, the stronger the correlation becomes. Generally, strong correlation is when $0.7 < r < 1$ and moderate, $0.3 < r < 0.7$ and weak when $r < 0.3$. For analysis, when ECt value could not be determined (non-sensitizer), then ECt value was assumed to be 100. Statistical analysis was done with SAS version 9.3 (SAS Institute Inc, Cary, NC, USA).

3. Result

ECt values for 22 reference substances with LLNA and its non-radioisotopic variants were presented in Table 1. These substances cover the various chemical classes, physicochemical properties and wide range of potencies, and were enlisted in OECD TG 429 performance standards for the evaluation of newly developed variants of LLNA. With the obtained ECt, substances were classified and sub-categorized according to UN GHS rev 4.0 (i.e., Cat 1A, 1B or No category when ECt is $\leq$ 2%, 2% < ECt < 100% or = 100%, respectively) as shown in Table 2.

When compared with the results of traditional LLNA as a gold standard, LLNA: DA produced 2 false positives (chlorobenzene, salicylic acid) and 1 false negative (xylene) while LLNA: BrdU-ELISA had 2 false positives (chlorobenzene, lactic acid). LLNA: BrdU-FCM also produced 2 false negatives (2-mercaptobenzothiazole, methyl methacrylate) while LNCC determined 4 among 6 non-sensitizers