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Chemical applicability domain of the Local Lymph Node Assay (LLNA) for skin sensitisation potency. Part 2. The biological variability of the murine Local Lymph Node Assay (LLNA) for skin sensitisation☆

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ABSTRACT

The Local Lymph Node Assay (LLNA) is the most common *in vivo* regulatory toxicology test for skin sensitisation, quantifying potency as the EC3, the concentration of chemical giving a threefold increase in thymidine uptake in the local lymph node. Existing LLNA data can, along with clinical data, provide useful comparator information on the potency of sensitisers. Understanding of the biological variability of data from LLNA studies is important for those developing non-animal based risk assessment approaches for skin allergy. Here an existing set of 94 EC3 values for 12 chemicals, all tested at least three times in the same vehicle have been analysed by calculating standard deviations (SD) for logEC3 values. The SDs range from 0.08 to 0.22. The overall SD for the 94 logEC3 values is 0.147. Thus the 95% confidence limits (2xSD) for LLNA EC3 values are within a factor of 2, comparable to those for physico-chemical measurements such as partition coefficients and solubility. The residual SDs of Quantitative Mechanistic Models (QMMs) based on physical organic chemistry parameters are similar to the overall SD of the LLNA, indicating that QMMs of this type are unlikely to be bettered for predictive accuracy.

1. Introduction

The Local Lymph Node Assay (OECD, 2010) is the most common *in vivo* regulatory toxicology test for skin sensitisation along with the guinea pig maximisation test (OECD, 1992). Currently a peptide reactivity assay and two cell-based *in vitro* assays have been formally validated (OECD, 2015a, b) or are at an advanced stage of validation (EURL-ECVAM, 2015; Reisinger et al., 2015), and integrated testing strategies (ITS) or data integration procedures (DIP) for using them in combination to identify sensitisation potential or predict sensitiser potency are being developed (Patlewicz et al., 2014; van der Veen et al., 2014; Natsch et al., 2015; Takenouchi et al., 2015; Jaworska et al., 2015; Hirota et al., 2015; Urbisch et al., 2015). However these non-animal approaches, ITS or DIP have not considered the underlying biological variability within their *in vivo* benchmark datasets when communicating their predictive capacity.

Consideration of variability within *in vivo* benchmark datasets is important to enable the uncertainty of a non-animal approach prediction to be explicitly communicated and appropriately benchmarked. Furthermore, characterization of variability within *in vivo* assays enables the continued use of such historical data to inform model-based, non-animal risk assessment approaches for skin sensitisation where one of the benefits is that model uncertainty can be explicitly visualised (MacKay et al., 2013; Maxwell et al., 2014).

Compared with the guinea pig maximisation test (GPMT), the LLNA uses fewer animals, it is quantitative, and it gives a numerical prediction of potency. Potency is quantified in terms of the EC3 value, this being the concentration of test chemical that, when applied under the LLNA protocol, would give rise to a threefold increase in thymidine uptake in the local lymph node. Several large (>100 chemicals) databases of LLNA potency values are now available (Gerberick et al., 2004, 2005; Kern et al., 2010) and the

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LLNA is therefore a valuable source of benchmarks which, along with clinical data, provides useful comparator information on the potency of sensitisers when developing non-animal approaches. This raises the questions of the applicability domain of the LLNA (i.e. what is the range of chemicals for which the LLNA result is a realistic reflection of potency in humans) and also the biological variability of the potency value.

This paper addresses the latter question by analysing a dataset of chemicals tested 3 or more times in the LLNA.

2. Evaluation of data on multiple testing

Basketter et al. (2007) published EC3 values from 94 assays on 12 chemicals. The number of assays per chemical ranges from 3 to 31. Each chemical was tested in the same vehicle on all occasions.

Although this is not usually explicitly stated, potency classification is based on a logarithmic scale of EC3 values:

It is therefore appropriate to use logEC3 values for statistical analysis of the data published by Basketter et al. (2007).

For each of the 12 compounds, the mean logEC3 value and the standard deviation were calculated, as illustrated for DNCB in Table 1.

Means and standard deviations were calculated in the same way for the remaining 11 compounds in the dataset. The antilog of the $logEC3_{mean}$ is the log-average mean (also known as geometric mean) EC3 value. Doubling the standard deviation gives the 95% confidence limit (as a ±value) on logEC3, and the antilog of this value gives the factor for 95% confidence on the EC3 value.

The results of this analysis are shown in Table 2, in which the log-average mean EC3 values are also compared against those previously published by Gerberick et al. (2005) with the aim of providing a curated *in vivo* potency database.

The compounds cover the potency range from extreme to weak, and include direct acting compounds (e.g. DNCB, cinnamaldehyde), compounds that require either biotic or abiotic activation (e.g. PPD, isoeugenol, eugenol, abietic acid) and compounds whose chemical mechanisms of action are not definitively established (e.g. $K_2Cr_2O_7$).

Summing the $(X_m\text{-}X_i)^2$ values (see footnote to Table 1) for all 94 assays, dividing by 93 and taking the square root, give an overall SD of 0.147. This corresponds to a factor of 2 for 95% confidence limits on EC3.

It may be noted that there is no strong correlation between potency and standard deviation. The two weakest sensitisers have SDs of 0.08 and 0.14, and the two strongest sensitisers have SDs of 0.17 and 0.22.

2.1. Non-animal based prediction of EC3 values

The factor of 2 for 95% confidence limits on EC3 values is not significantly greater than the reproducibility of many physical parameters, e.g. partition coefficient, solubility (Hansch and Leo, 1979). It is consequently not valid to simply attribute poor concordance between potency estimates derived in non-animal methods (e.g. *in vitro* assay, *in silico* prediction) and LLNA EC3 values to variability in the *in vivo* biological data.

Conversely, provided that appropriate non-animal parameters are used, good correlation with the EC3 should be obtainable in Quantitative Mechanistic Models (QMMs). This is illustrated by the *s* values (standard deviation of the residuals) for published QMMs for skin sensitisation potency (expressed as pEC3) based on reactivity parameters:

QMM for the Michael acceptor domain (Roberts and Natsch, 2009)

$$pEC3 = 0.24(\pm0.04)\ \log k + 2.11\ (\pm0.24) \quad (1)$$

$n = 10,\ R^2 = 0.836,\ s = 0.11$

QMM for the Schiff Base domain (Roberts et al., 2006)

$$pEC3 = 1.12(\pm0.07)\ \Sigma\sigma^* + 0.42(\pm0.04)\ \log P - 0.62(\pm0.13) \quad (2)$$

$n = 16,\ R^2 = 0.952,\ s = 0.12$

QMM for the S_NAr electrophiles domain (Roberts and Aptula, 2014)

$$pEC3 = 2.82\ RP - 5.44\ (RP = \Sigma\sigma^- + 0.24\ \sigma^*) \quad (3)$$

$n = 10\ R^2 = 0.987,\ s = 0.13$

In the first example the reactivity parameter log *k*, is based on the rate constant measured in a kinetic variant of the Direct Peptide Reactivity Assay (DPRA) (Roberts and Natsch, 2009). However it may be noted that in the next two examples (eqs. (2) and (3)) the reactivity parameters are based on physical-organic chemistry substituent constants derived not from peptide assays, but ultimately from kinetic and equilibrium measurements (Isaacs, 1995) on chemicals unrelated to those modeled in the QMM. This illustrates that the "high fidelity" approach of using models resembling skin proteins as closely as possible is not necessary in the context of non-animal prediction of skin sensitisation potency.

2.2. Comparison with the NICEATM database

While this paper was in preparation, a paper was published in which, *inter alia*, a similar study on a different database, the NICEATM (NTP Interagency Center for the Evaluation of Alternative Toxicological Methods) database, was reported (Hoffmann, 2015). In this database Hoffmann found 27 chemicals for which at least three EC3 values in the same vehicle had been reported. Standard deviation values on logEC3 ranged from 0.137 to 1.048, with a median standard deviation of 0.252. Clearly there is much wider variation in the NICEATM dataset than in the Basketter dataset

Table 1
Variation of EC3 values for DNCB, tested in AOO (Acetone Olive Oil).

EC3, %	logEC3	$(X_m-X_i)^2$
0.04	−1.40	2.199E-05
0.02	−1.70	0.0878
0.05	−1.30	0.0103
0.03	−1.52	0.0145
0.03	−1.52	0.0145
0.02	−1.70	0.0878
0.06	−1.22	0.0327
0.03	−1.52	0.0145
0.06	−1.22	0.0327
0.05	−1.30	0.0103
0.05	−1.30	0.0103
0.06	−1.22	0.0327
0.05	−1.30	0.0103
n	13	
Mean	−1.40	
SD	0.170	$= \sqrt{(\sum (X_m - X_i)^2/12)}$

$(X_m\text{-}X_i) = logEC3_{mean}$ - $logEC3_{exp}$.

$logEC3 < -1$	Extreme
$-1 < logEC3 < 0$	Strong
$0 < logEC3 < 1$	Moderate
$1 < logEC3 < 2$	Weak
$2 < logEC3$	Non-sensitiser

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