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Tiered application of the neutral red release and EpiOcular™ assays for evaluating the eye irritation potential of agrochemical formulations

Raja S. Settivari [a, *], Ricardo Acosta Amado [b], Marco Corvaro [c], Nicolo R. Visconti [a], Lynn Kan [a], Edward W. Carney [a], Darrell R. Boverhof [a], Sean C. Gehen [b]

[a] The Dow Chemical Company, Midland, MI, USA
[b] Dow AgroSciences LLC, Indianapolis, IN, USA
[c] Dow Chemical Services UK Ltd, Milton Park, Abingdon, Oxon, UK

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ABSTRACT

Agrochemical formulations have been underrepresented in validation efforts for implementing alternative eye irritation approaches but represent a significant opportunity to reduce animal testing. This study assesses the utility of the neutral red release assay (NRR) and EpiOcular™ assay (EO) for predicting the eye irritation potential of 64 agrochemical formulations relative to Draize data. In the NRR, formulations with an NRR50 value $\leq$ 50 mg/mL were categorized as UN GHS Cat 1 and those >250 mg/mL were classified as UN GHS Non Classified (NC). The accuracy, sensitivity, and specificity were 78, 85 and 76% and 73, 85 and 61% for identifying UN GHS 1 and NC formulations, respectively. Specificity was poor for formulations with NRR50 > 50 to $\leq$250 mg/mL. The EO (ET-40 method) was explored to differentiate formulations that were UN GHS 1/2 and UN GHS NC. The EO resulted in accuracy, sensitivity, and specificity of 65%, 58% and 75% for identifying UN GHS NC formulations. To improve the overall performance, the assays were implemented using a tiered-approach where the NRR was run as a first-tier followed by the EO. The tiered-approach resulted in improved accuracy (75%) and balanced sensitivity (73%) and specificity (77%) for distinguishing between irritating and non-irritating agrochemical formulations.

1. Introduction

Assessment of acute eye irritation potential is required for registration of agrochemical active substances and formulations prior to commercialization (EPA, 2007; Sanco, 2010). Eye irritation data can be obtained with accepted test methods such as those adopted by the Organization for Economic Cooperation and Development (OECD). The acute hazard characterization data derived from these tests is used for hazard communication purposes including classification and labeling and for defining the appropriate personal protection equipment (PPE) required for safe use of the product (EPA, 2014).

Historically, the rabbit Draize ocular irritation test has been considered the global standard for assessing eye irritation potential of chemicals. Per the most recent OECD test guideline (OECD TG 405), chemical-mediated ocular effects are evaluated in up to three albino rabbits. The effects are graded according to (a) the severity of lesions produced in the cornea, iris, and conjunctiva and (b) the duration over which the lesion(s) persist. While it has served as the standard approach for many years, the Draize test has several limitations including subjectivity related to qualitative scoring of effects, biological variability in animal response as well as anatomical differences between rabbit and human eyes like nictitating membrane, cornea thickness and lack of sufficient tearing effects (Williams et al., 1982; Scott et al., 2010; Adriaens et al., 2014; Barroso et al., 2016). Considering the above concerns as well as the ethical considerations related to animal testing, increased emphasis has been placed on developing mechanism-based non-animal alternatives to the Draize test.

While there are several modes of action by which chemicals can cause ocular injury upon direct contact, most often the degree of ocular irritation is driven primarily by direct cytotoxicity (Scott

* Corresponding author.
E-mail address: rssettivari@dow.com (R.S. Settivari).

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et al., 2010; Hackett and McDonald, 1991; Fox and Boyes, 2008). A number of alternative model systems have been developed to assess the cytotoxic response of chemicals in different culture systems. These can be divided into (i) organotypic models: Bovine Corneal Opacity and Permeability (BCOP) assay; Isolated Chicken Eye (ICE) test and Hen's Egg Test-Chorioallantoic Membrane (HETCAM) assay, etc. (ii) 2-dimensional cell culture models: Neutral Red Release (NRR); Neutral Red Uptake (NRU); Short Time Exposure (STE) test; Fluorescence Leakage (FL) test; Cytosensor Microphysiometer (CM) test, etc. (iii) 3-dimensional cell models: EpiOcular™ (EO) assay; Human Corneal Epithelial Model (HCE), etc. While some of these methods have undergone formal validation and have internationally adopted guidelines, others are still under development. In each of these test systems, the test material is applied directly to the cells or tissue to mimic human topical exposure and then cell viability or membrane integrity is determined as an indicator of potential ocular effects. These alternative methods have many potential advantages including reduction in animal use, permitting robust concentrations-response evaluation, shorter turnaround time etc. With all of these alternative methods, accuracy in comparison to *in vivo* data-sets needs to be determined. While the ultimate goal is to maximize both sensitivity and specificity, it is of particular importance to reduce occurrence of false negative predictions especially for severe eye irritants (UN GHS Cat 1; which may cause extensive ocular damage) so as to ensure that resulting hazard communication materials are adequately health protective (for appropriate PPE usage). Ultimately, if products are misclassified, the appropriate PPE and precaution may not be indicated and therefore could represent an issue for safe handling and use by down-stream users.

The NRR assay is a cytotoxicity-based alternative test method that uses mouse fibroblasts or human keratinocytes to identify potential ocular irritants. In this assay, the cells are incubated with a water-soluble weak cationic dye, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red; NR), which selectively accumulates within lysosomes (due to the pH difference in lysosomes and cytoplasm) of healthy cells. Subsequent exposure of the NR dye-loaded cells to potential ocular irritants results in cell membrane damage and release of the NR dye which is quantified to correlate to the eye irritation potential of the chemical (Reader et al., 1990). The NRR assay has relatively short exposure period (i.e. 1 min) and permits evaluating test chemicals at concentrations up to 100%. The NRR assay was included in several inter-laboratory studies and external validation studies, including an ECVAM retrospective validation study (Zuang, 2001). It was one of six alternate test methods that had better correlation with the *in vivo* Draize ocular test for surfactants and hydro-alcohol formulations belonging to Global Harmonization System (UN GHS) category 1, however, it tended to over-predict UN GHS category 2 and category Non Classified (NC) substances (Gettings et al., 1994, 1996).

The EpiOcular assay is another *in vitro* cytotoxicity-based assay for determining eye irritation potential of chemicals. The tissues used in this assay are reconstructed from primary human keratinocytes, which are cultured for several weeks to form a highly differentiated, multi-layered stratified squamous epithelium that is morphologically similar to that found in the human cornea. In the EpiOcular assay, the test chemical is applied topically and the exposure time required to reduce tissue viability by 40% of controls is measured (ET-40 method) to discriminate chemicals belonging to UN GHS NC from ocular irritants. The performance of the EpiOcular Eye Irritation Test (EIT) was evaluated by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and Cosmetic Europe between 2008 and 2013. Recently an OECD test guideline was published for the EIT for the identification of UN GHS Cat NC compounds from ocular irritants (OECD TG 492).

Both the NRR and the EpiOcular assays have been evaluated for their performance primarily using single compounds or a few simple mixtures in their respective validation studies. Agrochemical formulations are diverse and often complex mixtures containing one or more active ingredients and various co-formulants (e.g. solvents, surfactants, etc.). The combination and concentrations of co-formulants are specific to agrochemical formulations and serve a function in delivering the active ingredient effectively in field applications (adjuvants, antifoam, biocides etc). Often the agrochemical formulations consist of high concentrations of active ingredients and co-formulants as they are intended to be diluted several-fold before their use. These characteristics may impact osmolarity and surface tension thereby presenting challenges to successfully apply these alternative methods. Successful application of alternative methods for formulations would provide a major advancement for the agrochemical industry in reducing animal use since one active ingredient can be formulated to make multiple formulated products, each requiring an evaluation of eye irritation potential prior to registration. The purpose of the current study was to evaluate the performance of the NRR and EpiOcular assays alone and in combination for a variety of agrochemical formulations. In this study, a total of 64 and 51 agrochemical formulations were evaluated in the NRR and EpiOcular assays, respectively (Table 1). In each case, *in vivo* ocular irritation data was already available for these formulations. As the NRR and EpiOcular assays cover different regions of the eye irritation spectrum, these assays when used in a tiered–approach are expected to complement each other and provide better predictions compared to the existing *in vivo* Draize eye irritation data. Therefore, the data from both assays were also applied in tiered–approach and the results were compared to existing *in vivo* Draize data. Based on the performance of the selected test methods on the agrochemical formulations included in this study, the NRR and EpiOcular assays were concluded to provide encouraging performance for assessing ocular irritation potential when applied in a tiered-manner.

2. Materials and methods

2.1. Test materials

All prototypical compounds (sodium dodecyl sulfate, benzalkonium chloride, Tween™ 80, Triton™ X-100, polyethylene glycol 400, ammonium lauryl sulfate, benzethonium chloride, and Tween™ 20) used to demonstrate technical proficiency of the conducting laboratory (i.e., The Dow Chemical Company) were purchased from Sigma-Aldrich, St. Louis, MO. In the main study, 64 agrochemical formulations corresponding to eleven formulations types (Tables 1A and 1B) were assessed. All agrochemical formulations (liquids and solids) were of commercial quality, obtained from Dow AgroSciences, Indianapolis, IN and represented all categories (Cat) of eye irritation potential according to the UN GHS classification and labeling (ST/SG/AC.10/30/Rev.5 – UN – New York and Geneva, 2013) (Table 1B). Results of *in vivo* rabbit studies were used in order to establish the classification according to the UN GHS criteria. For the scope of this study, the following definitions were used: 'Severe Irritant' for UN GHS Cat 1, 'Moderate Irritant' for UN GHS Cat 2A, 'Mild irritant' for UN GHS Cat 2B, 'non classified' for those formulations which do not meet UN GHS criteria for classification (UN GHS Cat NC) and a general descriptor of 'Irritants' for UN GHS Cat 2 (Cat 2A and 2B). All the *in vivo* studies for the selected agrochemical formulations have been previously performed as part of the regulatory requirements and no additional animal experiments were conducted for the purpose of this study.

Cell culture reagents including Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's phosphate buffered saline (DPBS)