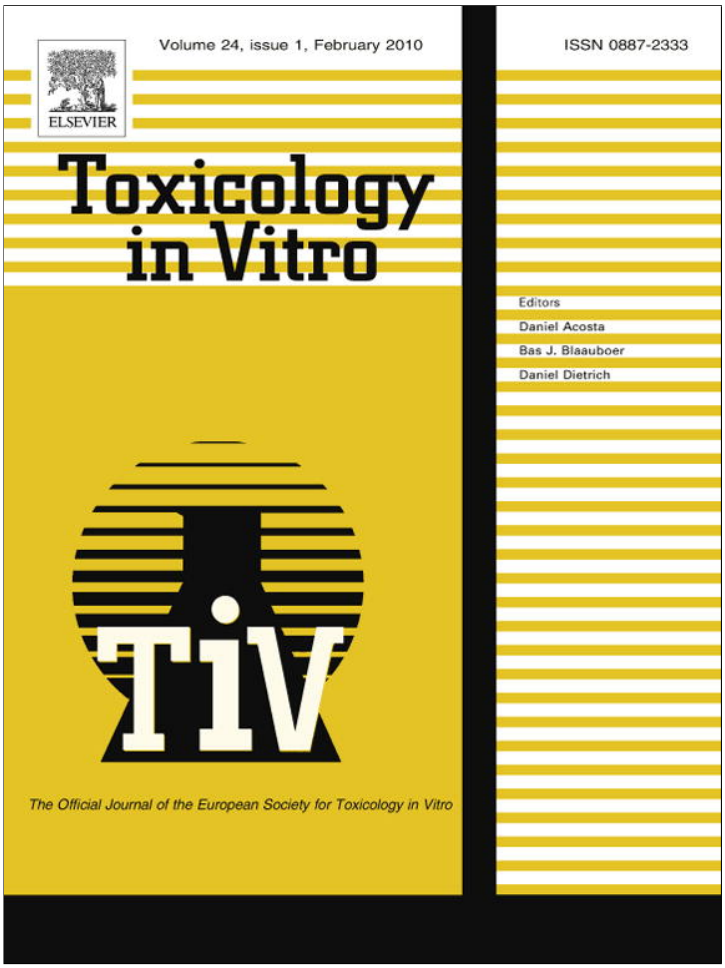

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Review

A proposed eye irritation testing strategy to reduce and replace *in vivo* studies using Bottom–Up and Top–Down approaches ☆

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ABSTRACT

In spite of over 20 years of effort, no single *in vitro* assay has been developed and validated as a full regulatory replacement for the Draize Eye Irritation test. However, companies have been using *in vitro* methods to screen new formulations and in some cases as their primary assessment of eye irritation potential for many years. The present report shows the outcome of an Expert Meeting convened by the European Centre for the Validation of Alternative Methods in February 2005 to identify test strategies for eye irritation. In this workshop test developers/users were requested to nominate methods to be considered as a basis for the identification of such testing strategies. Assays were evaluated and categorized based on their proposed applicability domains (e.g., categories of irritation severity, modes of action, chemical class, physicochemical compatibility). The analyses were based on the data developed from current practice and published studies, the ability to predict depth of injury (within the applicable range of severity), modes of action that could be addressed and compatibility with different physiochemical forms. The difficulty in predicting the middle category of irritancy (e.g. R36, GHS Categories 2A and 2B) was recognized. The testing scheme proposes using a Bottom–Up (begin with using test methods that can accurately identify non-irritants) or Top–Down (begin with using test methods that can accurately identify severe

☆ Disclaimer: The authors of this document participated as individuals, and the opinions expressed do not represent the official positions of any government agency or other organization. Affiliations given were those current at the time of the Expert Meeting.

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irritants) progression of *in vitro* tests (based on expected irritancy). Irrespective of the starting point, the approach would identify non-irritants and severe irritants, leaving all others to the (mild/moderate) irritant GHS 2/R36 categories.

Contents

1. Introduction

The Draize eye irritation test continues to be the primary method accepted by regulatory agencies worldwide. Most regulatory agencies divide responses into categories of irritation based on specific tissue scores and the duration over which the lesions persist. While scoring of the specific tissue lesions is similar across most agencies, the various hazard categorization schemes have distinct differences (EPA,1998; ECC, 1967; EU, 2001, 2004; OECD, 2002; UN/ECE, 2003). Over the past decades, there have been substantial investments into both the development of *in vitro* methods and execution of validation/evaluation studies to assess the reliability and reproducibility of these test methods to predict the eye irritation responses in the Draize Test (Bruner et al., 1991; Balls et al., 1995; Brantom et al., 1997; Gettings et al., 1991, 1992, 1994, 1996; Spielmann et al., 1993, 1996; Bradlaw et al., 1997; Ohno et al., 1999; ICCVAM, 2007, 2006a,b,c,d). Despite these efforts, no *in vitro* test has successfully been validated to fully replace the Draize eye irritation test for regulatory purposes.

Despite the lack of formally validated in vitro eye irritation test methods for regulatory purposes, in vitro eye irritation tests have a long history of use and acceptance by industry for specific purposes (Harbell and Curren, 2001; Curren and Harbell, 2002; Eskes et al., 2005). More recently, there has been limited acceptance by regulatory agencies for the prediction of severe eye irritants (EC, 2004; NIEHS, 2008). In February 2005, an Expert Meeting was convened by ECVAM to critically evaluate the limitations and advantages of selected in vitro test methods with a view to identify proposed applicability domains where reliable and relevant results may be obtained, based on expertise from in-house experience and from the various evaluation studies which took place in the last decades. From these analyses, it was envisioned that an in vitro eye irritation testing strategy could be developed to reduce, and ultimately replace, animal use (Goldberg and Silber, 1992; Rougier et al., 1992; Balls et al., 1999; Eskes et al., 2005).

2. Eye irritation expert meeting objectives

To progress this concept, ECVAM invited test method developers and users to nominate in vitro eye irritation test methods that could be considered as a basis for a testing strategy. Specifically, participants were asked to provide parallel in vivo and in vitro data to support the usefulness of the nominated in vitro test method for a specific applicability domain (e.g., range of eye irritation severity, chemical class, mechanisms of irritation).

On the 8th–11th of February 2005, over thirty scientists from academia, government, non-profit organizations, private industry (including contract test laboratories), as well as international validation experts and regulators met in Ispra, Italy to share data in support of in vitro eye irritation test method(s) nominated for consideration.

The objectives of this activity were to:

1. Obtain in vitro eye irritation test method nominations, with supporting data, for a specific applicability domain(s).
2. Clarify how each nominated method is currently being used (e.g., screening to guide Research & Development (R&D) efforts, hazard classification, risk assessment).
3. Identify partners to progress promising testing methods into validation.
4. Identify gaps where to focus future research and method development efforts.
5. Propose in vitro testing strategies or approaches that could be further developed to validation by ECVAM in their overall efforts to reduce, and ultimately replace, animal use for eye irritation hazard identification and classification in accordance with Annex V of Directive 67/548/EEC (the "Dangerous Substances Directive") (ECC, 1967; EU, 2001, 2004).

Fourteen test methods were nominated for which supporting data was submitted to ECVAM for review (Fig. 1). Each participant provided an overview of the optimized test method(s) used within their organizations, described how each method was currently used for specific purposes and submitted data to support their proposed applicability domain. Based on the knowledge acquired and the expert's experience, a testing strategy approach to reduce, and hopefully replace, animal use for eye irritation was proposed.

3. Results

A large variety of test methods was nominated for use within a testing strategy (Fig. 1) including isolated corneas/eyes (bovine, porcine, rabbit, chicken), chorioallantoic membrane methods (HET-CAM and CAM-TBS), reconstructed human tissues (RHT) engineered with either transformed human corneal epithelial cells (Human Corneal Epithelium (HCE or SkinEthic™HCE) or normal human foreskin keratinocytes (EpiOcular™), cytotoxicity assays (Neutral Red Release & Red Blood Cell Lysis), cell function-based assays (Fluorescein Leakage & Cytosensor™ microphysiometer), simulated corneal opacity models (Irritection®) and the slug muco-

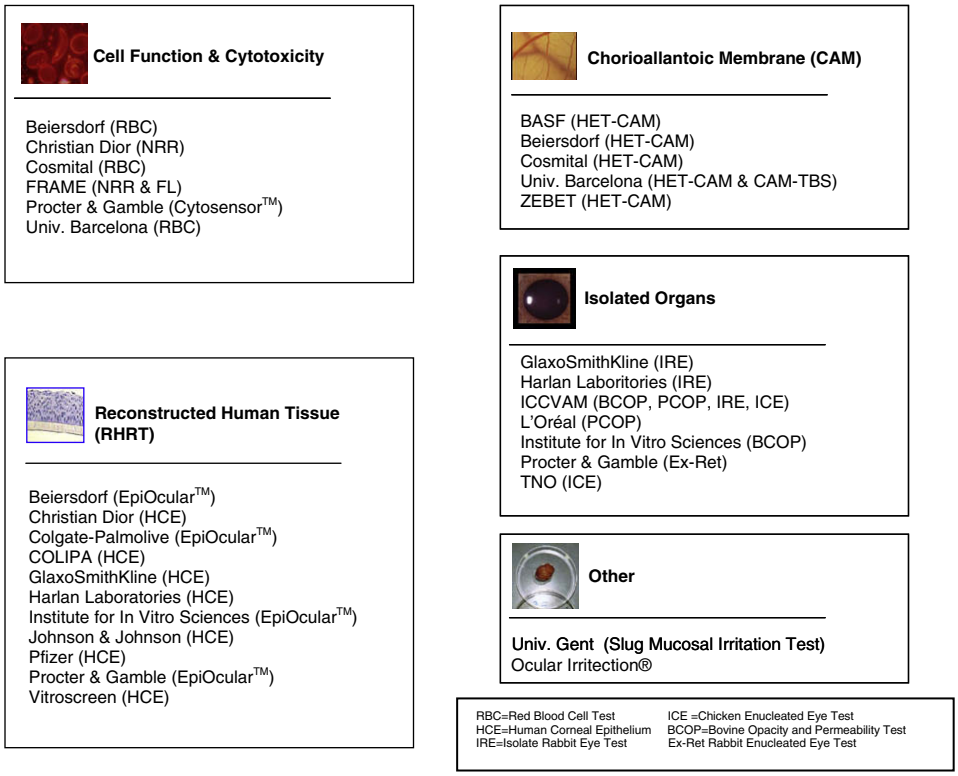

Fig. 1. Test methods nominated for use in testing strategy. This figure illustrates the broad participation in the expert meeting and diverse test methods nominated for use within a testing strategy to reduce, and ultimately replace, *in vivo* eye irritation testing. The methods and organizations mentioned above were represented at the Expert Meeting on 8th-11th February 2005 in Ispra, Italy.

sal irritation (SMI) test. These in vitro methods had defined protocols and Standard Operating Procedures (SOPs) that include controls which could be used to confirm that the assays were conducted correctly. The in vitro test methods were currently used for various purposes including: R&D screening, risk assessment of finished products, in-house characterization of chemical hazard, and for chemicals classification and labeling of severe eye irritants in accordance with the European Union's regulatory acceptance for this purpose (EC, 2004). The primary use of the nominated test methods has been to evaluate the eye irritation potential of consumer products (e.g., cosmetics, cleaning and detergent products, pharmaceutical/ OTC, beauty care, ophthalmic products).

The general approach used by several industry organizations to ensure eye safety from intended use and foreseeable misuse (e.g., occupational and consumer accidental exposure) of a consumer product is represented in Fig. 2. Following the evaluation of all existing data (e.g., structure activity relationships, skin irritation data, physicochemical properties, other in vivo data provided by the supplier) an in vitro eye irritation test, optimized for the product type and category of interest, was conducted. For some affiliations, a tiered testing approach, with two or more in vitro tests, was conducted. Often an upper benchmark of similar composition to the test formula or ingredient was included in each in vitro test. The benchmark served as an internal control to identify the maximum in vitro response acceptable for a given category (Cuellar et al., 2002; Rispin et al., 2006). Benchmarks were selected based on a variety of data that included historical in vivo data, consumer in-use studies and market experience. Based on the risk assessment approach outlined in Fig. 2, it was reported that years of safe marketing experience (based on corporate or National post-market surveillance), for diverse finished product categories, had been obtained in the absence of in vivo animal data. As a reduction and refinement approach, several contributors have developed and qualified in vitro tests to identify and classify/label ingredients as severe eye irritants, labeled as R41 according to the EU classification scheme. For these contributors, in vivo eye irritation testing was primarily restricted to those chemicals not regarded as severe irritants based on the results of in vitro assays.

Based on available data and the experience of the participants, methods were proposed for identifying and classifying substances as R41/GHS cat.1 or not-classified (see Tables 1A and 1B), and deemed suitable for use within a testing strategy and defined applicability domain(s) for the purpose of hazard identification and labeling in accordance with Annex V of the "Dangerous Substances Directive" 67/548/EEC (ECC, 1967; EU, 2001, 2004).

4. Applicability domains for eye irritation testing strategies

In order to identify test methods for a defined applicability domain, parameters from which a domain could be established were discussed. The domains included the severity of the eye irritation response, mechanism of induced eye irritation and physicochemical properties of the chemicals/formulations.

There was general agreement that obtaining harmonized and generally accepted chemical categories to define appropriate applicability domains for an in vitro test method might be difficult. Therefore, it was proposed to expand this concept to define the applicability domain as a function of the mechanism of eye irritation induced. The four modes of action discussed included: cell membrane lysis, saponification, coagulation and actions on macromolecules (Maurer et al., 2002; Jester, 2006). *Membrane Lysis:* breakdown of membrane integrity as might occur from exposure to membrane active materials (e.g., surfactants). *Coagulation*: precipitation/denaturation of macromolecules (particularly protein), characteristic of acid, alkali, or organic solvent exposure. *Saponification*: breakdown of lipids by alkaline action. The outward signs

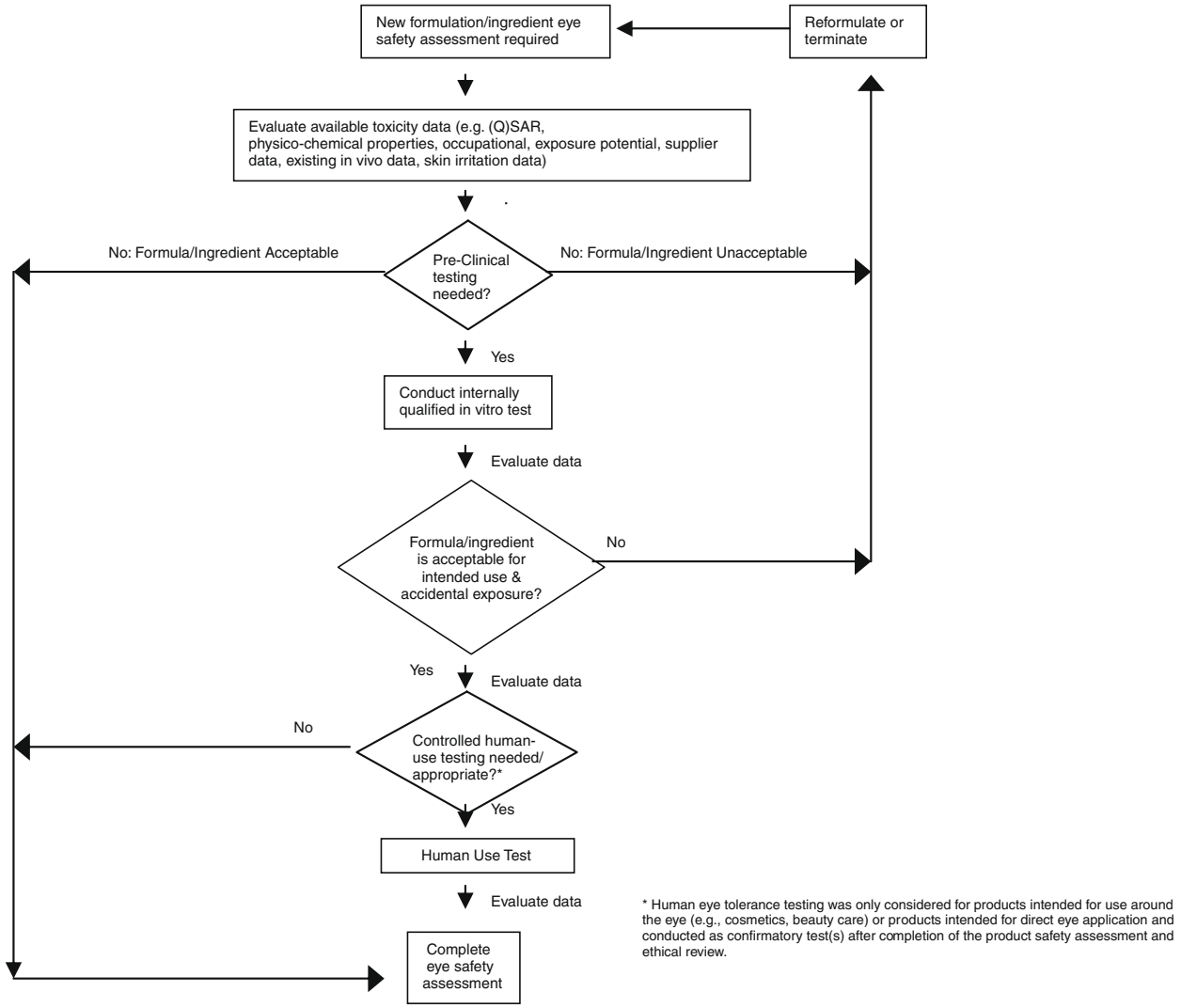

Fig. 2. *In vitro* eye irritation testing approach for risk assessment. This schematic provides an overview of the approach generally adopted by some industry organizations to assess the eye irritation potential of finished products via the use of internally qualified in vitro eye irritation test methods.

Table 1A
EU criteria for classification (EU, 2001). All scores at each of the reading times (24, 48 and 72 h) for an effect should be used in calculating the respective mean values.

	R36 Irritant to the eyes[a]	R41 Risk of serious damage to eyes[b]
Corneal opacity	$2.0 \leqslant$ mean score < 3.0 or $2.0 \leqslant 2$ animals (of 3) < 3.0	mean score $\geqslant 3.0$ or 2 animals (of 3) $\geqslant 3.0$
Iris lesion	$1.0 \leqslant$ mean score $\leqslant 1.5$ or $1.0 \leqslant 2$ animals (of 3) < 2.0	mean score > 1.5 or 2 animals (of 3) $= 2.0$
Redness of conjunctivae	mean score $\geqslant 2.5$ or 2 animals (of 3) $\geqslant 2.5$	Included in the persistence measurements
Oedema of conjunctivae (chemosis)	mean score $\geqslant 2.0$ or 2 animals (of 3) $\geqslant 2.0$	Included in the persistence measurements

[a] R36 classification also applies to organic peroxides (except if evidence to the contrary) and substances causing significant ocular lesions in humans.
[b] R41 classification also applies to ocular lesions still present at the end of the observation time (a maximum of 21 days), substances causing irreversible colouration to eyes and substances causing severe ocular lesions in humans.

may be similar to membrane lysis and coagulation but the action tends to be progressive with time through the tissue. *Actions on macromolecules:* Chemicals that react with cellular constituents/ organelles (e.g., alkylation, oxidative attack on macromolecules such as essential proteins or nucleic acids) that may or may not lead to overt lysis or coagulation. Concern with this group is the lack of immediate response in the tissue (delayed onset). Examples include peroxides, mustards and bleaches. These modes of action were selected based on the mechanism of irritation known to be induced by various chemical classes (Table 2). The participants proposed the mechanism of action each test method is known or anticipated to detect (Table 3).

Relative to the severity of eye irritation, there was general agreement with the following principles:

- In general, methods that included a stroma (isolated organ methods) have the greatest potential to distinguish severe eye irritants (EU R41/GHS cat.1) from other classes (EU R36 / GHS cat.2 or not labeled). This view is consistent with the mechanistic understanding reported by (Maurer et al., 2002; Jester, 2006),

Table 1B
GHS Criteria for Classification (UN/ECE, 2003). Based on mean scores following grading at 24, 48 and 72 h after installation of the test material.

	Category 2A Irritant to the eyes[a]	Category 1 Irreversible effects on eye[b]
Corneal opacity	At least 2 animals (out of 3) ⩾ 1.0	At least 2 animals (out of 3) ⩾ 3.0
Iris lesion	At least 2 animals (out of 3) ⩾ 1.0	At least 2 animals (out of 3) > 1.5
Redness of conjunctivae	At least 2 animals (out of 3) ⩾ 2.0	Included in the persistence measurements
Oedema of conjunctivae (chemosis)	At least 2 animals (out of 3) ⩾ 2.0	Included in the persistence measurements

[a] Cat.2 classification: All effects have to fully reverse within 21 days after treatment. This single hazard category provides the option to identify within the category a sub-category for substances inducing eye irritant effects reversing within an observation time of 7 days (category 2B, mildly irritating to eyes).
[b] Cat.1 classification also applies to at least in one animal effects on cornea, iris or conjunctiva that are not expected to reverse or have not fully reversed within an observation period of normally 21 days. Animals with grade 4 cornea lesions and other severe reactions (e.g., destruction of cornea). Persistent corneal opacity, discoloration of the cornea by a dye substance, adhesion, pannus and interference with the function of the iris or other effects that impair sight. Persistent lesions are those which are not fully reversible within an observation period of normally 21 days.

Table 2
Mechanism of Eye Irritation and Chemical Categories.

	Mechanism of Eye Irritation			
	Cell Membrane Lysis	Coagulation	Saponification	Actions on Macromolecules
Chemical Categories	• Surfactants • Organic solvents • Ketones • Alcohols • Volatile liquids • Ethers • Polyethers • Ester(s) • Aromatic amines	• Acids • Cationic surfactants • Organic solvents	Alkalis	• Peroxides • Mustards • Alkyl halides • Epoxides • Bleaches (oxidizers)

Example of chemical categories known or anticipated to act through one or more modes of action to induce eye irritation. Mechanistic considerations, by chemical category, are suggested as one approach from which to define and ultimately select test methods for use within an eye irritation testing strategy.

Table 3
Proposed modes of action addressed by the nominated test methods.

Method	Membrane Lysis	Coagulations	Saponification	Actions on Macromolecules[a]
HCE	x	x	x	?
EpiOcular	x	x	x	?
Irritection	x	Some limits	Some limits	No
HET-CAM	x	x	x	More data needed
CAM-TBS	x	x	x	More data needed
ICE	x	x	x	With histology
IRE	x	x	x	With histology
PCOP	x	x	x	With histology
BCOP	x	x	x	With histology
RBC	x	Some limits	Some limits	No
NRR	x	Some limits	Some limits	No
Cytosensor	x	No	No	No
SMI	x	x	x	?
FL	x	?	?	?

The table shows the mode(s) of action each nominated test method is anticipated to detect. It is important to note however, that substances working through a mode of action not directly addressed by a test method may still be well predicted by that test method. Validation efforts are required to determine the overall applicability domain of the assays. A question mark indicates that additional evaluation of the data is required.
[a] Chemicals that react with cellular constituents / organelles that may or may not lead to over lysis or coagulation.

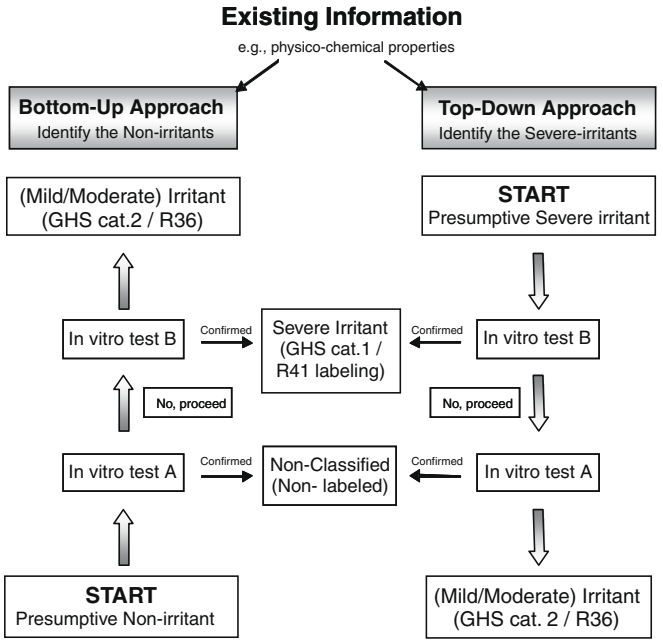

Fig. 3. Bottom–Up and Top–Down *in vitro* testing strategy approach for eye irritation. This figure illustrates the proposed testing strategy approach from which to develop an eye irritation testing strategy. If the test material is expected to be a no-low eye irritant the Bottom–Up approach is initiated. Conversely, the Top–Down approach is initiated if the test material is expected to be a moderate to severe eye irritant. Validated methods would be used in a two-step procedure to determine if a test material is a non-classified or severe irritant (EU R41/GHS cat.1). A default EU R36/GHS cat. 2 classification could be assigned if neither a non-classified or severe irritancy assignment were made.

which demonstrated that the area and depth of cell injury observed microscopically paralleled the general level of macroscopic damage and irritation observed and time to recovery, if it occurred. These studies showed that damage to the cornea from materials not classified for eye irritancy was limited to the epithelium while those classified as eye irritants produced corneal damage that extended into the stroma with depth of stromal (and possibly endothelial) damage identifying the severity of the eye irritant (Fig. 4).

- Epithelial models (Human Corneal Epithelial, HCETM or EpiOcularTM) and test methods which assessed the potential of a test material to disrupt cellular membranes (Red Blood Cell Assay, Neutral Red Release, Fluorescein Leakage and CytosensorTM) or measured protein coagulation/vasculature changes (HET-CAM) have the greatest potential to distinguish non-classified substances from irritants.

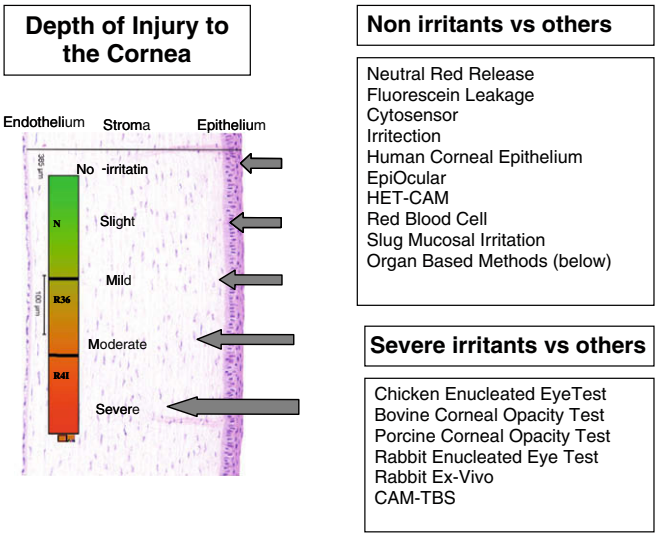

Fig. 4. Potential applicability domain of test methods according to severity of eye irritation. Each method was nominated by the test sponsor for an applicability domain based on the severity of eye irritation responses. Methods were nominated based on in-house experience on the performance of the test method to detect non-classified substances (NC) from other classes (EU R36/GHS cat.2 and R41/GHS cat.1 test materials) or severe eye irritants from other classes (EU R36/GHS cat.2 and NC).

- In general, it will be more difficult to distinguish the middle range of irritation for test materials, i.e., test materials which induced irritation (EU R36/GHS cat.2 labeling) from those which induced severe eye irritation / corrosivity (EU R41/GHS cat.1 labeling) and from the not classified substances.
- Information on the physicochemical properties of a test material/formula should be considered to determine, if feasible, the potential severity of irritation, mechanism(s) of irritation and compatibility (e.g., solid, liquid, solubility profile), for selecting the appropriate in vitro test methods. The type of information typically required for a new chemical notification and therefore available for inclusion in a testing strategy would include physical form, MW, pH, octanol/water partition coefficient, ionization potential, melting and boiling point, vapor pressure, water solubility and surfactant critical micelle concentration. The test method should also not alter the acid/alkaline potential of the ingredient or formulation.

Based on: (1) in-house experience, (2) data from previous evaluation studies, (3) expert opinion, (4) the data presented and (5) agreement to the above principles, the following was recommended as a plausible approach from which a testing strategy could be developed and validated.

5. Bottom–Up and Top–Down Approach: severity of response

The Bottom–Up and Top–Down Approach illustrated in Fig. 3 depicts the general approach recommended from which a testing strategy could be developed for hazard identification and labeling in alignment with Annex V of *Directive 67/548/EEC*. In this approach, the applicability of a test method is based on the range of irritation severity for which reliable and relevant results can be demonstrated. Validated *in vitro* tests capable of accurately and reliably distinguishing either non-irritants (NI or non-classified) from classifiable (EU R36/GHS cat.2 and EU R41/GHS cat.1) eye irritants or severe eye irritants/ocular corrosives (EU R41/GHS cat.1) from other classes (EU R36/GHS cat.2 and NI) could result in a significant reduction in animal use. Based on the physicochemical properties of the test material and other existing data (e.g., skin irritation data) the eye irritancy potential is estimated. If the test material is anticipated to be of no to low eye irritancy potential, the Bottom–Up approach would be initiated (Fig. 3). In contrast, test materials estimated to have high eye irritancy potential would initiate the Top–Down approach (Fig. 3).

With the Bottom–Up approach, test materials estimated to have a no to low eye irritancy potential would be initially tested using an appropriate test method validated and highly accurate and reliable for identifying non-classified from classified (EU R36/GHS cat.2 and EU R41/GHS cat.1) substances (Fig. 3). If test results indicate that the material is non-classified, no additional testing would be needed and the material would be considered as non-classified (Fig. 3). In contrast, if the test material was identified as classifiable, an additional appropriate validated and highly reliable test to identify EU R41/GHS cat.1 materials from other classes (EU R36/GHS cat.2) would be used. A result predicting severe irritation potential would lead to EU R41/GHS cat.1 classification and a non-severe result would result in EU R36/GHS cat.2 labeling.

In contrast, the Top–Down approach would be initiated if the test material was estimated to have high eye irritancy potential (Fig. 3). Initiating the Top–Down or Bottom–Up approach would streamline the testing series to avoid unnecessary *in vitro* studies and related costs.

Based on this approach, participants also identified the most suitable potential placement for each nominated method within the proposed testing strategy (Fig. 4). The proposed test method placement would serve as a starting point to guide ECVAM's efforts, working in partnership with the test sponsor(s), to evaluate and ultimately validate reliable and relevant *in vitro* test methods useful for the proposed or an alternative applicability domain in accordance with the proposed testing strategy.

6. Discussion

The suggested Bottom–Up and Top–Down approach provides a reasonable framework from which to develop and validate an *in vitro* eye irritation testing strategy. In this approach, highly sensitive or specific test methods are used to identify either EU R41/GHS cat.1 or non-classified substances, respectively, for regulatory purposes without necessarily the need to use *in vivo* testing.

In November 2004 ECVAM completed an evaluation of the New Chemical Database to establish the prevalence of eye irritation according to the EU classification scheme (Hoffmann, personal communication). Of the 2497 chemicals registered and evaluated in this review, 78.9% of the chemicals were non-irritant (non-classified). If the prevalence of future submissions is consistent with the New Chemical Database repository evaluated, the validation of methods that can accurately identify non-irritants from classified substances (R36/R41) could reduce the need for animal eye irritation testing up to approximately 80%. Additional validation of test methods for R41classification could further reduce the animal use by 16%. As reflected in the database, only a very small percentage of chemicals are classified as R36. Therefore, focusing on test methods that can accurately predict non-classified and severe irritant categories versus others could eventually lead to elimination of the reliance on animal testing. To ultimately implement this approach, it is important that international regulatory authorities accept the scientific validity of assays capable of accurately identifying negative results, without enforcing the unnecessary need for confirmatory animal results. The continued recognition of expert judgment, concurrent with the acceptance of scientifically sound weight of evidence approaches that integrate and accept the use of all evidence for hazard classification will be important considerations for future test strategy developments.

The technical challenges associated with the development of *in vitro* test systems that reproducibly mimic the complex biological responses of the *in vivo* eye are recognized. While this paper illustrates a new approach to further advance the use of *in vitro* test methods for classification purposes, it is foreseen that targeted development and inclusion of *in vitro* test methods required to obtain the specific degree of sensitivity or specificity required will be necessary to enable the future development of test batteries that can distinguish all UN GHS categories, as required by various international authorities.

In addition, factors associated with the Draize *in vivo* test were identified that contribute to validation difficulties. Variation in the animal to animal responses within replicate test subject groups, variations in the responses over time in single laboratories, and variations in responses across laboratories responses are well documented (Wiel and Scala, 1971; Marzulli and Ruggles, 1973; Griffith et al., 1980). The variability in these studies resulted from factors such as an indeterminate effective exposure (volume over time) during treatment, differences in rabbit size (and therefore size of the eye), inconsistent use of restrain to prevent pawing of the irritated eye, and subjective scoring differences among graders. Prinsen (2006) reported that the contact time for materials placed in the cul-de-sac of the rabbit eye can vary from minutes to twenty-four hours, leading to diverse animal responses as a result of sustained exposure and/or mechanical abrasion. The range of animal scores, introduced from the limitations of the test method design and standardization, can be further compounded by those regulatory categorization schemes that are based on the most severe response of a single animal rather than the overall responses of the group of animals treated. These variables can confound an understanding of the test material's true range of irritancy potential relative to its *in vivo* categorization by a single assay (Jackson, 1984; Prinsen, 2006).

Concerns have also been raised with regard to the relevance of the Draize eye irritation test procedures to humans. This concern is the result of the method of test material instillation, the volume administered and the increased sensitivity of the rabbit to eye irritation as compared to humans (Freeberg et al., 1986, Lambert et al., 1993, Nussenblatt et al., 1998; Bagley et al., 2006).

In several past validation projects, the selection of Draize *in vivo* eye irritation data has primarily been based on the Maximum or Modified Maximum Average Scores (MAS or MMAS). This approach was used in recognition of the continuous nature of the degrees of eye irritation. However, most regulatory authorities classify eye irritants according to the duration and severity of effects on specific ocular tissues (cornea, iris, conjunctiva). As such, validation efforts now tend to compare *in vitro* data with the regulatory accepted *in vivo* classifications based on the Draize test data (often using several distinct regulatory schemes). It is recognized that the biological responses, when reduced to classification bands, are discontinuous and not necessarily linear in their progression. Thus, it is difficult to distinguish misclassification by the *in vitro* test from variability of the *in vivo* results that may occur at the borderline of distinct classification bands. Since accuracy is required of the *in vitro* assay in the validation process, the borderline responses present an additional challenge in the effort to replicate predicted *in vivo* irritation category.

In order to develop and finalize a testing strategy to progress into validation, data compilation from all available sources and subsequent analyses are required. Furthermore, it is important to recognize the need for development of statistical approaches/tools that enable integrated analysis of data from *in vitro* assays used in combination. To progress the validation efforts on single assays, an ECVAM Retrospective Weigh-of-Evidence Validation Study took place on four cytotoxicity- and cell function-based assays (Neutral Red Release, Fluorescein Leakage, Cytosensor Microphysiometer and Red Blood Cell Assay). ECVAM also started a prospective validation study on reconstructed human tissue models (EpiOcular™ and SkinEthic HCE™). In addition, several meeting participants have submitted or are currently designing validation studies for execution in collaboration with ECVAM. ICCVAM, on the other hand has completed an evaluation of the usefulness and limitations of four organotypic methods (BCOP, Isolated Chicken Eye, Isolated Rabbit Eye, and HET-CAM) for identifying severe ocular irritants and corrosives based on BRD's (ICCVAM, 2006a,b,c,d, 2007). Two of the methods (BCOP, ICE) were endorsed as scientific valid and recommended for regulatory use (ESAC, 2007; ICCVAM, 2008). These assays have been accepted by EU and US regulatory authorities (EC, 2004; NIEHS, 2008; EHP, 2008). Pending the integration of all existing information, data analysis will be initiated to identify and assess the feasibility of validating a testing strategy using the Top–Down and Bottom–Up approach developed from this expert meeting.

To date, the use of physicochemical descriptors have not been fully explored or required to define the applicability domain of *in vitro* test methods. However, as testing strategies are further developed and deployed, the integration of such tools is envisioned. The BfR decision support system has attempted to compile and evaluate structural alerts and physicochemical descriptors for which relevant predictions can and cannot be made (Gerner et al., 2005). Pending their validation and larger distribution expert evaluation of the data is still required to determine if there is a scientific relevance for these results. In addition, efforts should be initiated to determine if physicochemical descriptors can be developed and be used to define the applicability domain of the various *in vitro* test methods. The integration of knowledge acquired as a result of this in-depth evaluation is expected to provide new insight into the mechanism and advantages/limitations of the various *in vitro* test methods. Furthermore, software to aid in the calculation of these descriptors to assist users and regulators in both the test method selection and determining if results are valid, may prove invaluable tools to the future success and validation of an eye irritation testing strategy for regulatory purposes.

Finally, additional efforts were also recommended to define the boundaries for applicability domains and to develop the decision criteria to guide users through the test strategy ultimately developed.

7. Conclusion

The proposed Bottom–Up and Top–Down testing strategy is a plausible approach for which *in vitro* test methods can be developed and validated in an attempt to reduce animal studies in the near term. To fully eliminate animal use and improve the relevance of *in vitro* eye irritation methods to predict human responses will require continued research to advance *in vitro* systems and to improve our mechanistic understanding of eye irritation. Additional human test data, where it can be ethically collected, and accidental human exposure data would also be helpful. The usefulness of such data could be improved by establishing uniform methods of recording data and incorporating methods to assess the reversibility of a response, sensory(pain) attributes, discoloration and approaches from which quality human eye irritation data can be obtained. Furthermore, a systematic compilation and evaluation of high quality existing Draize eye irritation data is needed to serve as reference data for future validation studies of *in vitro* methods, and to improve comparative understanding of human and rabbit responses where parallel testing data is available. An aligned approach among the scientific community developing *in vitro* eye irritation test methods for regulatory purposes will also be necessary to identify and develop prediction models to meet regulatory agency needs.

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