Critical Reviews in Toxicology, 2010; 40(1): 50–83

informa
healthcare

REVIEW ARTICLE

Acute toxicity testing of chemicals—Opportunities to avoid redundant testing and use alternative approaches

Stuart Creton[1], Ian C. Dewhurst[2], Lesley K. Earl[3], Sean C. Gehen[4], Robert L. Guest[5], Jon A. Hotchkiss[6], Ian Indans[7], Michael R. Woolhiser[6], and Richard Billington[8]

[1]National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), London, UK, [2]Health and Safety Executive, York, UK, [3]Huntingdon Life Sciences, Huntingdon, UK, [4]Dow AgroSciences LLC, Indianapolis, Indiana, USA, [5]Harlan Laboratories Ltd., Shardlow, UK, [6]The Dow Chemical Company, Midland, Michigan, USA, [7]Health and Safety Executive, Bootle, UK, and [8]Dow AgroSciences Ltd., Abingdon, UK

Abstract

Assessment of the acute systemic oral, dermal, and inhalation toxicities, skin and eye irritancy, and skin sensitisation potential of chemicals is required under regulatory schemes worldwide. In vivo studies conducted to assess these endpoints can sometimes be associated with substantial adverse effects in the test animals, and their use should always be scientifically justified. It has been argued that while information obtained from such acute tests provides data needed to meet classification and labelling regulations, it is of limited value for hazard and risk assessments. Inconsistent application of in vitro replacements, protocol requirements across regions, and bridging principles also contribute to unnecessary and redundant animal testing. Assessment of data from acute oral and dermal toxicity testing demonstrates that acute dermal testing rarely provides value for hazard assessment purposes when an acute oral study has been conducted. Options to waive requirements for acute oral and inhalation toxicity testing should be employed to avoid unnecessary in vivo studies. In vitro irritation models should receive wider adoption and be used to meet regulatory needs. Global requirements for sensitisation testing need continued harmonisation for both substance and mixture assessments. This paper highlights where alternative approaches or elimination of tests can reduce and refine animal use for acute toxicity requirements.

Keywords: *3Rs; acute toxicity; animal testing; irritation; regulatory; sensitisation*

Contents

Address for Correspondence: Dr Stuart Creton, PhD, National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), 20 Park Crescent, London, W1B 1AL, United Kingdom. E-mail: stuart.creton@nc3rs.org.uk

(Received 11 June 2009; revised 07 October 2009; accepted 09 October 2009)

ISSN 1040-8444 print/ISSN 1547-6898 online © 2010 Informa UK Ltd
DOI: 10.3109/10408440903401511

1. Introduction

In vivo toxicological testing designed to assess the acute oral, dermal, and inhalation toxicities, skin and eye irritation, and skin sensitisation potential of chemicals and chemical preparations (the commonly termed 'six pack' of acute toxicity studies) is required by regulatory authorities around the world for the purposes of classification and labelling, risk assessment, and risk management of substances, in support of public health protection. However, the value of some data derived in acute toxicity studies may be limited, particularly where multiple routes of exposure and/or species are tested for the same substance.

While acute toxicity testing meets the needs of regulatory requirements, such as for classification and labelling purposes, which are primarily focused on hazard identification, the utility of the data obtained in these tests for the purpose of predicting the likelihood of effects occurring in humans has been questioned (Balls, 1991; Basketter et al., 1997; Langley, 2005; York et al., 1996; York and Steiling, 1998). In the pharmaceutical sector, a recent evidence-based review of the use of acute systemic toxicity studies during drug development, involving 18 European companies and coordinated by the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), concluded that stand-alone acute toxicity studies to determine a lethal dose are not useful to support first clinical trials in humans. This study highlighted that the utility of information obtained from acute toxicity studies is generally very limited, providing an estimate of minimum lethal and/or maximum non-lethal dose without an assessment of other parameters that are typically used in risk assessment. Instead, the information needed can be obtained from other tests employing non-lethal endpoints that are already carried out as part of the development process (Robinson et al., 2008). The findings of the review have led to changes in practice under the current regulations by the companies involved, reducing the numbers of animals used in acute lethality tests by 70%. The findings have also been incorporated into the revised international regulations, effectively ending the regulatory requirement for acute lethality studies for new medicines prior to clinical trials (ICH, 2009).

Clearly the replacement and/or reduction of unnecessary in vivo tests would have significant animal welfare

benefits and in some cases would also result in lower testing costs. Full replacement methods have not yet been accepted internationally for most of the endpoints under discussion in this paper. Nevertheless opportunities to reduce or avoid in vivo testing do exist. Recognising the potential benefits of reducing animal testing for acute toxicity, not only for the pharmaceutical sector but also for the chemical industry (i.e. plant protection products, biocides, industrial chemicals, and consumer products), the NC3Rs convened an expert working group to consider where there are opportunities to waive acute toxicity test requirements or to use alternative approaches that can replace, reduce, or refine animal use for chemicals through the identification of areas of 'redundant' animal testing—i.e. where there is duplication of testing, or testing is not required due to the availability of alternative approaches for obtaining the required data. Some tests may be redundant due to a lack of scientific justification for performing the study or because the information needed can be derived from existing data on similar chemicals, and waiving the need to perform such tests can make a significant contribution to reducing animal use.

This paper reviews existing arguments for redundancy in acute toxicity testing of chemicals and chemical preparations, and the potential for use of alternative methodologies in the generation of acute toxicity data, giving consideration to the sometimes disparate regulatory approaches in place across various industry sectors. A list of abbreviations commonly used in this paper is provided in Table 1.

2. Regulatory framework

Acute toxicity testing is required by regulatory bodies around the world. A major driver for these studies is for the classification and labelling of chemicals and chemical preparations based on their hazardous properties, although data from particular acute studies also contribute to elements of risk assessment and risk management. Substances that require classification and labelling include industrial chemicals, biocides, active pesticide ingredients and final formulations, isolated pharmaceutical intermediates, new food additives, cosmetic ingredients, and consumer products. Legislative controls around the world differ slightly with regard to their requirements and the broad hazard categories. However, the basic purpose of acute toxicity testing is the same: to allow substances to be categorised according to their potential hazards and the dose required to cause toxicity (e.g. categorisation under the Globally Harmonised System of Classification and Labelling of Chemicals [GHS]) is the same.

Test requirements can vary internationally, and also depend on the type of chemical under regulation. For example, EU and US regulations for pesticides require data to be generated for acute systemic toxicity by the oral, dermal, and inhalation routes, and also for skin and eye irritancy and skin sensitisation potential; the so-called 'six-pack'. For industrial chemicals, however, the EU's REACH regulation (Registration, Evaluation, Authorisation and restriction of CHemicals) sets out the toxicological information that is required depending on the production volume of the

Table 1. List of frequently used abbreviations.

ATC	Acute Toxic Class methods for assessing acute toxicity
BCOP	Bovine corneal opacity and permeability test
CxT	Concentration × Time protocol for acute inhalation toxicity
DPD	EU Dangerous Preparations Directive
ECVAM	European Centre for the Validation of Alternative Methods
EPA	US Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FDP	Fixed Dose Procedure
GHS	Globally Harmonised System of Classification and Labelling of Chemicals
GPMT	Guinea pig maximisation test
HET-CAM	Hen's egg test—chorioallantoic membrane
HSE	UK Health and Safety Executive
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICE	Isolated chicken eye test
IRE	Isolated rabbit eye test
ITS	Intelligent Testing Strategy
LLNA	Local lymph node assay
LVET	Low-volume eye irritation test
NC3Rs	UK National Centre for the Replacement, Refinement and Reduction of Animals in Research
NICEATM	National Toxicology Program Interagency Centre for the Evaluation of Alternative Toxicological Methods
NTP	US National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
PSD	UK Pesticides Safety Directorate
QSAR	Quantitative structure-activity relationship
REACH	Registration, Evaluation, Authorisation and restriction of CHemicals
RHE	Reconstructed human epidermis
UDP	Up-and-Down Procedure for acute oral toxicity

chemical concerned (EC, 2007). An acute oral toxicity study and an assessment of skin sensitisation potential, plus in vitro studies for skin irritation and corrosion and eye irritation potential are required for all chemicals produced at volumes of ≥1 tonne per year. Acute toxicity testing by a second exposure route and in vivo skin and eye irritation testing are also required for chemicals with an annual production volume ≥10 tonnes. Requirements can also vary in terms of the accepted testing methods and approaches for a particular endpoint.

International variation in testing requirements can be a cause of duplication in toxicity testing. For example, within the EU the local lymph node assay (LLNA) is the preferred method for assessing skin sensitisation potential, whereas guinea pig assays are still preferred in other regions of the world (e.g. China). In addition, agencies can differ in their preference based on the substance being regulated. In the case of the US Environmental Protection Agency (EPA), the LLNA has been a stand-alone test to evaluate chemical substances since 2002, but at the time of preparing this paper, guinea pig assays are still the requested study type when testing formulated products for sensitisation potential (personal communication, US EPA).

Therefore, the same substance may be tested under multiple protocols, all essentially providing very similar information on the same endpoint, resulting in excessive use of animals, time, and financial resources. Examples of this lack of harmonisation are discussed in more detail in the relevant endpoint-specific sections of this paper.

Recent changes to European legislation have substantial implications for the use of animals in acute toxicity testing. The Seventh Amendment to the Cosmetics Directive has resulted in the marketing of cosmetic products containing ingredients tested on animals being banned for the majority of endpoints, including acute toxicity tests, from 2009 (EC, 2003), whereas it has been estimated that REACH may require evaluation of around 30,000 to over 68,000 existing chemicals over a period of 11 years, potentially requiring the use of large numbers of animals (Hartung and Rovida, 2009; Pedersen et al., 2003). At present, it is unclear how many substances will require testing for the acute toxicity endpoints covered in this paper, as data may already have been generated for many substances to meet classification and labelling requirements. REACH also has a number of mechanisms in place to minimise new testing, but it is estimated that a proportion of existing substances will need to be tested, as will new substances entering the market (van der Jagt et al., 2004). Both of these regulatory changes have produced an urgent need for alternative approaches. This need is most obvious in the case of cosmetic ingredients, but REACH also states that whenever possible information should be generated by means other than testing on vertebrates. Moreover, it has been argued that the current toxicity testing paradigm involving extensive in vivo testing is not fit for the purpose of testing such a large number of chemicals within the required time frame, and therefore alternative approaches and strategies are needed (Schaafsma et al., 2009). Given the EU ban on testing of cosmetic ingredients, these are not specifically focused on in this review, although activities led by the cosmetics industry in response to the regulatory change should be expected to contribute to the development of alternative approaches for acute toxicity testing.

3. Options for waiving test requirements

3.1. Waiving opportunities highlighted in regulatory test guidelines

It is impossible to discuss redundancy and opportunities for waiving in acute toxicity testing of chemicals without considering the impact of the implementation of REACH. As already noted, animal testing should only be performed as a last resort under this legislation, and a number of generic and endpoint-specific opportunities for waiving of toxicity tests are set out in the guidance documents accompanying the REACH legislation[1]. Chapter R.5 provides generic advice on adaptation of information requirements, and lists three main options for waiving of tests: where there is absent, unlikely, not relevant or not significant exposure; when testing does not appear scientifically necessary (discussed in more detail below); and where testing is not technically possible, for example due to physicochemical characteristics such as solubility or volatility.

Advice specific to acute toxicity testing is detailed in chapter R.7, where it is stated that the potential to avoid acute toxicity testing should be carefully exploited and an intelligent testing strategy (ITS) is provided for determining whether in vivo testing is required. The first stage of this strategy is the review of existing data including human, animal, and in vitro test data, physicochemical properties, structure-activity relationships (SARs), quantitative structure-activity relationships (QSARs), and chemical grouping methods such as read-across. Evaluation of the existing information is then performed to enable a decision to be made on the testing needs for the chemical. Notably, if a substance is considered likely to be corrosive, no acute toxicity testing should normally be conducted, and where information on corrosivity is not available a validated in vitro corrosivity test (e.g. OECD Test Guideline 430, 431, or 435; see section on skin corrosivity and irritation potential) should be performed prior to any other testing. Another important point to note is that although acute toxicity testing by a second route is a standard requirement for compounds produced at volumes greater than 10 tonnes per annum (tpa), the guidance states that information on only one route of exposure may be sufficient and justified, based on physicochemical, toxicokinetic, or human data and review of all possible exposure scenarios.

Following evaluation of the available data, consideration is given as to whether the information is adequate for hazard

[1] Available at: http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_en.htm?time=1236269339

characterisation, or if additional information is required. A number of reasons why the substance may be excluded from acute toxicity testing on the basis of a lack of scientific justification are set out. Testing may not be required if

- A weight of evidence (WOE) analysis demonstrates that the available information is sufficient for an adequate hazard characterisation and the exposure to the substance is adequately controlled.
- The substance is not bioavailable via a specific route and possible local effects are adequately characterised (e.g. no dermal absorption for dermal route).
- For inhalation, no testing is required if it is not technically possible to generate a test atmosphere, the vapour pressure is very low (<0.1 Pa at 20°C) or the particle size is >100 µm.
- The data meet the requirements for classification for toxic effects or the substance has already been classified for acute toxic effects.

Other international regulatory test guidelines also include options for minimising the need for animal testing for acute toxicity, and endpoint-specific examples of where in vivo testing can be avoided are included at the relevant sections of this paper.

3.2. Calculation of toxicity of mixtures

One of the most important ways in which acute toxicity testing can be waived is through the use of calculation methods to assess the toxicity of mixtures of chemicals based on the properties of the individual components, thereby avoiding the need to test the mixtures themselves. The World Health Organization's guidelines on *Classification of Pesticides by Hazard* state that while the preferred method of classification of pesticide formulations is through assessment of toxicity data on the actual mixture, classification can also be based on the most hazardous constituent of the mixture as if that ingredient were present as the total concentration of all the active components, or can be based on calculations from the lethal dose $(LD)_{50}$ values of the ingredients, taking into account the percentage of each active ingredient in the formulation (WHO, 2005).

Within the EU, the Dangerous Preparations Directive (DPD; 1999/45/EC) sets out similar calculation methods for the evaluation of human and environmental health hazards of chemical preparations including industrial chemicals, pesticides, and biocides (EC, 1999). These methods enable the hazard classification of a preparation to be made on the basis of the percentage of each substance and their toxicological properties. In providing these calculation methods, the DPD makes clear that there is no obligation to conduct further experiments on animals for the classification of preparations.

The Globally Harmonized System of Classification and Labelling of Chemicals (GHS) has been developed over the last decade to provide an internationally harmonised approach to the classification and labelling of chemicals and chemical mixtures (GHS, 2007). The GHS is in various stages of implementation around the world, and in the next few years is expected to supersede most if not all regional or national regulations on classification and labelling, including the DPD. Importantly, the GHS states that tests and experiments that do not require the use of animals are to be preferred, and guidance is provided on bridging principles and calculation methods that can be used to classify mixtures without in vivo testing of the actual formulation, both when data are available for all the ingredients, and when data are unavailable for one of more components of the mixture.

3.3. Read-across, grouping of chemicals, and QSARs

Grouping of chemicals that are expected to have similar structural, chemical, and toxicological characteristics, and applying read-across, may allow the properties of a chemical for which there are few or no data to be predicted, and this approach can support a reduction in animal testing. Provision for read-across is included in international regulatory programmes including those of REACH and the US EPA (EC, 2008a; EPA, 2002), and has already been used in the US High Production Volume (HPV) challenge programme and the UK Notification of New Substances (NONS) Regulations to reduce animal testing and industry costs (EPA, 2004; Hanway and Evans, 2000). Experience of this approach within the UK has revealed the importance of a number of factors that should be taken into account when considering the suitability of using read-across: (i) the similarity of the purity and impurity profiles of the chemicals, (ii) their physicochemical properties, (iii) their likely toxicokinetics, and (iv) the significance of reading across results obtained from outdated test methods.

QSARs are another non-testing approach that can be used in the evaluation of chemicals, helping to provide information for use in priority setting, guide the design of a testing strategy, provide mechanistic information that could support grouping of chemicals, and fill data gaps. To make the use of QSARs more readily accessible, the Organisation for Economic Co-operation and Development (OECD) has developed the QSAR application toolbox, software that provides a transparent and reproducible method for grouping and evaluating chemicals and applying read-across. The first version of this software is now available as a free download from the OECD's Web site[2]. In addition, the OECD has published principles and guidance on validation of QSARs to help enhance the regulatory acceptance of QSAR models for assessing chemical safety (OECD, 2007).

At present, QSAR models have not been sufficiently developed or validated to enable them to be used as stand alone alternatives to animal testing. However, QSAR information can be used to supplement experimental test data as part of a weight of evidence or ITS approach (ECHA, 2008a).

[2] http://www.oecd.org/document/23/0,3343,en_2649_34379_33957015_1_1_1_1,00.html

3.4. Changes in manufacturing site

In some regions, registration of a pesticide active ingredient from a new manufacturing source triggers new acute toxicity, skin and eye irritation, and skin sensitisation testing in order to demonstrate equivalence of the two sources. This is an area where there is clear redundancy and opportunities for waiving of testing. In the absence of any new toxicological information, when materials are shown to be chemically equivalent by analysis, additional testing should not be required.

3.5. Waiving of test requirements for granular pesticide products

In 2001, the US EPA issued guidance aimed at streamlining the acute toxicity evaluation and classification process for granular pesticide products, including those with granular fertilisers in the product (EPA, 2001). Based on an extensive historical database, the EPA concluded that an effective precautionary labelling policy can be implemented for granular pesticide products without testing of each product, taking into account the EPA acute toxicity Categories for the source products, which are based on results of the six-pack of acute toxicity tests.

For granular pesticide products, defined as products composed of <10% of a registered active ingredient(s), >90% of clearly recognised innocuous inert carrier(s), and ≤5% of sticker/binder, if the acute toxicity profile of the registered source product(s) is in EPA Toxicity Category III (acute oral, dermal, or inhalation LD_{50}/LC_{50} of >500–5000 mg/kg, >2000–5000 mg/kg, and >0.5–2 mg/L, respectively; moderate skin irritation at 72 hours; eye irritation clearing in ≤7 days) or IV (acute oral, dermal, or inhalation LD_{50}/LC_{50} of >5000 mg/kg, >5000 mg/kg, and >2 mg/L, respectively; mild/slight skin irritation at 72 hours; minimal eye irritation clearing in <24 hours) for all endpoints, then the acute toxicity profile of the source product may be applied to the granular product without any need for testing. Furthermore, for oral, dermal, and inhalation systemic toxicities, Category III results for the source product may be extrapolated down to Category IV for the granular product, on the assumption that the innocuous inert components do not contribute to toxicity and therefore act as diluents. However, if the acute oral, dermal, and/or inhalation effects of the source product are classified as Category I (acute oral, dermal, or inhalation LD_{50}/LC_{50} of ≤50 mg/kg, ≤200 mg/kg, and ≤0.05 mg/L, respectively) and/or II (acute oral, dermal, or inhalation LD_{50}/LC_{50} of >50–500 mg/kg, >200–2000 mg/kg, and >0.05–0.5 mg/L, respectively), it is not possible to use extrapolation to lower the classification, and testing of the new granular product would be required to achieve a lower classification.

Skin and eye irritation classifications for the registered product may also be used for the granular product, but should not be extrapolated down. For skin sensitisation, if the granular product contains any known sensitisers, it is required to be labelled as a sensitiser, while if the product is not a dermal sensitiser and there are no known sensitisers in the product, a skin sensitisation study may be waived and the product will not require labelling for this effect.

Similar guidelines are in place for granular fertiliser pesticide products, defined as products comprising <10% of a registered active ingredient(s), >90% of granular fertiliser components plus innocuous inert carrier(s), and ≤5% of sticker/binder. However, on the basis of evidence indicating that fertiliser products can be more irritating to the eye than comparable non-fertiliser products, eye irritation studies must be performed on the granular fertiliser pesticide product.

4. Acute oral toxicity testing

Acute systemic toxicity testing is required to provide information on the adverse health effects that could arise following short-term exposure to a chemical by a particular route (oral, dermal, or inhalation). For many chemicals, the most likely route leading to significant systemic exposure may be oral. Such exposure may be accidental or deliberate, and could potentially occur in the workplace, as a bystander, or at home; for example due to inadvertent ingestions following unexpected splashes or sudden release, or through consumption of chemical-containing foods, drinks, or other products such as those used for cleaning or personal care.

Acute oral toxicity data are a common requirement under many regulatory frameworks around the world to provide classification and labelling warning of the possible consequences of oral exposure to a chemical. For example in the EU, REACH requires acute oral toxicity data on all chemicals produced in volumes ≥1 tonne per annum (tpa), while EU, US, and Japanese regulations require assessment of the acute oral toxicity of pesticide active ingredients and formulated products.

4.1. Current test methods for assessing acute oral toxicity

The acute oral toxicity test was devised to identify the median dose killing half the animals in a test group (lethal dose [LD_{50}]) after a single dose (or multiple doses within a 24-hour period) and thereby allows a simple point estimate numerical comparison between substances, enabling a ranking of potency to be made. By 1981, the LD_{50} test had been modified to reduce the number of animals required and developed into a new OECD test guideline, TG 401 (OECD, 1981a). Using a sighting study or historical data, three dose groups of at least 5 animals/sex/group were selected to identify the LD_{50}. An upper dose limit of 5000 mg/kg was also introduced to this new guideline to limit the unnecessary use of animals. A further refinement of the method in 1987 allowed the use of only one gender, with testing in the other sex only conducted at the LD_{50} dose, and the reduction of the limit test to 2000 mg/kg unless there were exceptional circumstances.

Soon after the implementation of the 1981 OECD test guideline, a working party of the British Toxicology Society (BTS) proposed a new method for assessing acute oral toxicity that used a smaller number of animals and

also avoided the use of death as an endpoint, replacing it with the observation of evident clinical signs of toxicity to provide a range estimate of the LD_{50} (BTS, 1984). The proposed method was validated for the purpose of ranking and classifying compounds according to the EU system of classification (van den Heuvel et al., 1990). The new test guideline, the Fixed Dose Procedure (FDP; OECD TG 420), was adopted in 1992 and became the first accepted alternative to the LD_{50} test. Subsequent to this, two further methods that also sought to reduce animal numbers, the Acute Toxic Class (ATC; TG 423) and the Up-and-Down Procedure (UDP; TG 425), were adopted (OECD, 2001b, 2001c, 2008b). It has been indicated that most FDP tests will be completed using 5–7 animals, while the ATC method on average uses 7 animals and the UDP about 6–9 animals. Both of these latter methods use death or impending death as an endpoint for determining an estimate of the LD_{50}. The OECD Test Guideline 401 was finally deleted in 2002. The result has been that since the 1970s when a large proportion of animals used in toxicity testing were used in acute toxicity testing, the number of animals per test has dramatically reduced from approximately 100 to 5–9. The OECD guidance document 24 on acute oral toxicity testing sets out the properties of the three methods, and characteristics of the methods are detailed in Table 2 (OECD, 2001a).

Whilst the information obtained from guideline compliant acute oral toxicity tests is of value for hazard identification and thus classification and labelling of chemicals, it rarely if ever includes target organ effects, mode of action, or toxicokinetic data, limiting the usefulness of the data obtained for other purposes.

4.2. Refinement of acute oral testing—use of evident toxicity

As noted above, hazard classification for acute oral toxicity is either driven by estimating the LD_{50}, or is based upon the dose producing evident toxicity, i.e. clear signs of toxicity following administration of a test substance, such that an increase to the next highest dose would be expected to result in the development of severe toxic signs and probably mortality (OECD, 2001a). The ATC method and the UDP employ mortality or impending death as the endpoint. Animals that are moribund or obviously in pain and showing signs of severe and enduring distress should be humanely killed, as outlined in an OECD guidance document on humane endpoints, with animals humanely killed regarded as treatment-dependent deaths (OECD, 2000). Identification of dead animals or those in a moribund condition requires no judgement in estimating toxicity, and the simplicity of assessing the lethality of the substance and expressing it as a numerical value is one of the fundamental barriers to moving away from this approach. On the surface, lethality is a very convenient way to categorise and rank substances and the estimation of hazard using dosage-dependent death as an endpoint has a simplicity that gives the regulator a sense of familiarity and security.

The FDP employs evident signs of clinical toxicity and not necessarily death or impending death as the endpoint. With this comes a judgement made during the study about which clinical signs of toxicity, or combination of clinical signs, may be considered 'evident'. This requires more skill and experience but is eminently achievable. The non-lethal endpoint also has the advantage that it allows information on the recovery from clinically manifest toxicity.

In some regions, the FDP is less commonly used than the ATC method because of the perceived greater uncertainty associated with identifying evident clinical signs of toxicity rather than death. There is also concern that the use of the non-lethal endpoint might result in overestimation of toxicity purely based on clinical signs. However, information on doses that induce toxicity, rather than those that cause death, are likely to be of greater value in risk assessment and management, supporting the prediction, prevention, recognition, and treatment of symptoms in human cases of acute toxicity.

Table 2. Comparison of OECD acute oral toxicity test guidelines*.

Test guideline	TG 420 (2001)	TG 423 (2001)	TG 425 (2001)
Method	Fixed Dose Procedure (FDP)	Acute Toxic Class (ATC)	Up-and-Down (UDP)
Major endpoint	Evident toxicity	Mortality	Mortality
Major objective	Range estimate of LD_{50}	Range estimate of LD_{50}	Point estimate of LD_{50} with confidence intervals
Use of data	– Classification and labelling – Risk assessment – Dose selection for repeat-dose studies	– Classification and labelling – Risk assessment – Dose selection for repeat-dose studies	– Classification and labelling – Risk assessment – Dose selection for repeat-dose studies
	Animals tested		
Limit test	Up to 5 animals	Up to 6 animals	Up to 5 animals
Sighting study	1 animal per dose step (single sex used)	N/A	N/A
Main study	5 animals per dose step (single sex used)	3 animals per dose step (single sex used)	Single animals per dose step (single sex used)
Total animals used in a non-limit study (average)	5–7	7	6–9

*Adapted from the OECD guidance document on acute oral toxicity testing (OECD, 2001a).

Furthermore, several regulatory guidelines, including the European directive on the use of animals for experimental and other scientific purposes, require that where testing in animals is necessary, the method that incurs least severity is selected (EEC, 1986). On this basis, the FDP should be the preferred method for acute oral toxicity testing in the EU.

4.3. Replacement or reduction of in vivo acute toxicity testing

A number of approaches to reduce or replace animal use for acute oral toxicity testing have been suggested. One reductionist approach has been to initiate tiered in vitro testing based on the premise that acute toxicity can be broken down into a number of biokinetic, cellular, and molecular elements, each of which can be identified and quantified in appropriate model systems. The various elements can then be used in different combinations to model large numbers of toxic events (Walum, 1998).

The US National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) jointly evaluated two proposed in vitro cytotoxicity methods in their search for a replacement for the acute oral toxicity test. The 3T3 (mouse fibroblasts) neutral red uptake method (NRU) and the NHK (human keratinocytes) NRU method were selected based on their common use in cytotoxicity assessments. The study showed that the 3T3 and NHK NRU test methods are not sufficiently accurate as stand-alone methods to predict acute oral toxicity for the purpose of regulatory hazard classification. However, based on computer simulations for the reference substances tested in this study, it was recommended that either of these two in vitro basal cytotoxicity test methods should be used as part of a weight-of-evidence approach for the selection of starting doses for rodent acute oral toxicity testing of many compounds. This has the potential to reduce the number of animals used per test and for relatively toxic substances, may reduce the number of animals that die or are humanely killed due to severe toxicity (ICCVAM, 2006a; Stokes et al., 2008). As a result of these findings, a draft OECD guidance document on the use of these cytotoxicity assays to estimate starting doses for acute oral systemic toxicity testing has been prepared for consideration.

The ACuteTox research project, which started in 2005, is funded under the 6th Framework Programme in the EU and currently has 35 partners in 13 European countries (www.acutetox.org). The overall objective is to develop an in vitro testing strategy to replace acute toxicity testing in vivo and at the same time decrease costs and improve the scientific validity of the results. Fundamental to this project is the development of high-quality in vivo and in vitro databases; analysis of the correlation of in vivo test data with in vitro data and/or human lethal blood concentrations; investigation of the role of metabolism, kinetics, and target organ toxicity; and a subsequent iterative process in an attempt to improve the predictive capability and the efficiency of testing (Clemedson, 2008; Clemedson et al., 2007). Innovative tools and cell systems to identify novel endpoints and strategies to predict animal and human toxicities are also being investigated. Data mining and analysis to identify the most appropriate methods for use in an alternative testing strategy are ongoing (Kinsner-Ovaskainen et al., 2009).

Recently, Bulgheroni et al. (2009) evaluated the accuracy of predicting acute oral toxicity in rodents by using repeat-dose 28-day toxicity data. The authors analysed data from the EU New Chemicals Database, selecting chemicals for which both acute oral LD_{50} data and 28-day repeat-dose no observed adverse effect level (NOAEL) data were available. Using an empirical approach, a repeat-dose NOAEL of ≥200 mg/kg was selected as a threshold, with the expectation that compounds with a NOAEL ≥200 mg/kg would be considered as non-toxic (LD_{50} >2000 mg/kg) after acute oral exposure. This threshold enabled a correct identification of 63% of non-acutely toxic chemicals (those with an LD_{50} >2000 mg/kg), while less than 1% of chemicals in the database were misclassified as non-toxic when they actually had an LD_{50} ≤2000 mg/kg. Overall, the 'false negative' rate (i.e. chemicals with a NOAEL ≥200 mg/kg but an LD_{50} ≤2000 mg/kg) was 13%. However, for substances with a NOAEL less than 200 mg/kg, a substantial amount of false positives (i.e. chemicals with a NOAEL <200 mg/kg but an LD_{50} >2000 mg/kg) were generated, with 37% of chemicals with an LD_{50} >2000 mg/kg falling into this group, although the data set was highly populated with non-toxic substances (86.8%), making the ability to predict toxic substances difficult to test.

These data suggest there may be opportunities to use this information as part of intelligent testing strategies to reduce or replace the need for acute oral toxicity testing. Over 80% of compounds in the database had LD_{50} values >2000 mg/kg, and if it were possible to predict which chemicals would be expected to have an LD_{50} of this value, for example using in vitro data, in vivo acute toxicity testing could be avoided for many chemicals and only performed on the minority of substances predicted to have an LD_{50} ≤2000 mg/kg. In view of this, the researchers are currently undertaking further analysis to assess whether the validated 3T3/NRU cytotoxicity assay could be used to identify compounds that would be expected to have an LD_{50} greater than 2000 mg/kg (Kinsner-Ovaskainen et al., 2009). Historical data, information on physicochemical properties, and read-across approaches may also be of value in predicting which chemicals are likely to be non-toxic. In addition, Bulgheroni et al. proposed the use of a decision tree approach to acute toxicity testing, with substances with a NOAEL ≥200 mg/kg in a 28-day study (where available) being classified as non-acutely toxic, and substances with a NOAEL <200 mg/kg undergoing acute toxicity testing. They suggested that this approach may be of particular value for cosmetic ingredients, given that acute toxicity testing has been banned from March 2009 but repeat-dose studies on ingredients will be permitted until 2013 (Bulgheroni et al., 2009).

4.4. Opportunities for waiving

As noted in the introduction, an analysis recently undertaken by the pharmaceutical industry has led to a successful improvement in the requirements for acute (lethality) toxicity testing prior to first in man studies, thus leading to a reduction in animals used (Robinson et al., 2008). The working group, coordinated by the UK NC3Rs and comprising 13 international pharmaceutical companies and 5 contract research organisations, looked at the value and need for acute toxicity studies in drug development. They shared company data on 74 compounds to determine the extent of acute toxicity testing and the value placed upon the data produced. The group found that acute toxicity data are not used to terminate drugs from development, to support dose selection for repeat-dose studies in animals, or to set doses in the first clinical trials in humans. Furthermore, it was indicated that the information needed on acute toxicity can be assessed from any short-term or dose-escalation studies performed by the clinical route of exposure at more relevant doses for humans, and these studies are already conducted as part of the drug development process. Therefore the acute toxicity tests (often oral) were redundant.

The conclusions of the expert group were subsequently considered and endorsed by regulators and scientists from the EU, USA, and Japan at a workshop in London, November 2006 (Chapman and Robinson, 2007). As a result of this data sharing initiative, a 70% reduction in animal use for acute toxicity testing has been achieved by the companies in the collaboration, and the impact is expected to be realised worldwide as the recommendations of the collaboration have been incorporated into the revised International Conference on Harmonisation (ICH) M3 guidelines and the European Medicines Agency draft position paper on acute toxicity studies.

This successful approach clearly raises questions for non-pharmaceutical substances where similar information is obtained to inform about risks from acute human exposure. It may be possible to take a similar approach and use data from short-term range-finding studies that are used to support dose-setting in 28-day studies for chemicals. The example that the pharmaceutical companies have set is a powerful demonstration of how rapid change can come about once there is good agreement. As a result of this success, the European Partnership for Alternative Approaches to Animal Testing (EPAA) has established a cross-sector task force to examine the value of acute toxicity testing in the chemicals industry[3].

Avoidance of animal testing is clearly encouraged under REACH, and the Regulation sets out circumstances where it is justifiable to waive acute toxicity testing. In order to do this, the scientific argument must be made in the registration documentation with adequate scientific background pertinent to the chemical in question. In addition to the general options for waiving of acute toxicity testing, testing by the oral route specifically may be avoided where it is not technically feasible to administer a known dose by the oral route.

As discussed earlier, the toxicity of chemical mixtures such as pesticide formulations can be predicted using information on the individual components. Bridging principles and calculation methods to do this, such as those set out in by the EU DPD, WHO, and GHS, as well as the US EPA's guidance on acute toxicity data requirements for granular pesticide products, can therefore make a significant contribution to avoiding acute oral toxicity testing of chemical preparations.

5. Acute dermal toxicity testing

Dermal exposure occurs in a wide variety of occupational settings, and the spectrum of exposure can range from small quantities of substances accidentally splashed on small areas of the skin to repeated immersion of hands and forearms (Semple, 2004). Skin exposures can also occur outside the workplace. Chemicals that are absorbed through the skin enter the circulation and could potentially cause systemic toxicity, and therefore acute dermal toxicity testing has routinely been performed on many groups of chemicals under regulatory schemes around the world, supporting classification and labelling and indicating whether particular safe handling procedures including the use of protective clothing may be required.

The skin is the primary occupational exposure route for most pesticides, and assessment of acute dermal toxicity of agrochemicals is a requirement in the EU, USA, and Japan. For industrial chemicals, there is no absolute requirement for the acute dermal test under REACH, with only acute oral toxicity data required for substances produced in quantities of 1–10 tpa. However, acute toxicity testing by a second exposure route in addition to the oral route is required for chemicals produced in volumes ≥10 tpa. For these substances, testing for acute dermal toxicity, rather than acute inhalation toxicity, is appropriate under defined conditions: where (i) inhalation of the substance is unlikely, (ii) skin contact in production and/or use is likely, and (iii) the physicochemical and toxicological properties suggest potential for significant rate of absorption through the skin.

5.1. Value of acute dermal toxicity data

The data generated in an acute dermal toxicity study generally have limited utility. Due to the prescribed level of investigation (i.e. observations for clinical signs, mortality, body weights, gross pathological examination, and in some cases the microscopic examination of organs showing gross lesions), the predominant use is for classification and labelling only. The results from a standard study are of minimal if any use in risk assessment. Occasionally the acute dermal toxicity study might be informative regarding irritation potential without separately performing a skin

[3] http://ec.europa.eu/enterprise/epaa/3_activities/3_5_3rs_in_regulation/wg4_acute_tox_for_cross_sector

irritation study. However, due to the extended exposure time in the dermal toxicity study (24 versus 4 hours), there is a potential to over-predict irritation risk. In addition, in vitro assays for dermal irritation are becoming available, as discussed in the later section on skin irritation, so any benefits of using the acute dermal toxicity study for this purpose are declining.

One of the key functions of skin is to provide a barrier to dermal uptake. There is a limit to the volume of material that will remain in contact with an area of skin before it runs off. If a compound has a dermal LD_{50} of 400 mg/kg body weight (bw), a 10-kg infant would need to be exposed to 4 g of material in order to be exposed to the LD_{50}. Work performed for the US EPA showed that following immersion of the hand in viscous fluids (33–160 cSt), with no wiping to remove the film, the thickness of the fluid film was ca 0.01 cm (Cinalli et al., 1992). To obtain a total skin loading of 4 g with a film 0.01 cm thick, an area of 400 cm^2 would need to be covered. Such an area (20 cm × 20 cm) is very unlikely to result from unintentional exposure. Therefore unless there are indications that the intended use of the substance is likely to result in prolonged exposure to a significant area of skin, dermal acute toxicity studies are of limited relevance to typical human exposure scenarios.

Given that most of the acute dermal tests are limit tests (see below and Table 2), there is potential to reduce animal usage by modifying the current requirements of OECD Test Guideline 402. Currently five animals of each sex are required for a limit test according to TG 402. However, there appears to be no reason why it cannot be revised in line with the new acute oral toxicity tests, TG 420, 423, and 425, to use fewer animals, for example by limiting the group size to five animals of a single sex (females, unless there is good evidence to support the use of males) in total. Such a change could potentially halve the numbers of animals used in acute dermal toxicity tests.

5.2. Impact of deleting the acute dermal toxicity study

In the vast majority of cases, the acute dermal toxicity study does not provide information that is useful beyond an indication of the dose causing lethality and the applicable classification. Regulatory schemes for plant protection products, biocides, and industrial chemicals all require information on acute oral toxicity, and therefore if the toxicity categorisation and associated pictogram (i.e. harmful, toxic, not harmful) of a compound can be identified by studying only oral administration, there is arguably no benefit in performing an acute dermal toxicity study for additional classification and labelling purposes. If the systemic toxicity of a compound were greater by the oral route than via the skin, the resulting classification and labelling may be overly stringent for dermal toxicity, but there would not be a loss in public health protection. However, if there are a significant number of compounds that are classified as a potential hazard only via the dermal route, then the loss of the study could be seen as reducing protection.

To investigate the possibility of relying on an oral study alone for acute toxicity classification for both oral and dermal routes, retrospective analyses have been performed on pesticide active substances and new chemical entities submitted for the Notification of New Substances Regulations.

5.2.1. Pesticide active substances

The UK Pesticides Safety Directorate (PSD; now Chemicals Regulation Directorate) has access to an extensive collection of information (reports and summaries) on toxicity studies performed on pesticide active substances. PSD undertook an evaluation of acute toxicity results and classifications for active substances in an effort to determine the potential impact of deleting the acute dermal toxicity test from the basic requirements (Thomas and Dewhurst, 2007).

Within PSD's databases, the LD_{50} values of 240 active substances were identified and tabulated, all but 3 of which had undergone acute oral and dermal toxicity testing (Table 3). The LD_{50} values were compared with classification criteria using the UK Chemicals (Hazard Information and Packaging for Supply) Regulations 2002 (Anon, 2002) and the likely classification determined. For internal consistency, where a range of LD_{50} values was cited, the lowest value was used to derive the classification, and in the three cases where data were unavailable, the substances were assumed to be unclassified. The LD_{50}s in the summary documents were taken at face value and no effort was made to confirm them independently. Given the relatively large number of entries, it was considered that any overall conclusions would not be unduly confounded by an occasional error in the production of the summaries.

The relationship between dermal acute toxicity and oral acute toxicity was correlated and is summarised in Table 4. Of the 240 substances analysed, 121 were unclassified by both the oral and dermal routes, while the remaining 119 were classifiable by either or both routes. Only 2 of the 240 compounds (0.8%) received a classification via the dermal route which was more severe than that applicable by the oral route. While one additional compound had a lower LD_{50} value dermally than orally, this would not have altered the classification.

One of the active substances with a more severe classification via the dermal route, cadusafos, an organophosphate pesticide used as an insecticide and nematicide on bananas, was classified as 'very toxic' in contact with skin but only 'toxic' orally. Cadusafos is also 'very toxic by inhalation'. Notably, the dermal data were generated using rabbits and no dermal acute data are available for rats, the species used for the oral study. The ranges of LD_{50}s by the oral and dermal routes were similar (37–80 mg/kg bw orally; 11–42 mg/kg bw dermally).

The second compound with a more severe classification by the dermal route was dodemorph, a morpholine fungicide used on roses, which is classified as harmful in contact with skin but unclassified orally. Dodemorph produced marked local skin effects and a classification of

Table 3. Acute oral and dermal LD_{50} and classification data on pesticide active substances obtained from PSD's databases.

Data from European Community Coordination (ECCO) meetings				
	LD_{50} (mg/kg) and classification			
Active substance	Oral LD_{50}	Oral classification	Dermal LD_{50}	Dermal classification
2,4-D	425–764	Harmful	>2000	Unclassified
2,4-DB	1470	Harmful	>2000	Unclassified
Acephate	100–1400	Toxic	>2000	Unclassified
Alachlor	1350	Harmful	4982	Unclassified
Aldicarb	0.5	Very Toxic	218	Toxic
Alpha cypermethrin	57	Toxic	>2000	Unclassified
Amitraz	600	Harmful	>1600	Harmful
Amitrole	>5000	Unclassified	>2500	Unclassified
Atrazine	>2000	Unclassified	>2000	Unclassified
Azafenidin	>5000	Unclassified	>2000	Unclassified
Azimsulfuron	>5000	Unclassified	>2000	Unclassified
Azinphos-methyl	4–26	Very Toxic	72–250	Toxic
Azoxystrobin	>5000	Unclassified	>2000	Unclassified
Beflubutamid	>5000	Unclassified	>2000	Unclassified
Benalaxyl	<2000 (R22)	Harmful	>2000	Unclassified
Benomyl	>10000	Unclassified	No data, used carbendazim	Unclassified
Bentazone	1400–1800	Harmful	>5000	Unclassified
Beta-cyfluthrin	77–1369	Toxic	>5000	Unclassified
Bromoxynil	80–300	Toxic	>2000	Unclassified
Carbendazim	>10000	Unclassified	>2000	Unclassified
Carfentrazone-ethyl	>5000	Unclassified	>4000	Unclassified
CGA 245704	>2000	Unclassified	>2000	Unclassified
Chlorfenapyr	626	Harmful	>2000	Unclassified
Chlorothalonil	>5000	Unclassified	>2000	Unclassified
Chlorotoluron	>10000	Unclassified	>2000	Unclassified
Chlorpropham	4200	Unclassified	>2000	Unclassified
Chlorpyrifos	66–195	Toxic	1250–2000	Harmful
Chlorpyrifos-methyl)	2814	Unclassified	>2000	Unclassified
Chlozolinate	>4500	Unclassified	Data requirement	Unclassified
Cinidon ethyl	>2200	Unclassified	>2000	Unclassified
Cyclanilide	208	Harmful	>2000	Unclassified
Cyfluthrin	16–155	Very Toxic	>5000	Unclassified
Cyhalofop-butyl	>5000	Unclassified	>2000	Unclassified
Cypermethrin	287	Harmful	>2000	Unclassified
Daminozide	>5000	Unclassified	>5000	Unclassified
Deltamethrin	87	Harmful	>1000 (?, 21-d study)	Harmful
Desmedipham	>5000	Unclassified	>2000	Unclassified
Dinocap	1212	Harmful	>2000	Unclassified
Dinoterb	26	Toxic	150	Toxic
Diquat	220	Harmful	>424	Harmful
DNOC	24–85	Very Toxic	43–>2000	Very Toxic
Endosufan	10–27	Very Toxic	500	Harmful
Esfenvalerate	89	Toxic	>5000	Unclassified
Ethofumsate	>5000	Unclassified	>2000	Unclassified
Ethoxysulfuron	2910	Unclassified	>4000	Unclassified
Etoxazole	>5000	Unclassified	>2000	Unclassified
Famoxadone	>5000	Unclassified	>5000	Unclassified
Fenarimol	2500	Unclassified	>2000	Unclassified
Fenhexamid	>5000	Unclassified	>5000	Unclassified
Fenthion	343–556	Harmful	562–800	Harmful
Fentin acetate	140–298	Toxic	No value	Unclassified
Fentin hydroxide (EAS)	160 (toxic)	Toxic	127 (rabbit; toxic)	Toxic
Ferric phosphate (data on SO4)	1487	Harmful	>2000	Unclassified

Table 3. Continued on next page.

Table 3. Continued.

Active substance	LD_{50} (mg/kg) and classification			
	Oral LD_{50}	Oral classification	Dermal LD_{50}	Dermal classification
Flazasulfuron	>5000	Unclassified	>2000	Unclassified
Flufenacet	589–1617	Harmful	>2000	Unclassified
Flumioxazine	>5000	Unclassified	>2000	Unclassified
Flupyrsulfuron-methyl	>5000	Unclassified	>2000	Unclassified
Fluroxypyr	>2000	Unclassified	>2000	Unclassified
Flurtamone	>5000	Unclassified	>2000	Unclassified
Flusilazole	>2000	Unclassified	>2000	Unclassified
Forchlorfenuron	4917	Unclassified	>2000	Unclassified
Fosthiazate	57–73	Toxic	853–2372	Harmful
Glyphosate	>2000	Unclassified	>2000	Unclassified
Glyphosate trimesium	750	Harmful	>2000	Unclassified
Imazalil	227–371	Harmful	>2000	Unclassified
Imazamox	>5000	Unclassified	>4000	Unclassified
Imazosulfuron	>5000	Unclassified	>2000	Unclassified
Iodosulfuron	2678	Unclassified	>2000	Unclassified
Ioxynil	114–165	Toxic	1050–>2000	Harmful
Iprodione	>2000	Unclassified	>2500	Unclassified
Isoproturon	>2000	Unclassified	>2000	Unclassified
Isoxaflutole	>5000	Unclassified	>2000	Unclassified
Kresoxim-methyl	>5000	Unclassified	>2000	Unclassified
Lambda-cyhalothrin	56–79	Toxic	632–960	Harmful
Lindane	163	Toxic	~1600	Harmful
Linuron	1146–1508	Harmful	>2000	Unclassified
Maleic hydrazide	>5000	Unclassified	>5000	Unclassified
Mancozeb	>5000	Unclassified	>2000	Unclassified
Maneb	>5000	Unclassified	>2000	Unclassified
MCPA	962	Harmful	>4000	Unclassified
MCPB	4300	Unclassified	>2000	Unclassified
Mecoprop	1166	Harmful	>4000	Unclassified
Mecoprop-P	431- 1050	Harmful	>4000	Unclassified
Mepanipyrim	>5000	Unclassified	>2000	Unclassified
Metalaxyl-M	375–953	Harmful	>2000	Unclassified
Methamidophos	11	Very Toxic	50	Very Toxic
Methoxyfenozide	>5000	Unclassified	>5000	Unclassified
Metiram	>5000	Unclassified	>2000	Unclassified
Metsulfuron	>5000	Unclassified	>2000	Unclassified
Milbemectin	456	Harmful	>5000	Unclassified
Molinate	483	Harmful	4350	Unclassified
Monolinuron	1430–2490	Harmful	>2000	Unclassified
Oxadiargyl	>5000	Unclassified	>2000	Unclassified
Oxasulfuron	>5000	Unclassified	>2000	Unclassified
Paraquat	100	Toxic	>600	Harmful
Parathion	2–22	Very Toxic	71–100	Toxic
Parathion-methyl	3–20	Very Toxic	46–491	Very Toxic
Pendimethalin	>5000	Unclassified	>2000	Unclassified
Pethoxamid	983	Harmful	>2000	Unclassified
Phenmedipham	>8000	Unclassified	>2000	Unclassified
Picoxystrobin	>5000	Unclassified	>2000	Unclassified
Procymidione	>5000	Unclassified	>5000	Unclassified
Profoxidim	>5000	Unclassified	>4000	Unclassified
Prohexadione calcium	>5000	Unclassified	>2000	Unclassified
Propiconazole	~1500	Harmful	>4000	Unclassified
Propineb	>5000	Unclassified	>5000	Unclassified
Propoxycarbazone -sodium	>5000	Unclassified	>5000	Unclassified
Propyzamide	>5000	Unclassified	>2000	Unclassified

Table 3. Continued on next page.

Table 3. Continued.

Active substance	LD_{50} (mg/kg) and classification			
	Oral LD_{50}	Oral classification	Thiamethoxam to >2000	Dermal classification
Prosulfuron	986	Harmful	>2000	Unclassified
Pymetrozine	>5000	Unclassified	>2000	Unclassified
Pyraclostrobin	>5000	Unclassified	>2000	Unclassified
Pyraflufen-ethyl	>5000	Unclassified	>2000	Unclassified
Pyrazophos	151	Toxic	>2000	Unclassified
Pyridate	>2000	Unclassified	>2000	Unclassified
Quinoxyfen	>5000	Unclassified	>2000	Unclassified
Quintozene	>5000	Unclassified	>5000	Unclassified
Simazine	>2000	Unclassified	>2000	Unclassified
Spinosad	>2000	Unclassified	>5000	Unclassified
Spiroxamine	374–595	Harmful	1068–1600	Harmful
Sulfosulfuron	>5000	Unclassified	>5000	Unclassified
Tecnazene	1256	Harmful	>2000	Unclassified
Tepraloxydim	>5000	Unclassified	>2000	Unclassified
Thiabendazole	3100	Unclassified	>2000	Unclassified
Thiamethoxam	1563	Harmful	2000	Unclassified
Thiram	1800–2700	Harmful	>2000	Unclassified
Thifensulfuron(-methyl)	>5000	Unclassified	>2000	Unclassified
Thiocloprid	400–800	Harmful	>2000	Unclassified
Thiophanate-methyl	>5000	Unclassified	>2000	Unclassified
Triasulfuron	>5000	Unclassified	>2000	Unclassified
Trifloxystrobin	>5000	Unclassified	>2000	Unclassified
Tritosulfuron	4700	Unclassified	>2000	Unclassified
Vinclozolin	>15000	Unclassified	>5000	Unclassified
Warfarin	1.6	Very Toxic	40	Very Toxic
Ziram	267	Harmful	>2000	Unclassified
Zoxamide	>5000	Unclassified	>5000	Unclassified

Data from EFSA Pesticides Peer Review Co-ordination (EPCO) Draft Assessment Reports

Active substance	LD_{50} (mg/kg) and classification			
	Oral LD_{50}	Oral classification	Dermal (mg/kg)	Dermal classification
1-MCP	No data—gas	Unclassified	No data—gas	Unclassified
1,3-Dichloropropene	110–250	Toxic	333–1200	Toxic
Benalaxyl-M	>2000	Unclassified	>5000	Unclassified
Benfuracarb	205	Harmful	>2000	Unclassified
Benthiavalicarb-isopropyl	>5000	Unclassified	>2000	Unclassified
Bispyribac-sodium	>2000	Unclassified	>2000	Unclassified
Cadusafos	**37–80**	**Toxic**	**11–42 (rabbit)**	**Very Toxic**
Captan	>2000	Unclassified	>2000	Unclassified
Carbaryl	614	Harmful	>5000	Unclassified
Carbofuran	7	Very Toxic	>1000	Harmful
Carbosulfan	138	Toxic	3700	Unclassified
Clodinafop-propargyl	1392	Harmful	>2000	Unclassified
Clopyralid	>5000	Unclassified	>2000	Unclassified
Cyprodinil	>2000	Unclassified	>2000	Unclassified
Diazinon	1100	Harmful	>2000	Unclassified
Dichlorprop-P	567	Harmful	>2000	Unclassified
Dichlorvos	80	Toxic	120	Toxic
Dimethenamid	397	Harmful	>2000	Unclassified
Dimethoate	245	Harmful	>2000	Unclassified
Dimethomorph	3900	Unclassified	>2000	Unclassified
Dimoxystrobin	>5000	Unclassified	>2000	Unclassified
Diuron	437	Harmful	>5000	Unclassified
Ethephon*	1560-2210	Harmful	983-1390 (causes burns)	Harmful
Ethoprophos	40–80	Toxic	226–1280	Toxic

Table 3. Continued on next page.

Table 3. Continued.

Active substance	LD_{50} (mg/kg) and classification			
	Propamocarb to >2000	Oral classification	Dermal (mg/kg)	Dermal classification
Fenamiphos	6	Very Toxic	72	Toxic
Fenitrothion	330–1720	Harmful	890	Harmful
Fipronil	92	Toxic	>2000	Unclassified
Fluoxastrobin	>2000	Unclassified	>2000	Unclassified
Folpet	>2000	Unclassified	>2000	Unclassified
Formetanate	15–26	Very Toxic	>2000	Unclassified
Fosetyl-Al	>7000	Unclassified	>2000	Unclassified
Glufosinate	1510	Harmful	>2000	Unclassified
Haloxyfop-R	>300 (?)	Harmful	>2000	Unclassified
Malathion	1778	Harmful	8790	Unclassified
Metconazole	595	Harmful	>2000 (rabbits)	Unclassified
Methiocarb	13–135	Very Toxic	5000	Unclassified
Methomyl	30	Toxic	>2000	Unclassified
Metrafenone	>5000	Unclassified	>5000	Unclassified
Metribuzin	322	Harmful	>5000	Unclassified
Oxamyl	3	Very Toxic	>2000 (rabbit)	Unclassified
Oxydemeton-methyl	61	Toxic	112	Toxic
Phosalone	120	Toxic	1530	Harmful
Phosmet	113	Toxic	>5000	Unclassified
Pirimicarb	142	Toxic	>2000	Unclassified
Pirimiphos-methyl	1414	Harmful	>2000	Unclassified
Propamocarb	2000	Unclassified	>2000	Unclassified
Pyrimethanil	4149	Unclassified	>5000	Unclassified
Rimsulfuron	>5000	Unclassified	>2000	Unclassified
Spirodiclofen	>2500	Unclassified	>2000	Unclassified
Thiodicarb	50–100	Toxic	>2000	Unclassified
Tolclofos-methyl	>2000	Unclassified	>5000	Unclassified
Tolyfluanid	>5000	Unclassified	>5000	Unclassified
Triazamate	50–200	Toxic	>5000	Unclassified
Tribenuron methyl	>5000	Unclassified	>5000	Unclassified
Trichlorfon	212	Harmful	>5000	Unclassified
Triclopyr	633–729	Harmful	>2000	Unclassified
Trifluralin	>5000	Unclassified	>2000	Unclassified
Trinexapac	4210	Unclassified	>4000	Unclassified
Triticonazole	>2000	Unclassified	>2000	Unclassified

Data from EFSA Pesticide Risk Assessment Peer Review (PRAPeR) conclusions

Active substance	LD_{50} (mg/kg) and classification			
	Oral LD_{50}	Oral classification	Dermal (mg/kg)	Dermal classification
2,3-Dichlorobenzoic acid-methyl ester	1030	Harmful	>10000	Unclassified
Abamectin	8.7	Very Toxic	>330	Toxic
Acequinocyl	>5000	Unclassified	>2000	Unclassified
Acetochlor	1929	Harmful	>2000	Unclassified
Aclonifen	>5000	Unclassified	>5000	Unclassified
Amidosulfuron	>5000	Unclassified	>5000	Unclassified
Benfluralin	>5000	Unclassified	>5000	Unclassified
Bensulfuron	>5000	Unclassified	>2000	Unclassified
Bifenox	>5000	Unclassified	>2000	Unclassified
Bromuconazole	328	Harmful	>2000	Unclassified
Buprofezin	>2000	Unclassified	>2000	Unclassified
Chloridazon	2140	Unclassified	>2000	Unclassified
Clomazone	1369	Harmful	>2000	Unclassified
Copper compounds	299	Harmful	>2000	Unclassified
Cymoxanil	960	Harmful	>2000	Unclassified
Cyromazine	3387	Unclassified	>3100	Unclassified
Diflufenican	>5000	Unclassified	>2000	Unclassified

Table 3. Continued on next page.

Table 3. Continued.

Active substance	LD_{50} (mg/kg) and classification			
	Oral LD_{50}	Oral classification	Dermal (mg/kg)	Dermal classification
Dimethachlor	1600	Harmful	>2000	Unclassified
Dodemorph	**>4000**	**Unclassified**	**>1640**	**Harmful**
Epoxiconazole	5000	Unclassified	>2000	Unclassified
Fenoxaprop	>3150	Unclassified	>2000	Unclassified
Fenpropidin	1452	Harmful	>4000	Unclassified
Fenpropimorph	1670	Harmful	>4000	Unclassified
Fenpyroximate	245	Harmful	>2000	Unclassified
Fluazinam	>4100	Unclassified	>2000	Unclassified
Fludioxonil	>5000	Unclassified	>2000	Unclassified
Flurprimidol	709	Harmful	>5000	Unclassified
Flutolanil	>10000	Unclassified	>5000	Unclassified
Fuberidazole	>300	Harmful	5000	Unclassified
Imidacloprid	~500	Harmful	>5000	Unclassified
Mepiquat	270	Harmful	>1160*	Harmful
Metamitron	1183	Harmful	>5000	Unclassified
Metazachlor	>2000	Unclassified	>2000	Unclassified
Napropamide	>5000	Unclassified	>2000	Unclassified
Nicosulfuron	>5000	Unclassified	>2000	Unclassified
Penconazole	<2000 (971 rabbit)	Harmful (rabbit—Harmful)	>3000	Unclassified
Phosphides	8.7	Very Toxic	460	Harmful
Prosulfocarb	1820	Harmful	>2000	Unclassified
Prothioconazole	>6200	Unclassified	>2000	Unclassified
Quinoclamine	200–500	Toxic	>2000	Unclassified
Spiromesophen	>2000	Unclassified	>2000	Unclassified
Sulcotrione	>5000	Unclassified	>4000	Unclassified
Tebuconazole	1700	Harmful	>2000	Unclassified
Tetraconazole	1031	Harmful	>2000	Unclassified
Tralkoxydim	934	Harmful	>2000	Unclassified
Triadimenol	689	Harmful	>5000	Unclassified

Note. This table is based on one developed by PSD for an internal exercise and is made available to provide general information on pesticides. Although steps were taken to ensure the data in this table are reliable, the values have not been independently confirmed by PSD.
Classification values have been assigned as part of the present analysis. Classification values were assigned according to the UK Chemicals Hazard Information and Packaging for Supply Regulations 2002 Criteria. Acute oral toxicity: Very toxic, $LD_{50} \leq 25$ mg/kg; Toxic, >25 to ≤200 mg/kg; Harmful, >200 to ≤ 2000 mg/kg; Unclassified, >2000 mg/kg. Acute dermal toxicity: Very toxic, $LD_{50} \leq 50$ mg/kg; Toxic, >50 to ≤400 mg/kg; Harmful, >400 to ≤ 2000 mg/kg; Unclassified, >2000 mg/kg.
*Limit test performed with salt at 2000 mg.

Table 4. Summary of comparison of acute oral and dermal classifications for pesticide active substances evaluated within the EU since 1996.

Active substances reviewed with oral and dermal acute toxicity data	240
LD_{50} indicates acute toxicity classification by any route	119
LD_{50} indicates classification by the dermal route	35
Dermal classification the same as for the oral route	18
Dermal classification less severe than for the oral route	15
Dermal classification more severe than for the oral route	2

'causes burns' has been proposed. The compound with a lower dermal LD_{50} value than oral, but for which classification was the same, ethephon, produced similar local effects and has also had a classification of 'causes burns' proposed. It is unclear if death of animals in the studies on dodemorph or ephethon was due to systemic toxicity caused by the substances following dermal administration, or the humane killing of animals due to the severe local effects. It is also unclear if damage of the skin facilitated increased systemic exposure to the substances. In accordance with current best practices to minimise animal use, pain, and distress, an assessment of dermal corrosivity and/or irritancy should be undertaken before deciding whether to conduct an acute dermal study. Substances having the potential to cause severe local effects, such as dodemorph and ethephon, would now be considered exempt from testing in an acute dermal toxicity study.

5.2.2. Industrial chemicals

In 1979, an EU Directive established a notification scheme for new industrial chemicals, which in the UK was enforced (replaced by REACH in 2008) by Notification of New Substances Regulations, with the Health and Safety Executive (HSE) acting as the Competent Authority. Before placing a new substance on the market, a manufacturer was required to notify in the appropriate Member State. Notification

required a manufacturer to produce a dossier describing various toxicological tests conducted on the substance, and a classification and labelling proposal based on the results. For a significant number of substances, information from standard (OECD-compliant) acute toxicity studies is available for both the oral and dermal routes of exposure. These studies were used to determine whether the acute dermal toxicity study contributed to the overall hazard classification (Indans et al., 1998).

The identity of the chemicals analysed is no longer available, but a summary of the analysis is provided in Table 5. Of the 438 base-set notifications with oral and dermal acute toxicity data, 348 compounds were unclassified by both the oral and dermal routes, 90 were classified for acute oral toxicity, and 4 of these were also classified for acute dermal toxicity. Only 1 of the 438 compounds (0.2%) had a more severe classification by the dermal route than for the oral route.

5.3. Implications of the comparison of acute oral and dermal classification data

These analyses suggest that the routine conduct of acute dermal testing in addition to oral testing is of minimal value for hazard classification, at least for pesticide active substances and industrial chemicals. Acute oral data should generally be sufficient for classification and labelling for both routes, with the oral classification being used to inform whether protective clothing and safe handling procedures are required. This finding could potentially have a significant impact in reducing the use of animals in acute testing, and therefore warrants further investigation.

Only three substances (two pesticide active ingredients and one chemical) had a more severe classification by the dermal route, and it would be useful to explore whether there is any particular explanation for this, such as physicochemical properties, toxicokinetic factors, or local effects, although experimental error or variability could also provide an explanation. If any property(s) were found to be indicative of the potential for increased toxicity with skin administration, it might be possible to use this as a screen, with acute dermal toxicity testing being performed only on compounds that possess the property of concern.

Based on our evaluation, we would recommend that an acute dermal toxicity study should only be performed in cases where it is suspected that the acute toxicity of the material might be greater by the dermal route than by the oral route. Such circumstances might exist when there is an indication of acute systemic toxicity in skin irritation and/or sensitisation studies that is not identified in an acute oral toxicity study, or if toxicity is observed in an acute oral toxicity test and there is a potential for high dermal absorption, as determined using an in vitro test such as OECD TG 428 (OECD, 2004a). An acute dermal study may also be justified if information on mechanism of action or toxicokinetics suggests that acute toxicity might be greater by the dermal rather than oral route.

Table 5. Summary of acute toxicity classifications for industrial chemicals in the HSE Notification of New Substances Database.

Chemicals tested for acute oral and dermal toxicity	438
Substances classified for acute oral toxicity	90
Substances classified for acute dermal toxicity (in addition to acute oral toxicity)	4
Substances with a more severe classification for acute dermal toxicity than for acute oral toxicity	1

6. Acute inhalation toxicity testing

Many chemicals exist in the form of gases, volatile liquids, or aerosols/particles (mists and dusts), and for these compounds, depending on their physicochemical properties, the primary route of exposure may be via the respiratory system. Inhalation of chemicals occurs as a result of their presence in air in a variety of occupational or environmental settings, which may arise due to a number of reasons, including accidental or deliberate release, as a by-product of industrial processes, from combustion of fuels, or the use of aerosols in the workplace or home. Acute toxicity may occur following inhalation exposure either as a result of local effects in the respiratory tract, or due to systemic effects following absorption from the lungs.

Chemical regulations around the world include requirements for acute inhalation toxicity testing, where appropriate, to inform classification and to determine whether any particular safe handling procedures or equipment may be required. While the oral route is mandatory for acute toxicity testing under most regulatory frameworks, selection of a second route of exposure (dermal or inhalation) is based on expert judgement, which may take into account the inherent acute toxicity of the chemical and the primary route of exposure when handling the material. The inhalation route should be selected for powders having particles in the inhalable range (≤100 µm mass median aerodynamic diameter [MMAD]) and for substances with high vapour pressure at ambient temperatures. The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) requires acute inhalation studies 'if the product consists of, or under conditions of use will result in, an inhalable material (e.g., gas, vapour, or aerosol/particulate)' (CFR, 1998), while REACH states that inhalation should be selected as the second route of exposure for acute toxicity testing if human exposure is possible via this route, or if physicochemical properties indicate that such exposure may occur.

Although the dermal route is the primary occupational exposure route for most pesticides, there are exceptions. Pesticides with high vapour pressures (>1 Pa), such as fumigants, exist as a gas/vapour under normal environmental conditions so inhalation is the primary route of exposure. For materials with very low (<1%) dermal absorption, even small amounts of inhaled material can be a significant source of absorbed dose because absorption by the respiratory tract is generally more efficient than dermal absorption. Finally, materials that otherwise would not pose an inhalation hazard because of their physicochemical properties (e.g. liquid

or large particle-size solid pesticide preparations with a low vapour pressure) may be aerosolized for the purpose of application and thus become respirable and subject to acute inhalation testing.

6.1. Reduction and refinement approaches for acute inhalation testing

The current OECD acute inhalation Test Guideline 403 (TG 403) was adopted in 1981 (OECD, 1981b). Revision of this test guideline taking into account scientific progress, changing regulatory needs, and animal welfare considerations has recently been completed. The revised TG 403 (OECD, 2009b) was designed to use fewer animals while incorporating scientific advancements and providing maximum flexibility to characterise the entire range of the concentration-mortality relationship so that it can satisfy a variety of regulatory needs (NRC, 2001). The revised guideline allows a choice between two types of studies depending on regulatory and scientific needs: a traditional LC_{50} or a concentration × time (CxT) study. An alternative guideline, the Acute Toxic Class Method (TG 436), which is able to satisfy most regulatory needs, providing a range estimate of LC_{50} and GHS categorization, has also been adopted (OECD, 2009c). A further test guideline that has been proposed, the Fixed Concentration Procedure (TG 433), provides not only a reduction but also a refinement compared with the current test guidelines as, like the FDP for oral toxicity, it does not require lethality or impending death as an endpoint. The NC3Rs is currently coordinating collaborative efforts to develop the scientific information and evidence needed to achieve regulatory acceptance of this guideline.

A revised guidance document on Acute Inhalation Toxicity Testing (OECD, 2009a) describes the attributes of the various tests, and these are summarised in Table 6. The CxT protocol of TG 403 uses considerably more animals than the other two protocols, while the LD_{50} protocol of TG 403 will also use more animals than TG 436 in a non-limit study. Therefore, for animal welfare reasons, and bearing in mind that regulatory guidelines including the European directive on the use of animals require that where animal testing must be performed, the test that incurs least severity is selected (EEC, 1986), TG 436 should be selected in preference over either of the TG 403 protocols if the test is being conducted for the purposes of classification and labelling or other purposes where a range estimate of the LC_{50} is sufficient to satisfy regulatory or scientific needs. This is also stated in the OECD guidance document, while the REACH guidance states that TGs 436 or 433 (once adopted) should be performed where possible.

6.2. Opportunities to avoid acute inhalation toxicity testing

There are many potential sources of redundancy in acute inhalation toxicity testing. While redundancy in testing guidelines has been greatly reduced through harmonisation

Table 6. Comparison of draft OECD acute inhalation toxicity testing guidelines*.

Test guideline	TG 403 revised (2008) Traditional LC_{50} study	TG 403 revised (2008) CxT study	TG 436 (2008)
Method	Traditional LC_{50}	Concentration × time (CxT)	Acute Toxic Class (ATC)
Major endpoint	Mortality	Mortality	Mortality
Major objective	Concentration response for lethal and non-lethal endpoints (endpoints are system independent)	Concentration response for lethal and non-lethal endpoints (endpoints are system independent) Derivation of n in $C^n \times t$	Range estimate determination
Use of data	Classification and labelling Derivation of LC_x values for one exposure duration (usually 4 hours)	Classification and labelling Derivation of LC_x values for multiple exposure durations	Classification and labelling Range estimate of LC_{50} values for one exposure duration (usually 4 hours)
	Animals tested		
Limit test	3M and 3F (or 5 of susceptible sex)	In case of 1 animal/sex/(Cxt) point: Both sexes: 10; Susceptible sex: 10 In case of 2 animals/sex/(Cxt) point: Both sexes: 20; Susceptible sex: 20	3M and 3F (or 6 of susceptible sex)
Sighting study	≤ 3M and ≤ 3F (or ≤ 3 of susceptible sex) per concentration. At least 3M and 3F per concentration to test sex differences if unknown	≤ 3M and ≤ 3F per concentration	N/A
Main study	5M and 5F (or 5 of susceptible sex) per concentration	1 or 2 animals/sex/(Cxt) point (or 2 or 4 animals of susceptible sex per (Cxt) point 5 durations per concentration	3M and 3F (or 6 of susceptible sex)
Total animals used in a non-limit study	If 4 concentrations tested: Both sexes: 40 Susceptible sex: 20 (if used for classification and labelling)	If 4 concentrations tested: In case of 1 animal/sex/(Cxt) point: Both sexes: 40; Susceptible sex: 40 In case of 2 animals/sex/(Cxt) point: Both sexes: 80; Susceptible sex: 80	If 1 concentration tested: 6 If 2 concentrations tested: 12 If 3 concentrations tested: 18

*Adapted from draft OECD guidance document on acute inhalation toxicity testing (OECD, 2008c).

of existing and draft guidelines in the USA, Europe, and Asia-Pacific and other international regions, some variation in national/international requirements, and areas of redundancy, remain.

Unnecessary testing may result from having to repeat a study because of failure to meet guideline requirements of particle size or limit-test concentration, or due to improper calculation of the concentration of the active in a formulation. One cannot assume that the physical characteristics (particle size; particle/vapour phase equilibrium) of a test atmosphere remain constant at different chamber concentrations, therefore, additional care and analytical characterization of the test atmosphere may be critical in order to interpret test results. Inhalation exposure studies are expensive, technically challenging, and may require a significant number of animals, therefore, every effort must be made to ensure that each study is conducted correctly. If it is not possible to achieve the correct test conditions, performance of the test may not be appropriate.

In some cases, irrelevant testing is conducted on a form of the product that may not be representative of the product as used. Liquid formulations that are not specific, ready-to-use products are often diluted, often by many fold (e.g. 50-, 100-, or even 1000-fold), prior to application as a water-based spray. Therefore, potential exposure to the undiluted liquid formulation is limited only to its vapours, if any, and potential exposure to the components of the undiluted formulation by particle inhalation is greatly reduced by the extent of the dilution. Granular preparations that may not be respirable in their native form may require particle size reduction prior to testing to produce a respirable aerosol (1–4 µm MMAD) that is compliant with test guidelines. The 1–4 µm particle size requirement is designed to optimise deposition of the test material in the alveolar (gas-exchange) region and thus systemic exposure. In either case, the end result of testing an undiluted liquid or granular formulation that may not otherwise be inhalable per se, in a form that optimises or maximises inhalation exposure, is not relevant to the human exposure situation and therefore should not be undertaken.

As already noted, the REACH legislation requires that animal testing be only performed as a last resort. In addition to generic advice on opportunities for waiving test requirements, the REACH guidance also sets out endpoint-specific guidance that highlights instances where acute toxicity testing by the inhalation route should not be performed. Testing is not required if the particle size is >100 µm, the vapour pressure is very low (<0.1 Pa at 20°C), or if it is not technically possible to generate a testing atmosphere (ECHA, 2008b).

In addition, and as noted earlier, bridging principles and calculation methods such as those set out by the EU DPD, WHO, and GHS can make a significant contribution to avoiding acute inhalation toxicity testing of chemical mixtures, as can the US EPA's guidance on acute toxicity data requirements for granular pesticide products.

6.3. In vitro alternatives to acute inhalation studies

The structural and functional heterogeneity of the upper and lower respiratory tract complicates the development of any in vitro test system for assessing inhalation toxicity. There are at least 45 cell types, with widely varying phenotypes and unique roles and functions within the respiratory tract. Any cell along the respiratory tract may be a target of an inhaled toxicant, depending upon the properties of the inhalant and where it is absorbed or deposited. Sometimes cells are selectively affected because of their inherent function (e.g. metabolism), adding more complexity to the development of a screening bioassay. In addition, because cell-specific damage can be pivotal in disease pathophysiology, selection of the appropriate cell type for an in vitro study is typically linked to the question being asked (Costa, 2008). Given the complexity of the task, it is perhaps not surprising that no in vitro test system has yet been identified, let alone validated, for use as a substitute or adjunct to current in vivo acute inhalation toxicity testing procedures.

Despite these complexities, there is both interest in and progress toward the development of in vitro methods for respiratory toxicity assessment using relevant airway and alveolar lung cells, tissue slices, and explants and evaluation/implementation of target-specific endpoints (ICCVAM, 2001; Lambre et al., 1996). Despite the often disparate goals of those in the basic research and safety testing/risk assessment communities, collaborative efforts could yield a common, 'validated' testing paradigm that could be of benefit to all. While a single in vitro bioassay is unlikely, a battery of tests using several cell types and endpoints might provide sufficient dose-response data to set limits of potential toxicity sufficient for classification and labelling or to aid in selecting initial exposure concentrations used in standard in vivo inhalation toxicity studies. The use of cell systems maintained at an air-liquid interface is a significant advance in technology, which permits expression of a differentiated cell phenotype and exposures similar to in vivo exposures, and may simplify calculations of dose to the cell (Aufderheide and Mohr, 1999, 2000; Bakand et al., 2006a; Bakand et al., 2006b; Gerde, 2008; Kesimer et al., 2008; Lee et al., 2005; Lin et al., 2007; Park et al., 2007; Seagrave et al., 2007).

7. Skin corrosivity and skin irritancy potential

Exposure of the skin to chemicals not only can give rise to systemic toxicity, but also can result in irritation or corrosion at the site of contact. Corrosive substances may destroy tissues with which they come into contact, while irritants are generally non-corrosive substances that can cause inflammation through reaction with skin proteins and interference with lipids (ECHA, 2008b). Generation of information on skin corrosivity and skin irritancy potential is a regulatory requirement in the EU for industrial chemicals, biocides, and pesticides, while other regulatory authorities such as those in the USA and Japan require

similar data for a number of local regulatory schemes. The information is used to indicate whether specific safe handling procedures including protective clothing are required and to indicate the appropriate classification and labelling for the substance.

Historically, such information has usually been obtained from studies in animals. The most widely accepted standard protocol dates from 2002. The current in vivo OECD test guideline provides for the testing of skin corrosivity and skin irritation. Firstly, there is an assessment of corrosivity, either based on extremes of pH (<2 and >11.5) or an in vivo (or a validated in vitro) test. Where a substance is established as corrosive, no further testing is justified. Only when corrosivity can be excluded on pH grounds should in vivo testing be considered (OECD, 2002a). It should be noted that there are no similar agreed pH boundaries for skin irritation.

7.1. Validation and acceptance of in vitro tests for skin corrosivity and skin irritancy potential

In 2004, the OECD adopted two test guidelines for assessment of skin corrosivity based on successfully validated in vitro methods: OECD TG 430 (Transcutaneous Electrical Resistance (TER) Test (OECD, 2004b)) and OECD TG 431 (Human Skin Model Test (OECD, 2004c)). In 2006, the OECD adopted a third validated in vitro test for skin corrosivity, the in vitro Membrane Barrier Test, TG 435 (OECD, 2006), the test system being available commercially as Corrositex®. A positive finding from one of these tests can be used to classify a substance with respect to corrosivity. A negative result is usually followed up with a test for skin irritation, as these in vitro assays only inform on skin corrosivity and not skin irritancy potential.

The results of tests conducted according to the Membrane Barrier Test, TG 435, can be used to discriminate between the three GHS corrosivity subclasses (GHS Skin 1A, 1B, or 1C), and the three UN Transport Packing Groups (Packing Group I, II, or III), but notably, only for chemicals and chemical mixtures that qualify for testing—a limitation of the method is that depending upon the results of an initial compatibility test, many non-corrosive chemicals and chemical mixtures, and some corrosive chemicals and chemical mixtures, may not qualify for testing. The US Department of Transportation has granted authorisation of Corrositex® as an alternative to the in vivo corrosivity test specified in the Hazardous Materials Regulations, Title 49 CFR 173.136 and 173.137, for the following seven classes of material:

- Acids—inorganic and organic
- Acid derivatives (anhydrides, haloacids, salts, etc.), inorganic and organic
- Acyl halides
- Alkylamines and polyalkylamines
- Bases, inorganic and organic
- Chlorosilanes
- Metal halides and oxyhalides

At the time of writing this paper, four commercially available reconstructed human epidermis (RHE) models are considered by the ECVAM Scientific Advisory Committee (ESAC) to be scientifically validated for the assessment of skin corrosivity potential using OECD TG 431; EpiSkin™, SkinEthic's RHE, MatTek's Epiderm™, and CellSystems®' EST-1000 (ECVAM, 1998, 2000, 2006, 2009b). The ESAC statement for the Episkin™ human skin model for corrosivity concludes that it is able to distinguish between known EU R 35 (UN Packing Group I) and R 34 (UN Packing Group II and III) chemicals. For data generated using the other human skin model corrosivity models or the TER test, the EU Risk Phrase R 35, GHS 1A, and UN Packing Group I are applied as default for materials giving a positive result.

For assessment of skin irritancy potential, three RHE in vitro tests are considered by ESAC to be scientifically validated: EpiSkin™, SkinEthic RHE, and the modified EpiDerm™ skin irritation test (SIT) methods (ECVAM, 2007c, 2008, 2009a). These are all regarded as stand-alone replacements for the in vivo test, with sufficient accuracy and reliability for distinguishing between irritant and non-irritant chemicals. These methods have been adopted as part of the first adaptation to technical progress (ATP) of the EU Test Method Regulation (761/2009/EC, updating 440/2008/EC), as Test Method B.46, for use in several regulatory schemes including REACH, plant protection products, and biocides (EC, 2009). Another in vitro test for skin irritation, the LabCyte™ EPI-MODEL24, has undergone a validation study in Japan[4] and the OECD's WorkPlan for its Test Guidelines Program includes activities to explore the use of this method.

The EpiSkin™, SkinEthic RHE, and the modified EpiDerm™ SIT tests were only validated against the EU skin irritation criteria in Directive 67/548, but it is expected that in the future the United Nation (UN) GHS will become the standard for classification and labelling (GHS, 2007). This may be problematic as the UN GHS divides skin irritation into two separate classes (GHS Skin 2 or 3), whereas in the EU, the Classification Labelling and Packaging of Substances and Mixtures Regulation (EC, 2008b) merges the two into a single class (GHS Skin 2). This is not anticipated to be a major issue in the EU as the cut-offs for classification as a skin irritant in Directive 67/548 and in the Classification, Labelling and Packaging (CLP) Regulation are very similar, and after a re-evaluation of the performance of the three methods using the GHS cut-offs, ESAC has issued a statement indicating that the original statements relating to the scientific validity of these methods continue to be accurate and can be extended to use of the GHS cut-off values implemented under the CLP Regulation (ECVAM, 2009a). However, with non-EU submissions, it is possible that further in vivo testing may be required to discriminate between GHS skin Category 2 and 3. It should be noted that although no mixtures (preparations) were included in the validation trials of the in vitro skin irritation tests, there is no scientific reason to suggest that these tests cannot be used to inform on the skin irritation potential of mixtures.

[4] http://www.jacvam.jp/files/effort/04-002/jssareport_090415.pdf

The adoption of these methods as stand-alone methods for distinguishing between skin irritants and non-irritants in the EU represents a significant breakthrough in terms of replacement of animal testing for regulatory purposes. It is now possible in principle to obtain information on skin irritation using the validated in vitro protocols without the need for animal testing (Macfarlane et al., 2009). However, in vitro methods are not yet accepted as full replacements under US and Japanese regulatory guidelines and there is a need for the acceptability of these tests to be endorsed beyond the EU, through the development and harmonisation of internationally acceptable guidelines. A draft proposal for an OECD guideline on in vitro assessment of skin irritation is currently under discussion[5]. In addition to the animal welfare drivers for using in vitro approaches for assessing skin irritation, it is also important to consider that the predictivity of the in vivo methods for effects in humans has been questioned (Basketter et al., 1997; York et al., 1996).

In the USA, there are opportunities to avoid animal testing for skin irritation, as the Consumer Product Safety Commission (CPSC) and EPA guidelines both state that animal testing is only required in certain circumstances (Table 7). Under the EPA guidelines, testing is not required if the substance is a strong acid or base, the compound is highly toxic in acute dermal studies, or if corrosive properties can be predicted from validated and accepted in vitro tests or structure-activity relationships, whilst animal testing under CPSC is only required if hazard determination cannot be made from physicochemical characteristics, expert opinion, prior human exposure, or previous animal testing.

Furthermore, bridging principles and calculation methods such as those set out by the EU DPD and GHS, as well as the US EPA's guidance on acute toxicity data requirements for granular pesticide products, can be employed to avoid in vivo testing for the skin irritation potential of chemical mixtures by making use of information on the individual components.

8. Eye irritation

The potential for accidental exposure of human eyes to a chemical substance or mixture can exist in almost any situation in which both the person and substance are present at the same time. The exposure can occur in three ways: by direct contact due to unexpected splashes or sudden release, by indirect contact during handling, or by contamination of the air. The risk of exposure can be minimised in specific situations by the wearing of suitable eye protection but cannot be eliminated from all situations because of unpredictability of release of substance and the lack of control over the correct use of eye protection. The gathering of information on the ocular irritancy potential of the chemical substance or mixture is therefore a standard regulatory requirement within all industrialised regions of the world in order to provide warning of the possible consequences of exposure of the eyes by appropriate classification and labelling, and to recommend safe handling procedures.

8.1. Limitations of the current in vivo method

The rabbit eye irritation test is the method of assessment of eye irritation potential currently accepted by all regulatory agencies. The use of rabbits was reported by a number of investigators in the 1940s, culminating in publication of the method of Draize et al., in which a numerical system for grading the response in the cornea, iris, and conjunctivae was described (Draize et al., 1944). The test was first adopted as a national regulatory requirement under the US Federal Hazardous Substances Labelling Act (FHSA) in 1960, and it forms the basis of all current test guidelines.

Despite the use of other species, including non-human primates and rodents, the rabbit, especially the albino rabbit, has proven particularly useful for assessment of eye irritation potential due to its widespread availability, ease of handling, and the relatively large surface area of ocular tissue available for exposure to the test item and subsequent evaluation of response. Its relevance for prediction of eye irritation potential has been justified by the fact that the gross effects on the eye following exposure can be easily observed, and the suggestion that the dosage used, coupled with the relatively high sensitivity of the rabbit eye to chemical irritants, will ensure that potential human eye irritants are successfully identified. Despite these considerations, there has been much criticism of the method, both for reasons of animal welfare and scientific relevance and reliability (York and Steiling, 1998). For example, there are anatomical and physiological differences between rabbit and human eyes (e.g. the presence of a large nictitating membrane in the rabbit). Also, evaluation of the ocular response is highly subjective, the reproducibility of results is poor, and data are generally over-predictive of human eye injury.

In addition, the standard amount of substance administered to the eye is 100 µl or 100 mg. Given that the maximum volume the conjunctival sac of the rabbit eye can accommodate is 30–50 µl (Mishima, 1981), the amount applied is considered to be excessive. A low-volume eye irritation test (LVET), which involves applying one tenth of the standard dosing volume directly to the surface of the cornea, was developed as an alternative approach (Griffith et al., 1980). Various investigations of the method have been conducted since the 1980s and findings have included a lower severity of response than in the Draize test and a better correlation with eye irritation in humans (Cormier et al., 1996; Gettings et al., 1996, 1998a, 1998b). However, a reduced ability to differentiate eye irritants of low irritancy potential was identified and despite a number of apparent advantages over the Draize test, at least with some categories of products, the LVET has never been adopted as an official test guideline.

[5] http://www.oecd.org/LongAbstract/0,3425,en_2649_34377_43664851_1_1_1_1,00.html

Table 7. Summary of major EU, US, and Japanese regulations and associated test guidelines for skin irritation.

Region	Chemical type	Legislation	Test guidelines	Opportunities for waiving, reduction, or refinement	Number of animals	Test details
EU	General chemicals	REACH	Commision regulation (EC) 440/2008: method B.4 + B.46	In vivo testing should not be performed until all relevant available data evaluated in a weight of evidence analysis (e.g. existing human/animal data, evidence of irritation/corrosivity of structurally related substances, pH and results from validated and accepted in vitro/ex vivo tests. B.46 in vitro methods adopted in early 2009: EpiSkin™, SkinEthic RHE, and Epiderm™ SIT. In vivo test first performed on one animal—no additional animals tested if corrosion or severe irritation observed. Although not specified in the test method, allowance of a minimum of 24 h between treatment of the first animal and subsequent animals is appropriate.	1, 2, or 3 for B.4 None using B.46	B.4: 4-h exposure period with observation up to 14 d; can be terminated earlier if reversibility observed, or at 72 h if no skin damage is observed. Sequential testing recommended: – Initial test in one animal, testing of additional animals not required if corrosive effects observed. – If corrosive effect not observed in initial animal, irritant or negative response confirmed in up to 2 additional animals. – If irritant effect seen in initial animal, two additional animals tested sequentially or simultaneously. If severe irritation/corrosion expected, initial testing to be performed in 1 animal with 3 test patches for increasing exposure periods; if no serious skin reaction after 3 min, second patch removed after 1 h; if observations indicate test can continue humanely, third patch removed after 4 h. B.46: Three-dimensional reconstructed human epidermis models based on assessment of cell viability Suitable for classifying UN GHS category 2 irritants
EU	Plant protection products	91/414/EEC	As above	As above	As above	As above
EU	Biocides	98/8/EC	As above	As above	As above	As above
USA	Household products (CPSC); Industrial chemicals (OSHA; for worker safety)	Federal Hazardous Substances Act (FHSA), 2004	CPSC code of federal regulations 1500.41	Tiered and sequential approach to testing recommended (49 FR 22522): animal testing performed only if hazard determination cannot be made from physicochemical characteristics, expert opinion, prior human exposure, or previous animal testing	Minimum of 6	24-h exposure period, observations at 24 and 72 h post-application
USA	Pesticides	Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)	EPA: OPPTS 870.2500	Testing not required if: – pH ≤2 or ≥11.5 (buffering capacity to be taken into account). – Compound is highly toxic in acute dermal studies, or if does not produce irritation at the limit dose of 2000 mg/kg body weight. – Corrosive properties can be predicted from well validated and accepted in vitro tests. – Corrosive potential can be predicted from structure-activity relationships. Existing human and animal data should also be reviewed. In vivo test first performed on 1 animal if severe irritation/corrosion expected.	At least 3 (unless justification for using fewer animals provided)	4-h exposure; observation period should be sufficient to evaluate the reversibility of effects, but should not exceed 14 d. If severe irritation/corrosion expected: – Initial testing in 1 animal with 3 test patches for increasing exposure periods; if no serious skin reaction after 3 min, second patch removed after 1 h; if observations indicate test can continue humanely, third patch removed after 4 h. – If corrosive effect seen in first animal, testing in additional animals not required. – If no corrosive effect in initial animal, test is completed using 2 additional animals. If severe irritation/corrosion not expected, test conducted on all 3 animals with 4-h exposure period.
USA	General chemicals	Toxic Substances Control Act (TSCA)	As above	As above	As above	As above

Table 7. Continued on next page.

Table 7. Continued.

Region	Chemical type	Legislation	Test guidelines	Opportunities for waiving, reduction, or refinement	Number of animals	Test details
Japan	Agricultural chemicals	Agricultural Chemicals Regulations Law	Guideline 2-1-4	In vivo test first performed on 1 animal if severe irritation/ corrosion expected.	3 or more	4-h exposure; observations up to 72 h, continued up to 14 d where needed to assess reversibility. If severe corrosion expected: – initial testing in 1 animal with 3 test patches for increasing exposure periods; if no serious skin reaction after 3 min, second patch removed after 1 h; if observations indicate test can continue humanely, third patch removed after 4 h. If severe irritation expected: – expose 1 animal for 4 h. If no severe irritation or corrosion observed in initial animal, test 2 additional animals. If severe irritation/corrosion not expected, test conducted on all 3 animals with 4-h exposure period.

8.2. Tiered approaches to reduce animal testing for eye irritation

An OECD test guideline for assessment of eye irritation potential was first adopted by the OECD in 1981 as Test Guideline 405. It has been revised on two occasions, the first time in February 1987 and the second time in April 2002 (OECD, 2002b). An important aspect of the 2002 version of OECD TG 405 is the inclusion of a tiered approach, or 'sequential testing strategy' for assessment of eye irritation, as recommended during an OECD workshop held in Solna, Sweden, in 1996. This is also a feature of the GHS. Although the sequential testing strategy appears as a supplement to TG 405 rather than as an integral part of the guideline, it is presented as the recommended approach that is considered to represent best practice, and provides an ethical benchmark for in vivo testing for eye irritation/ corrosion. The sequential strategy requires the consideration of all existing information, including human and animal data, physicochemical properties and chemical reactivity, structure-activity relationships (SARs), and the use of validated in vitro tests before any in vivo testing is conducted in order to avoid unnecessary animal use. Thus, if a test material can be classified using existing information no further testing is required.

It is also recommended that an in vivo skin irritation test be conducted before testing in the rabbit eye, although this may not be necessary if a validated in vitro replacement to the in vivo skin irritation test can be used instead, for example in the EU (see section on skin irritation). Materials that are known to be corrosive or severely irritant to skin can be considered to have the potential to cause similar effects in the eyes and should not be tested. Materials with extremes of pH, i.e. ≤2 and ≥11.5, are also recognised as possessing the potential to produce serious eye damage, especially when associated with significant buffering capacity, and should not be tested. In vitro alternatives that have been validated and accepted may be used to make classification decisions. It is recommended that all available information be taken into consideration in order to make a weight-of-the-evidence assessment of eye irritancy, ideally without conducting new animal tests.

Table 8 outlines the testing for eye irritation required under some of the major regulatory schemes in the EU, USA, and Japan. Many regulatory systems have adopted the tiered strategy recommended by OECD TG 405, including for assessment under REACH, assessment of plant protection products (Directive 94/414/EEC) and for biocidal products (98/8/EC). In the USA, the CPSC adopted a policy to reduce the number of animals tested and minimise the pain and suffering associated with testing (49 FR 22522-22523). Under this policy, eye irritation testing is not required if a product is a primary skin irritant. In addition, a tiered and sequential approach to testing is recommended such that testing in animals is only performed if the appropriate hazard determination cannot be made from physicochemical characteristics, expert opinion, prior human experience, or animal testing. The CPSC also advises that topical anaesthetics be applied to the eyes prior to in vivo testing. However, test guidelines of the Japanese Agricultural Chemicals Regulation Law have not yet been updated to comply with the requirements of the 2002 OECD TG. The test guideline of the US EPA Office of Prevention, Pesticides, and Toxic Substances (OPPTS 870.2400) has also not been updated, although it does state that strong acids or bases, and substances that have been found to be corrosive or severe irritants in dermal studies, need not be tested for eye irritation. In addition, results from well-validated and accepted in vitro test systems may identify irritants or corrosives that need not be tested in vivo.

Where animal testing is performed, EU test guidelines and those of the US EPA offer opportunities to reduce the numbers of animals used, by first testing one animal. If positive effects are seen in this animal, testing of subsequent animals need not be performed. Although not currently specified in any official test guideline, a common practice amongst test facilities is to allow a minimum of 24 hours

Table 8. Summary of major EU, US, and Japanese regulations and associated test guidelines for eye irritation.

Region	Chemical type	Legislation	Test guidelines	Opportunities for waiving, reduction or refinement	Number of animals	Test details
EU	General chemicals	REACH	Commission regulation (EC) 440/2008: method B.5	Sequential testing strategy recommended. In vivo testing should not be considered until all relevant available data evaluated in weight of evidence analysis, as per OECD + GHS guidance (e.g. existing human/animal data, evidence of irritation/corrosivity of structurally related substances, pH, results of validated in vitro/ex vivo tests for skin/eye corrosion or irritation). If in vivo testing needed, testing for dermal irritation/corrosion performed prior to decision on testing in eyes. In vivo test first performed on one animal. Additional animals tested only if no severe damage or response observed. Although not specified in the test method, allowance of a minimum of 24 h between treatment of the first animal and subsequent animals is appropriate. Local anaesthetics may be used. Use of a satellite group to investigate the influence of washing not recommended unless scientifically justified.	1, 2, or 3	Observation up to 21 d, can be terminated earlier if reversibility observed, or at 72 h if no ocular lesions develop. Sequential testing recommended: – If compound found to be corrosive or severe irritant, testing beyond initial animal should not be performed. – If corrosive/severe irritant effect not observed in initial animal, irritant or negative response confirmed in up to 2 additional animals. If irritancy seen in first animal, sequential testing in subsequent animals recommended: terminate test after second animal if corrosive or severe irritant effects observed.
EU	Plant protection products	91/414/EEC	As above	As above	As above	As above
EU	Biocides	98/8/EC	As above	As above	As above	As above
USA	Household products (CPSC); Industrial chemicals (OSHA; for worker safety)	Federal Hazardous Substances Act (FHSA), 2004	CPSC code of federal regulations 1500.42*	Tiered and sequential approach to testing recommended (49 FR 22522): animal testing performed only if hazard determination cannot be made from physicochemical characteristics, expert opinion, prior human exposure, or previous animal testing. Testing not required if substance is a skin irritant. Topical anaesthetics to be applied to eyes prior to testing.	6 (12 or 18 possible)	Observation up to 72 h (may be extended to 7 d) First test: If 1 animal +ve, test considered negative If ≥4 animals +ve, test considered positive If 2–3 animals +ve, test repeated Second test: If ≥3 animals +ve, test considered positive If 1-2 animals +ve, test repeated Third test: ≥1 animal +ve, test considered positive
USA	Pesticides	Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)	EPA: OPPTS 870.2400*	Testing not required if pH ≤2 or ≥11.5 (buffering capacity to be taken into account). Testing not required if corrosion or severe irritation observed in dermal study. Data from validated + accepted in vitro tests may be used to avoid in vivo testing. Test should be performed on 1 animal if marked effects anticipated; further testing may not be needed if severe irritation/corrosion observed Local anaesthetic may be used if thought substance may cause extreme pain.	1 to at least 3	Study may be ended at 72 h if no evidence of irritation. Extended observation to 7 or 21 d if persistent corneal involvement or ocular irritation. Additional testing for effectiveness of washing may be indicated for some substances shown to be irritating.
USA	General chemicals	Toxic Substances Control Act (TSCA)	As above	As above	As above	As above

Table 8. Continued on next page.

Table 8. Continued.

Region	Chemical type	Legislation	Test guidelines	Opportunities for waiving, reduction or refinement	Number of animals	Test details
Japan	Agricultural chemicals	Agricultural Chemicals Regulations Law	Guideline 2-1-5	Study should start with 1 animal if compound suspected to be a severe irritant. Additional animals not required if severe eye corrosion/ irritation observed. Local anaesthetic may be used if thought substance will cause severe pain.	3 or more	If severe irritation occurs, study of effectiveness of eye washing to be performed on at least 3 animals.

*BCOP and ICE assays have now been accepted by all US regulatory agencies for identification of severe ocular irritants and ocular corrosives as part of a tiered testing approach, with specific limitations for certain chemical classes and/or physical properties.

to elapse between treatment of the first and subsequent animals. This is because the ocular response will often not reach its maximum until a number of hours after treatment of the animal. In the United Kingdom, this practice is detailed in the UK Home Office Guidance on Eye Irritation Tests[6] and hence it is an expectation that all UK test facilities follow this approach. Although data are not available to demonstrate what influence this has had on the number of animals exposed to severe irritants, widespread adoption of the practice would offer the opportunity for reduction.

The use of humane endpoints, based upon the degree of pain exhibited by the animal and/or the severity of the ocular reactions, can be incorporated into the test as a method of refinement, and can be optimised by observation of animals more frequently than the times specified in the test guideline. These measures are also included in the UK Home Office guidance on eye irritation tests.

8.3. Alternatives to the rabbit eye irritation test

A diverse range of in vitro and ex vivo test methods have been investigated as potential alternatives to the rabbit eye irritation test, including tests utilising cell cultures, cell-function assays, bacterial assays, tests involving isolated cornea or eyes, tests using fertilised hens eggs, methods using invertebrates (e.g. the slug mucosal irritation assay (Adriaens et al., 2008)), and three-dimensional reconstructed human tissue models such as EpiOcular™. Many have been included in formal validation programmes and whilst some have proven useful for testing specific classes of materials, or materials with a limited range of irritancy, there are currently no individual methods or combination of methods that are considered to be acceptable as complete replacements to the rabbit eye irritation test. This is due in part to the difficulty in modelling the full range of mechanisms of action of chemical eye irritants in vitro, but also to the poor reproducibility of in vivo eye irritation data used in the validation process.

The OECD TG 405, Method B.5 of 440/2008/EC, OPPTS 870.2400, and the GHS all make provision for the use of in vitro tests to minimise animal use for assessment of eye irritancy use when validated. Within the EU, Commission Directive 86/609/EEC requires that "An experiment shall not be performed if another scientifically satisfactory method of obtaining the results sought, not entailing the use of an animal, is reasonably and practically available". This Directive is implemented through national law in various member states, for example in the UK through the Animals (Scientific Procedures) Act 1986.

In the EU, a positive result in one or more of the following four tests is accepted as evidence of severe irritation potential:

- Isolated rabbit eye (IRE) test (also known as the rabbit enucleated eye test or REET)
- Isolated Chicken eye (ICE) test
- Bovine corneal opacity & permeability (BCOP) test
- Hens egg test—chorioallantoic membrane (HET-CAM) test

Therefore in the EU, if a positive result is obtained with one of these tests, a substance can be considered a severe eye irritant and Risk Phrase R 41 should be applied with no further testing justified. Where a negative result is obtained, an in vivo test should subsequently be performed, as to date the tests have not been shown to adequately discriminate between eye irritants and non-irritants.

Furthermore, ICCVAM has recommended that there are sufficient data available to support the use of the BCOP and ICE in appropriate circumstances and, with certain limitations, as screening tests to identify substances as ocular corrosives and severe irritants in a tiered testing strategy as part of a weight-of-evidence approach (ICCVAM, 2006b). This conclusion was subsequently endorsed by the ECVAM Scientific Advisory Committee (ECVAM, 2007b). US regulatory agencies, including the CPSC and EPA, have indicated their support for the use of these two methods using this approach, and this should have a significant impact in reducing animal testing for this purpose (Hood, 2008). The identified limitations for the BCOP are testing of solids, alcohols, and ketones, while for the ICE, the limitations are for testing of alcohols, surfactants, and solids. OECD Test Guidelines have been developed for the BCOP and ICE methods for identification of severe ocular irritants

[6] http://scienceandresearch.homeoffice.gov.uk/animal-research/publications-and-reference/publications/guidance/hoguidance-eye-irritation-tests?view=Html

and corrosives, and these were adopted as official test guidelines by the OECD Council in September 2009 (OECD, 2009d, 2009e).

ICCVAM considers that the IRE and HET-CAM do not currently have sufficient performance and/or sufficient data to substantiate their use for regulatory hazard classification purposes, but may have applicability for other uses. ESAC has recommended that further investigations of protocol optimisation should be conducted before a statement on their validity can be made.

In 2005, ECVAM commissioned a workshop to allow developers and users of in vitro and ex vivo methods to nominate them for consideration as a basis for an overall testing strategy to reduce animal usage. A bottom-up/top-down approach was proposed, involving the sequential progression of in vitro tests, beginning with tests that can accurately identify non-irritants or severe irritants, respectively. Under this proposed approach, chemicals not identified as non-irritants or severe irritants would be given a default classification as a mild irritant (Scott et al., 2009). More recently, experts at a scientific meeting organised by the European Cosmetic Association COLIPA have argued that once additional in vitro assays have achieved acceptance, it should be possible to apply them in a tiered approach to evaluate eye irritation without in vivo testing. By using a combination of assays, it should be possible to obtain information over the entire range of irritancy for different classes of chemicals (McNamee et al., 2009).

In May 2009, NICEATM, in collaboration with ICCVAM, convened an independent scientific peer-review panel to evaluate several alternative ocular toxicity testing methods and approaches. Methods reviewed by the Panel included a testing strategy using in vitro test methods to assess the eye irritation potential of antimicrobial cleaning products, and the validation status of five in vitro/ex vivo test methods for identifying moderate and mild irritants and products that do not require labelling for eye hazards. The panel also evaluated, and agreed with, a proposal for the routine use of topical anaesthetics, systemic analgesics, and humane endpoints to avoid or minimize pain and distress during in vivo eye irritation safety testing (ICCVAM, 2009a). ICCVAM is reviewing the panel's conclusions and recommendations, along with comments from its scientific advisory committee and the public, as it works to develop final recommendations on these methods and approaches for US Federal Agencies[7].

Recently, the EPA has announced that it is to conduct an 18-month pilot study to evaluate the use of a non-animal testing approach to assess the eye irritation potential of antimicrobial products with cleaning claims. In this study, three assays will be evaluated: the BCOP, the EpiOcular™ model of the corneal epithelium, and the cytosensor microphysiometer assay, which involves measurement of the metabolic rate of treated cells in vitro[8].

[7] http://iccvam.niehs.nih.gov/methods/ocutox/PeerPanel09.htm

[8] http://www.epa.gov/oppad001/eye-irritation.pdf

8.4. Opportunities for waiving eye irritation testing

Unless the physical nature of a material precludes contact with the eyes, it will not be possible to completely eliminate the risk of accidental exposure, even when stringent control measures are put into place. Waiving of testing based on lack of potential exposure is therefore rarely achievable. Taking into consideration all current accepted regulatory test guidelines for assessment of eye irritation potential, together with the GHS/OECD and REACH testing strategies, there are a number of situations in which assessment of eye irritation potential in rabbits can be waived:

- Chemicals or mixtures for which reliable animal or human eye irritancy data already exist.
- The substance is a gas or vapour.
- Chemicals or mixtures that will degrade rapidly in contact with the air, including pyrophoric substances.
- Chemicals or mixtures classified as corrosive or severely irritating to skin, as it is accepted that these are likely to produce similar effects in the eyes.
- Chemicals or mixtures with extremes of pH, i.e. ≤2 and ≥11.5, which can be regarded as having potential to produce serious eye damage, especially when associated with significant buffering capacity.
- Chemicals possessing physicochemical properties that would be expected to induce irritation in the eyes, for example organic peroxides.
- Chemicals or mixtures with a close structural or compositional relationship to those of known eye irritancy potential.
- Existence of data from validated in vitro or ex vivo tests that allow classification of eye irritancy potential or absence of eye irritancy potential. It is feasible that this could also be extended to validated tests for eye irritancy potential that use invertebrates.
- Where a weight-of-the evidence assessment indicates that the material has the potential to cause severe eye irritancy.

In addition, calculation methods and bridging principles such as those set out by the EU DPD and GHS can be applied to avoid the need for assessing the eye irritation potential of chemical mixtures, as can the US EPA guidance on acute toxicity data requirements for granular pesticide products.

Although not included in test guidelines, a positive result in a non-validated test may sometimes be considered by a regulatory agency to be acceptable for classification of the material as an eye irritant. This is demonstrated by the willingness of EU authorities to accept a positive result in the IRE or HET-CAM tests as evidence of severe irritation potential despite their not having validation status at present.

9. Skin sensitisation

Whilst chemical contact with the skin has the potential to cause systemic toxicity, corrosion, or irritation, dermal exposures can also result in the induction of an allergic response in susceptible individuals, i.e. skin sensitisation. Evaluation of sensitisation potential is a requirement by most regulatory agencies around the world. Sensitisation assays support classification and labelling and indicate whether any specific handling procedures and protective clothing are required, including for consumers who are aware they are susceptible to particular skin sensitizers.

Traditionally, studies involving guinea pigs, such as the Buehler and Magnusson and Kligman (M&K) maximization methods (OECD, 1992), have been used for identification of chemical sensitisation potential. Over the past decade, the murine local lymph node assay (LLNA) (OECD, 2002c) has received considerable support as an alternative methodology, providing some technical advantages and animal welfare benefits relative to the guinea pig tests. However, while adoption of the LLNA as the method of choice can have a positive impact on animal welfare, increased use of the LLNA in some regions but slower adoption in others has recently increased the likelihood that redundant tests will be performed for the same chemical or formulated product to meet different regulatory needs.

9.1. Local lymph node assay

Table 9 outlines the test requirements set out in some of the major regulatory schemes in the EU, USA, and Japan. Although current test guidelines for skin sensitisation indicate that the adjuvant guinea pig maximization test (M&K) and non-adjuvant Buehler methods are acceptable, the LLNA has recently gained preference by regulatory agencies, particularly in the EU. Indeed, under REACH, the LLNA is specified as the method of choice, and the guinea pig test should only be used in cases where there is adequate scientific justification.

The LLNA differs from the traditional guinea pig assays in that it measures the induction phase of sensitisation, whereas the guinea pig tests assess elicitation as well as induction. All three assays are considered to provide reliable assessments of sensitisation potential, although each may have limitations as they relate to particular test substances (e.g. metals, irritants, dyes). The LLNA provides technical advantages as it relates to a quantifiable endpoint and its dose-response design allows for an assessment of relative potency, and LLNA dose metrics have demonstrated positive correlation with human patch test experience (Basketter et al., 2005). The guinea pig models offer the advantage of demonstrating specific elicitation responses.

The LLNA also provides clear animal welfare benefits relative to the Buehler or M&K methods. It minimises the duration and extent of pain and suffering in comparison to the guinea pig tests, particularly the M&K, which involves intra-dermal adjuvant injection. The LLNA does not require elicitation of a dermal skin reaction, fur removal, or application of occlusive dressing. The LLNA can reduce animal use by up to one half compared to guinea pig studies, and further reductions in animal use can be achieved by adoption of modified LLNA methodologies. More recently, a 'reduced' LLNA (rLLNA) protocol, which evaluates a single, high dose using as little as eight mice, has been developed (ECVAM, 2007a). The rLLNA is able to distinguish between skin sensitizers and non-sensitizers, but does not provide information on dose response. At the time of drafting this paper, ICCVAM has made recommendations to US Federal Agencies that the rLLNA be routinely considered before conducting the traditional multiple dose LLNA, and be used where appropriate[9].

Animal numbers can also be reduced in guinea pig tests. OECD TG 406 and OPPTS 870.260 indicate that 10 test and 5 control animals can be used in the M&K test if the substance is evaluated using a tiered approach and is determined to be a sensitiser. OECD TG 406 also presents this option when using the Buehler assay. Both guidelines recommend additional testing with another 10 test and 5 control animals if the initial results appear to be negative for sensitisation potential. From an animal welfare perspective, the M&K is the least desirable of the available choices as it causes the greatest potential for pain and suffering. While no test is perfect and each has benefits and drawbacks, the LLNA appears as a better option relative to balancing animal welfare considerations with the need for a scientifically sound, sensitive, and reliable test.

One drawback of the traditional LLNA is that it requires the use of radioactive materials. Alternative non-radioactive versions of the test have been developed, however, and a recent NICEATM-ICCVAM independent scientific peer-review panel assessed the performance of three such assays: the LLNA: DA (Daicel Adenosine Triphosphate), LLNA: BrdU-FC (Bromodeoxyuridine Detected by Flow Cytometry), and the LLNA: BrdU-ELISA (BrdU Detected by Enzyme-Linked Immunosorbent Assay) (ICCVAM, 2009b). The panel concluded that the available test data were sufficient to support the use of the LLNA: DA and the LLNA: BrdU-ELISA with certain limitations, while for the LLNA-BrdU-FC, the panel deferred a formal recommendation pending additional inter-laboratory validation. While ICCVAM has yet to make final recommendations to US Federal agencies on the use of these methods, the OECD has published draft test guidance for the LLNA: DA and the LLNA: BrdU-FC protocols.

9.2. Sources of redundancy in skin sensitisation testing

Currently, a lack of international harmonisation in preferences for and acceptance of sensitisation tests is a major contributor to redundant testing. Testing and animal use should be reduced with the development of global product formulations intended for registration in multiple regions. This strategy depends on global harmonization and falls short if testing requirements differ significantly from region to region. The LLNA is currently accepted in most regions

[9] http://iccvam.niehs.nih.gov/methods/immunotox/rLLNA.htm

Table 9. Summary of major EU, US, and Japanese regulations and associated test guidelines for skin sensitisation.

Region	Chemical type	Legislation	Test guidelines	Opportunities for waiving, reduction or refinement	Number of animals	Test details
EU	General chemicals	REACH	Commission regulation (EC) 440/2008: method B.6 + B.42	Buehler and GPMT assays under method B.6; LLNA under B.42; LLNA preferred method under REACH—scientific justification for use of guinea pig test must be provided. ECVAM Scientific Advisory Committee (ESAC) supports the use of the reduced LLNA (rLLNA) within tiered testing strategies to reliably distinguish between sensitisers and non-sensitisers (with certain conditions).	LLNA—minimum 4 per dose group [20 with 3 doses + positive and negative controls; 16 if no positive group included] Buehler—at least 30 (20 test and 10 control) GPMT—15 or at least 30	LLNA—study duration 6 d Buehler—study duration at least 29 d GPMT—study duration at least 23 d If not possible to conclude test substance is a sensitizer using less than 20 test and 10 control animals in the GPMT, testing of additional animals to give a total of at least 20 test and 10 control animals is strongly recommended. If necessary to clarify Buehler/GPMT results from first challenge, a second challenge should be considered 1 wk after first one. LLNA—option to prepare single-cell suspension of lymph node cells from pooled treatment groups ($n=4$) or for individual animals ($n=5$). Option for labs with available historical positive control data showing consistency of a satisfactory response to not include positive controls in each assay but at intervals no greater than 6 months. rLLNA uses only a negative-control group and equivalent of the high-dose group of a full LLNA. Buehler and GPMT—positive controls not routinely tested; labs should assess the performance of the technique used every 6 months using positive-control substances.
EU	Plant protection products	91/414/EEC	As above	As above—LLNA also preferred method in draft revisions to 91/414/EEC	As above	As above
EU	Biocides	98/8/EC	As above	As above, but no test method preference stated	As above	As above
USA	Household products (CPSC); Industrial chemicals (OSHA; for worker safety)	Federal Hazardous Substances Act (FHSA), 2004	CPSC code of federal regulations	No guideline under these regulations		
USA	Pesticides	Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)	EPA: OPPTS 870.2600 (March 2003)	LLNA, Buehler, and GPMT assays all included in test guideline; LLNA preferred alternative to guinea pig tests where applicable—may not be for certain metallic compounds, high MW proteins, strong dermal irritants + materials that don't adhere sufficiently to ear for an acceptable time period LLNA not accepted for testing of formulations	LLNA—minimum 5 per dose group [25 with 3 doses + positive and negative controls] Buehler—at least 30 (20 test and 10 control) GPMT—15 or at least 30	LLNA—study duration 6 d Buehler—study duration at least 29 d GPMT—study duration at least 22 d If not possible to conclude test substance is a sensitizer using less than 20 test and 10 control animals in the GPMT, testing of additional animals to give a total of at least 20 test and 10 control animals is strongly recommended. If Buehler/GPMT results from first challenge are equivocal, a second challenge may be conducted 1 wk after first one. LLNA—concurrent positive controls to be included in each test. Lymph nodes processed separately for individual animals (5 animals per dose group). Buehler and GPMT—positive controls not routinely tested; labs should assess the performance of the technique used every 6 months using positive control substances.

Table 9. Continued on next page.

Table 9. Continued.

Region	Chemical type	Legislation	Test guidelines	Opportunities for waiving, reduction or refinement	Number of animals	Test details
USA	General chemicals	Toxic Substances Control Act (TSCA)	As above	As above	As above	As above
Japan	Agricultural chemicals	Agricultural Chemicals Regulations Law	Guideline 2-1-6	GPMT and Buehler tests are set out in guideline. However, other test methods may be substituted if information on sensitisation can be obtained.	Buehler—30 (20 test and 10 control) GPMT—15 or at least 30	Buehler—study duration at least 35 d GPMT—study duration at least 22 d If not possible to conclude test substance is a sensitizer using less than 20 test and 10 control animals in the GPMT, desirable to conduct additional studies with at least 20 animals per test substance group and at least 10 controls. If necessary to further confirm results from first challenge, a second challenge may be conducted 1 wk after first one. Recent background data may be used in place of positive controls if available.

of the world, but considerable challenges remain to achieve global acceptance (Table 9).

The OECD adopted the mouse LLNA as a stand-alone protocol for testing skin sensitisation potential in April 2002. In 2003, the US EPA published an updated version of its skin sensitisation test guideline (OPPTS 870.2600) to indicate that the local lymph node assay was included as a recommended method to assess sensitisation hazard. Recognition of the LLNA as an acceptable sensitisation test followed extensive validation efforts by ICCVAM (NIEHS, 1999). More recently, however, the validity of the LLNA for the testing of mixtures and aqueous substances has been challenged and at the time of the writing of this paper, the EPA was no longer accepting the LLNA for testing of pesticide formulations unless the assay were to yield a positive result (personal communication, US EPA). Conversely, EU registrations are required to use the LLNA. Further validation of the applicability of the LLNA for mixtures (i.e. pesticide formulations) has been requested of ICCVAM.

A recent inter-laboratory evaluation of the LLNA for use with aqueous pesticide formulations demonstrated good predictability of hazard potential when compared with existing guinea pig data and experience with human exposure (Boverhof et al., 2008). However, to gain endorsement for this application, a broader data set appears necessary. Understandably, redundant LLNA and guinea pig data for the same formulation are not generally available. Given this, an approach for validation of mixtures may require a less traditional and more creative approach to analyzing sensitization data, which does not require new in vivo testing. Investigators have recently presented a retrospective assessment of LLNA ($N=52$), M&K ($N=32$), and Buehler ($N=31$) results for aqueous pesticide formulations tested since 2000 (Gehen et al., 2009).

The study found that positive results were obtained in 63.5%, 58.1%, and 19.4% of LLNA, M&K, and Buehler tests, respectively. Further consideration for the sensitization potential of the active ingredients indicated 78% of the formulations that contained R43-classified active ingredients tested positive using the LLNA. With the M&K, 82% of formulations with positive active ingredients tested positive, while the response rate was only 42% with the Buehler test. This new analysis clearly suggests that the LLNA is capable of predicting sensitisation potential of a 'mixture' and appears similar to the M&K and superior to the Buehler test in sensitivity for assessing hazard potential of pesticide formulations. Based on these results, as well as general acceptance of the LLNA as a sensitive test for sensitization potential, the LLNA should be accepted globally for sensitisation testing of substances, mixtures, and formulated products (Boverhof et al., 2008). A recent NICEATM-ICCVAM independent scientific peer-review panel has also concluded that the LLNA should be considered appropriate for testing pesticide formulations and other products (e.g. natural complex substances, dyes, and aqueous solutions), unless there is a biologically based rationale for exclusion (ICCVAM, 2009b). The panel's recommendations will be reviewed by ICCVAM before it makes final recommendations to US Federal agencies[10]. Failure to achieve harmonisation in the near future will result in unnecessary and redundant animal use. Since harmonisation is currently lacking, flexibility is needed in accepting guinea pig studies in the near future to avoid duplicate testing.

9.3. Opportunities to waive sensitisation testing

In addition to global harmonisation, another strategy to reduce the number of animals utilised in sensitisation testing is through the responsible application of testing waivers. In vivo testing of new substances and products for skin sensitisation potential will likely continue to be required under most circumstances globally; however, there may be opportunities to waive studies when sufficient information exists to predict the result if a study were to be performed. For example, European Commission Directive 94/79/EC indicates that skin sensitisation testing for agricultural

[10] http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm

chemicals "…must always be carried out except where the substance is a known sensitizer." Other global regulatory agencies are likely to take a similar pragmatic approach to waivers for known sensitizers. Under REACH, in vivo testing for skin sensitisation should not be conducted until an assessment of the available human, animal, and alternative data has been performed, and does not need to be conducted if the available information indicates that the substance should be classified for skin sensitisation or corrosivity, the substance is a strong acid (pH < 2.0) or base (pH > 11.5), or is flammable in air at room temperature. Where absence of bioavailability via the skin can be demonstrated, there is a strong case for waiving testing and for regarding the substance as non-sensitising.

While most agencies will accept waivers for known sensitizers, substances not thought to be sensitizers, or those with little or no existing sensitisation data, will need to be tested in most cases. There are opportunities to reverse such practices, however. In situations when negative data exist for all components of a formulation, a waiver could be acceptable. Waivers should also be considered for formulations that represent dilutions or minimal modifications of an existing formulation with negative sensitisation results. Individual sensitisation data for the components will be helpful in such a case, although such information is often not available for 'inert' co-formulants.

For formulations, in vivo testing for skin sensitisation can also be avoided by applying information on the individual ingredients, using bridging principles or calculation methods such as those set out by the EU DPD and GHS, as well as the US EPA's guidance on acute toxicity data requirements for granular pesticide products.

The key principle to avoiding unnecessary or redundant testing is to question the value and benefits from new testing based on existing information. If the result of the test can be reasonably predicted then a waiver should be provided. This principle is particularly true if a positive result is predicted. These decisions will need to be made on a case-by-case basis within the constraints of global regulatory frameworks and involve expert technical and regulatory opinions.

9.4. Advances in alternative approaches to sensitisation testing

Continued scientific advancement promises to further reduce or even eliminate animal use for sensitisation testing. Efforts to build sensitisation databases and improve the predictive value of computer modelling (QSAR) for skin sensitisation will be valuable in reducing animal use in the future. The development of a robust in vitro method has been difficult, in part due to the complex and specific nature of the sensitisation response. For the most part, investigators have tried to exploit the specific contributions of various cell types in the hope that they can identify chemical allergens. In vitro methods utilizing dendritic cells have shown promise in correctly identifying skin-sensitising chemicals (Ayehunie et al., 2009; Gildea et al., 2006; Hooyberghs et al., 2008; Kimber et al., 2004; Python et al., 2009; Ryan et al., 2007). Based upon the premise of protein reactivity as a necessary trait for chemical sensitisers, measurement of peptide reactivity as a means of identifying skin sensitisers is also the subject of much research (Aleksic et al., 2009; Gerberick et al., 2009; Mutschler et al., 2009; Natsch and Emter, 2008). At the time of writing this paper, ECVAM is tendering laboratories to participate in pre-validation studies to evaluate three in vitro test methods designed for the assessment of skin sensitisation potential: (1) a direct peptide reactivity assay, (2) a myeloid U937 skin sensitisation test (MUSST), and (3) a human cell line activation test (h-CLAT)[11].

With further development, in silico methods in combination with in vitro testing could be used as either a complete replacement or as part of a tiered approach to evaluate sensitisation potential. Depending on the results of this lower tiered testing or if predetermined exposure triggers are met, then an in vivo model (e.g. the LLNA) would be performed. Several groups are currently engaged in efforts to develop and validate these technologies (Hooyberghs et al., 2008; Maxwell et al., 2008; Natsch et al., 2009), including an EU project, Sens-it-iv (www.sens-it.iv.eu; Rovida et al., 2007).

10. Discussion and conclusions

Acute toxicity testing in vivo has been criticised on both scientific and animal welfare grounds. Information on potential acute toxic effects and associated symptoms in humans following chemical exposure is of course important, but it has been argued that the information obtained in the acute toxicity tests currently used, while providing the information needed to meet classification and labelling regulations, is of limited value as predictivity of effects in humans has not been adequately demonstrated (Balls, 1991; Basketter et al., 1997; Langley, 2005; York et al., 1996; Zbinden and Flury-Roversi, 1981). In addition to these important concerns, the time and cost demands of animal testing needed for assessment of the large number of chemicals requiring evaluation under international regulatory frameworks—particularly the need for assessment of around 30,000 existing chemicals under REACH—add to the drivers for development of alternative approaches that replace, reduce or refine the use of animals (Schaafsma et al., 2009; Ukelis et al., 2008).

Whilst methods accepted internationally as full replacements for in vivo testing for acute systemic toxicity testing and assessment of skin and eye irritation and skin sensitisation are not yet available, the disparate approaches taken across various regulatory frameworks around the world mean that there are a number of sources of redundant testing, and also opportunities for waiving of some tests under certain circumstances. In this paper we have sought to highlight where these opportunities exist, and where alternative approaches that can reduce and refine animal

[11] http://web.jrc.ec.europa.eu/callsfortender/index.cfm?action=app.tender&id=453&institute=6

use, and should therefore be considered best practice, are available.

Widespread validation and subsequent adoption of alternative test methods that confer scientific and animal welfare benefits can take an extremely long time, as with adoption of the revised test guidelines for acute oral toxicity, the LLNA, and most recently the acceptance of in vitro methods for skin irritation in the EU but not the rest of the world. Where tests to replace in vivo methods are under development, it is important to consider whether validation of these assays against in vivo approaches that have not been demonstrated to have predictivity for effects in humans is the most appropriate way of assessing their utility. Validation of a test method is of course important, but the primary goal should not be to ensure it predicts effects in test animals, but to ensure it provides adequate protection for human health. A more flexible approach to validation than a like-for-like comparison between in vivo and alternative methods is required. Indeed, there has been increasing recognition that the focus needs to change from direct replacement of an animal test to the identification of the information needed to make a decision on safety in humans, and then determining how this information can be obtained without in vivo testing (Fentem et al., 2004).

In addition to the development of replacement and reduction test methods, waiving of test requirements can also make a significant contribution to reducing animal use. General options for waiving are set out under REACH and other international regulations and the main points, which should be considered as best practice in determining when in vivo testing is required, are summarised in Table 10. Greater consideration of available data and development of intelligent testing strategies, including the use of read-across, as advocated under REACH, can support a reduction in animal testing and should be encouraged.

In many cases there is no need to test mixtures of chemicals such as pesticide formulations, as their acute toxicity can be predicted using information on the individual components. Bridging principles and calculation methods for this purpose are set out in the WHO guidelines on Classification of Pesticides by Hazard, the EU DPD and GHS, as well as the US EPA guidance on acute toxicity data requirements for granular pesticide products. These methods provide a significant opportunity to avoid in vivo testing while maintaining human and environmental health protection, and are routinely applied within the EU. The ongoing implementation of the GHS should provide an opportunity for greater use of bridging approaches worldwide, which would help avoid a substantial amount of redundant animal testing.

Whilst companies are encouraged to make the case for waiving of testing under REACH, notifiers do run the risk that justification for waiving will not be accepted by the competent authority, which could lead to a delay in assessment while testing proposals are put forward, testing is performed, and subsequent approval is completed. Increased dialogue between stakeholders to discuss acceptance of non-standard approaches is therefore desirable.

As this paper highlights, the regulatory landscape and acceptance of alternative test methods and approaches across industry sectors and geographical regions is very complex. There is a need for greater cross-sector, international dialogue to help promote a faster and more streamlined process for amending or adding new guidelines and widespread acceptance and adoption of these approaches.

Table 10. Best practice options for waiving in vivo acute toxicity testing.

General cases where acute toxicity testing should be waived
Substance likely to be corrosive based on pH, physicochemical properties, or result of validated in vitro assay.
WOE analysis demonstrates that the available information is sufficient for a hazard characterisation and exposure to substance is adequately controlled.
Substance is not bioavailable via a specific route and possible local effects are adequately characterised.
Data on related substances are available allowing read-across.
Bridging principles and calculation methods can be applied to classify mixtures of chemicals based on data available for the ingredients.
Specific cases for waiving acute inhalation toxicity testing
Particle size > 100 µm
Vapour pressure very low (<0.1 Pa at 20°C)
Not technically possible to generate a testing atmosphere

10.1. Acute oral toxicity testing

- Of the three in vivo tests currently accepted for acute oral toxicity, the FDP does not employ lethality as an endpoint and should be considered the preferred method, until such time that evident toxicity can be consistently applied to the ATC and UDP protocols.
- Generic and endpoint-specific options to waive requirements for acute oral toxicity testing should be employed where possible to avoid unnecessary performance of in vivo studies.

10.2. Acute dermal toxicity testing

- The data presented in this paper demonstrate that acute dermal toxicity testing very rarely provides information of value for hazard identification or assessment purposes when an acute oral study has been conducted.
- These findings suggest that acute dermal toxicity studies should not be performed except in exceptional circumstances, for example for chemicals where information on toxicokinetics or mechanism of action suggests that acute toxicity might be greater by the dermal rather than oral route.
- Where dermal testing is needed, limit tests should only be conducted in a group totalling five animals, in line with the newer acute oral guidelines. This could potentially halve the numbers of animals used in acute dermal toxicity tests.

10.3. Acute inhalation toxicity testing

- Of the available in vivo tests for acute inhalation toxicity, the acute toxic class method (OECD TG 436) requires the use of fewer animals than TG 403 and should be used apart from in those cases where it is unable to meet scientific or regulatory needs.
- The Fixed Concentration Procedure (draft TG 433) will not employ lethality as an endpoint and if adopted as a test guideline should be considered the preferred method.
- Generic and endpoint-specific options to waive requirements for acute inhalation toxicity testing should be employed to avoid unnecessary testing where possible.

10.4. Skin irritation testing

- In vitro models to replace the use of animals for skin irritation testing are now available and have been validated and accepted within the EU for distinguishing between irritant and non-irritant substances. Wider adoption by the OECD may occur in the near future. These tests should be used wherever they are accepted and meet regulatory needs.
- In regions where the in vitro models are not yet accepted, all available data should be evaluated before making any decision to perform an animal study. Best practice options, such as the initial use of just one animal and restriction of testing to the minimum number of animals necessary to achieve the objectives of the study, should be implemented when in vivo studies are conducted.

10.5. Eye irritation testing

- Tiered testing approaches, including use of the in vitro tests recently adopted by the OECD, should be implemented to reduce in vivo eye irritation testing to a minimum.
- When in vivo studies are required, best practice options such as the initial use of just one animal and restriction of testing to the minimum number of animals necessary to achieve the objectives of the study should be implemented.
- Use of local anaesthetics, systemic analgesics, and humane endpoints should also be considered to minimise pain and distress.

10.6. Skin sensitisation testing

- The analysis presented in this paper indicates that the LLNA performs at least as well as the guinea pig assays for predicting the sensitisation potential of formulations.
- The LLNA should therefore be accepted globally for sensitisation testing of substances, mixtures, and formulated products, apart from in cases where there is a scientific basis for exclusion.
- Available data should be evaluated before any decision to undertake in vivo testing is made, for example following the integrated testing strategy set out in the guidance accompanying REACH (ECHA, 2008b).

Declaration of interest

The manuscript of this paper was prepared by the authors during their normal course of employment as shown on the first page. The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) is an independent organisation established by the UK Government to accelerate the development and application of the 3Rs. The NC3Rs is funded by the UK Government, the Wellcome Trust, and the pharmaceutical and chemical industries, and its activities include a programme of work to advance the 3Rs in the regulatory use of animals by the chemical industry. The Health and Safety Executive is a non-departmental body funded by the UK Government. The Chemicals Regulation Directorate (CRD) of HSE is responsible for the regulation of biocides, plant protection products, detergents, and chemicals, providing UK competent authority functions within the EU legislation relating to these substances. CRD claims some fees from industry under UK Government full cost recovery rules. Huntingdon Life Sciences (HLS) is a contract research organisation providing in vivo and in vitro research services to the chemical, pharmaceutical, crop protection, food additive, and veterinary industries. Harlan Laboratories Ltd. provides pre-clinical and non-clinical contract research, in vitro and in vivo research models, animal diets, and services to the pharmaceutical, biotechnology, medical device, agrochemical, and chemical industries, as well as universities, government, and other research organisations. Dow Agrosciences and the Dow Chemical Company develop, manufacture, and market a broad range of chemicals, including chemicals that are routinely evaluated in a number of in vitro and in vivo toxicity assays to ensure they are safe when used according to the manufacturers' recommendations. The evaluations reported in this paper are expected to impact on the testing requirements adopted by regulatory agencies and, in turn, the testing programs of firms such as Dow that develop, manufacture, and market chemicals, and contract research organisations such as Harlan and HLS that undertake testing on behalf of industry. The authors alone are responsible for the content and writing of the paper.

References

Adriaens E, Bytheway H, De Wever B, Eschrich D, Guest R, Hansen E, Vanparys P, Schoeters G, Warren N, Weltens R, Whittingham A, Remon JP. (2008). Successful prevalidation of the slug mucosal irritation test to assess the eye irritation potency of chemicals. Toxicol In Vitro 22:1285–1296.

Aleksic M, Thain E, Roger D, Saib O, Davies M, Li J, Aptula A, Zazzeroni R. (2009). Reactivity profiling: Covalent modification of single nucleophile peptides for skin sensitization risk assessment. Toxicol Sci 108: 401–411.

Anon. (2002). Chemicals (Hazard Information and Packaging for Supply) Regulations 2002. Vol. No. 1689.

Aufderheide M, Mohr U. (1999). CULTEX—A new system and technique for the cultivation and exposure of cells at the air/liquid interface. Exp Toxicol Pathol 51: 489–90.

Aufderheide M, Mohr U. (2000). CULTEX—An alternative technique for cultivation and exposure of cells of the respiratory tract to airborne pollutants at the air/liquid interface. Exp Toxicol Pathol 52: 265–70.

Ayehunie S, Snell M, Child M, Klausner M. (2009). A plasmacytoid dendritic cell (CD123+/CD11c–) based assay system to predict contact allergenicity of chemicals. Toxicology 264: 1–9.

Bakand S, Winder C, Khalil C, Hayes A. (2006a). An experimental in vitro model for dynamic direct exposure of human cells to airborne contaminants. Toxicol Lett 165: 1–10.

Bakand S, Winder C, Khalil C, Hayes A. (2006b). A novel in vitro exposure technique for toxicity testing of selected volatile organic compounds. J Environ Monit 8: 100–5.

Balls M. (1991). Why modification of the LD_{50} test will not be enough. Lab Anim 25: 198–206.

Basketter DA, Clapp C, Jefferies D, Safford B, Ryan CA, Gerberick F, Dearman RJ, Kimber I. (2005). Predictive identification of human skin sensitization thresholds. Contact Dermatitis 53: 260–267.

Basketter DA, Reynolds FS, York M. (1997). Predictive testing in contact dermatitis. Irritant dermatitis. Clin Dermatol 15: 637–644.

Boverhof DR, Wiescinski CM, Botham P, Lees D, Debruyne E, Repetto-Larsay M, Ladics G, Hoban D, Gamer A, Remmele M, Wang-Fan W, Ullmann LG, Mehta J, Billington R, Woolhiser MR. (2008). Interlaboratory validation of 1% pluronic l92 surfactant as a suitable, aqueous vehicle for testing pesticide formulations using the murine local lymph node assay. Toxicol Sci 105: 79–85.

BTS (1984). A new approach to the classification of substances and preparations on the basis of their acute toxicity. A report by the British Toxicology Society working party on toxicity. Hum Toxicol 3: 85–92.

Bulgheroni A, Kinsner-Ovaskainen A, Hoffmann S, Hartung T, Prieto P. (2009). Estimation of acute oral toxicity using the No Observed Adverse Effect Level (NOAEL) from the 28 day repeated dose toxicity studies in rats. Regul Toxicol Pharmacol 53: 16–19.

CFR. (1998). Code of Federal Regulations, Title 40, Part 158, Section 340: Toxicology Data Requirements (Inhalation). Washington, DC: Office of the Rederal Register, National Archives and Records Administration.

Chapman K, Robinson S. (2007). Challenging the regulatory requirement for acute toxicity studies in the development of new medicines: A workshop report. NC3Rs, 2007.

Cinalli C, Carter C, Clark A, Dixon D. (1992). A laboratory method to determine the retention of liquids on the surface of hands. Washington, DC: US Environmental Protection Agency, Exposure Evaluation Division, Office of Pollution, Prevention and Toxics.

Clemedson C. (2008). The European ACuteTox project: A modern integrative in vitro approach to better prediction of acute toxicity. Clin Pharmacol Ther 84: 200–202.

Clemedson C, Kolman A, Forsby A. (2007). The integrated acute systemic toxicity project (ACuteTox) for the optimisation and validation of alternative in vitro tests. Altern Lab Anim 35: 33–8.

Cormier EM, Parker RD, Henson C, Cruse LW, Merritt AK, Bruce RD, Osborne R. (1996). Determination of the intra- and interlaboratory reproducibility of the low volume eye test and its statistical relationship to the Draize eye test. Regul Toxicol Pharmacol 23: 156–161.

Costa DL. (2008). Alternative test methods in inhalation toxicology: Challenges and opportunities. Exp Toxicol Pathol 60: 105–109.

Draize JH, Woodard G, Calvery HO. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Therapeutics 82: 377–390.

EC. (1999). Directive 1999/45/EC of the European Parliament and of the Council of May 1999. Concerning the approximation of the laws, regulations and administrative provisions of the Member States relating to the classifications, packaging and labelling of dangerous preparations.

EC (2003). Directive 2003/15/EC of the European Parliament and of the Council of 27 Feburary 2003 amending Directive 78/786/EEC on the approximation of the laws of the member states relating to cosmetic products. Off J Eur Union L 66: pp 26–35.

EC (2007). Corrigendum to Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending DirECtive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council DirECtive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC (OJ L 396, 30.12.2006). Off J Eur Union L 136, 50: pp 3–280.

EC. (2008a). Council Regulation (EC) No. 440/2008, laying down test methods pursuant to to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).

EC. (2008b). Council Regulation (EC. No 1272/2008 of the European Parliament and of the Council on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC. and amending Regulation (EC) No 1907/2006).

EC. (2009). Commission Regulation (EC) No 761/2009 of 23 July 2009 amending, for the purpose of its adaptation to technical progress, Regulation (EC) No 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).

ECHA. (2008). Guidance on Information Requirements and Chemical Safety Assessment. Chapter R6: QSARs and grouping of chemicals. Helsinki, Finland: European Chemicals Agency.

ECHA. (2008b). Guidance on Information Requirements and Chemical Safety Assessment. Chapter R7a: Endpoint specific guidance. Helsinki, Finland: European Chemicals Agency.

ECVAM. (1998). ESAC Statement, April 3, 1998: Scientific Validity of the EpiSkin Test (an In Vitro test for Skin Corrosivity).

ECVAM. (2000). ESAC Statement, March 21, 2000: Statement on the Application of the EpiDerm Human Skin Model for Skin Corrosivity Testing.

ECVAM. (2006). ESAC Statement, November 17, 2006: Statement on the Application of the SkinEthic Human Skin Model for Skin Corrosivity Testing.

ECVAM. (2007a). ESAC Statement, April 27, 2007: Reduced Local Lymph Node Assay (rLLNA) for Skin Sensitisation.

ECVAM. (2007b). ESAC Statement, April 27, 2007: Statement on the conclusions of the ICCVAM retrospective study on Organotypic in vitro assays as screening tests to identify potential ocular corrosives and sever irritants as determined by US EPA, EU (R41) and UN GHS classifications in a tiered testing strategy, as part of a weight of evidence approach.

ECVAM. (2007c). ESAC Statement, April 27, 2007: Statement on the Validity of In Vitro Tests for Skin Irritation.

ECVAM. (2008). ESAC Statement, November 5, 2008: Statement on the Scientific Validity of In Vitro Tests for Skin Irritation Testing.

ECVAM. (2009a). ESAC Statement, April 9, 2009: Statement on the Performance under UN GHS of Three In Vitro Tests for Skin Irritation Testing and the Adaptation of the Reference Chemicals and Defined Accuracy Values of the ECVAM Skin Irritation Performance Standards.

ECVAM. (2009b). ESAC Statement, June 12, 2009: Statement on the Scientific Validity of an In-Vitro Test Method for Skin Corrosivity Testing.

EEC. (1986). Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes.

EPA. (2001). Pesticide Registration Notice 2001-2. Acute toxicity data requirements for granular pesticide products, including those with granular fertilizers in the product. Washington, DC: US EPA. Office of Prevention, Pesticides and Toxic Substances.

EPA. (2002). Health Effects Test Guidelines. OPPTS 870.1000: Acute Toxicity Testing—Background.

EPA. (2004). Status and future directions of the high production volume challenge program. Washington, DC: US EPA, Office of Pollution Prevention and Toxics.

Fentem J, Chamberlain M, Sangster B. (2004). The feasibility of replacing animal testing for assessing consumer safety: A suggested future direction. Altern Lab Anim 32: 617–623.

Gehen SC, Wiescinski CM, Woolhiser MR, Billington R. (2009). Acceptability of the mouse local lymph node assay for pesticide formulation hazard assessment. Toxicologist 108: pp 316–317.

Gerberick GF, Troutman JA, Foertsch LM, Vassallo JD, Quijano M, Dobson RL, Goebel C, Lepoittevin JP. (2009). Investigation of peptide reactivity of prohapten skin sensitizers using a peroxidase-peroxide oxidation system. Toxicol Sci 112: pp 164–174.

Gerde P. (2008). How do we compare dose to cells in vitro with dose to live animals and humans? Some experiences with inhaled substances. Exp Toxicol Pathol 60: 181–184.

Gettings SD, Lordo RA, Demetrulias J, Feder PI, Hintze KL. (1996). Comparison of low-volume, Draize and in vitro eye irritation test data. I. Hydroalcoholic formulations. Food Chem Toxicol 34: 737–749.

Gettings SD, Lordo RA, Feder PI, Hintze KL. (1998a). A comparison of low volume, draize and in vitro eye irritation test data. II. Oil/water emulsions. Food Chem Toxicol 36: 47–59.

Gettings SD, Lordo RA, Feder PI, Hintze KL. (1998b). A comparison of low volume, Draize and in vitro eye irritation test data. III. Surfactant-based formulations. Food Chem Toxicol 36: 209–231.

GHS. (2007). Globally Harmonized System of Classification and Labelling of Chemicals. Second revised edition. Geneva: United Nations.

Gildea LA, Ryan CA, Foertsch LM, Kennedy JM, Dearman RJ, Kimber I, Gerberick GF. (2006). Identification of gene expression changes induced by chemical allergens in dendritic cells: Opportunities for skin sensitization testing. J Invest Dermatol 126: 1813–1822.

Griffith JF, Nixon GA, Bruce RD, Reer PJ, Bannan EA. (1980). Dose-response studies with chemical irritants in the albino rabbit eye as a basis for selecting optimum testing conditions for predicting hazard to the human eye. Toxicol Appl Pharmacol 55: 501–513.

Hanway RH, Evans PF. (2000). Read across of toxicological data in the notification of new chemicals. Toxicol Lett 116: 61.

Hartung T, Rovida C. (2009). Chemical regulators have overreached. Nature 460: 1080–1081.

Hood E. (2008). Alternative test models: Ocular safety assays accepted. Environ Health Perspect 116: A381.

Hooyberghs J, Schoeters E, Lambrechts N, Nelissen, I, Witters H, Schoeters G, Van Den Heuvel R. (2008). A cell-based in vitro alternative to identify skin sensitizers by gene expression. Toxicol Appl Pharmacol 231: 103–111.

ICCVAM. (2001). Report of the International Workshop on In Vitro Methods for Assessing Acute Systemic Toxicity. Bethesda, MD: NIH.

ICCVAM. (2006a). ICCVAM Test Method Evaluation Report (TMER): In Vitro Cytotoxicity Test Methods for Estimating Starting Doses For Acute Oral Systemic Toxicity Testing. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

ICCVAM. (2006b). ICCVAM Test Method Evaluation Report (TMER): In Vitro ocular toxicity test methods for identifying severe irritants and corrosives. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

ICCVAM. (2009a). Independent Scientific Peer Review Panel Report: Evaluation of the Validation Status of Alternative Ocular Safety Testing Methods and Approaches. Research Triangle Park, NC: National Institute of Environmental Health Sciences.

ICCVAM. (2009b). Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products. Research Triangle Park. NC: National Institute of Environmental Health Sciences, Research Triangle Park.

ICH. (2009). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and marketing authorization for Pharmaceuticals M3(R2). Recommended for adoption at step 4 of the ICH process on June 11, 2009.

Indans I, Fry T, Parsons P, Evans P. (1998). Classification and labelling of new industrial chemicals for acute toxicity, skin and eye irritation. Hum Exp Toxicol 17: 529.

Kesimer M, Kirkham S, Pickles RJ, Henderson AG, Alexis NE, Demaria G, Knight D, Thornton DJ, Sheehan JK. (2008). Tracheobronchial air-liquid interface cell culture: A model for innate mucosal defense of the upper airways? Am J Physiol Lung Cell Mol Physiol 296: pp L92–L100.

Kimber I, Cumberbatch M, Betts CJ, Dearman RJ. (2004). Dendritic cells and skin sensitisation hazard assessment. Toxicol In Vitro 18: 195–202.

Kinsner-Ovaskainen A, Bulgheroni A, Hartung T, Prieto P. (2009). ECVAM's ongoing activities in the area of acute oral toxicity. Toxicol In Vitro 23: pp 1535–1540.

Lambre CR, Auftherheide M, Bolton RE, Fubini B, Haagsman HP, Hext PM, Jorissen M, Landry Y, Morin JP, Nemery B, Nettesheim P, Pauluhn J, Richards RJ, Vickers AEM, Wu R. (1996). In vitro tests for respiratory toxicity. The report and recommendations of ECVAM workshop 18. Altern Lab Anim J 24: 671–681.

Langley G. (2005). European Coalition to End Animal Experiments: Acute Toxicity Testing Without Animals. London: ECEAE.

Lee MK, Yoo JW, Lin H, Kim YS, Kim DD, Choi YM, Park SK, Lee CH, Roh HJ. (2005). Air-liquid interface culture of serially passaged human nasal epithelial cell monolayer for in vitro drug transport studies. Drug Deliv 12: 305–311.

Lin H, Li H, Cho HJ, Bian S, Roh HJ, Lee MK, Kim JS, Chung SJ, Shim CK, Kim DD. (2007). Air-liquid interface (ALI) culture of human bronchial epithelial cell monolayers as an in vitro model for airway drug transport studies. J Pharm Sci 96: 341–350.

Macfarlane M, Jones P, Goebel C, Dufour E, Rowland J, Araki D, Costabel-Farkas M, Hewitt NJ, Hibatallah J, Kirst A, McNamee P, Schellauf F, Scheel J. (2009). A tiered approach to the use of alternative approaches for the safety assessment of cosmetics: Skin irritation. Regul Toxicol Pharmacol 54: pp 188–196.

Maxwell G, Aleksic M, Aptula A, Carmichael P, Fentem J, Gilmour N, Mackay C, Pease C, Pendlington R, Reynolds F, Scott D, Warner G, Westmoreland C. (2008). Assuring consumer safety without animal testing: A feasibility case study for skin sensitisation. Altern Lab Anim 36: 557–68.

McNamee P, Hibatallah J, Costabel-Farkas M, Goebel C, Araki D, Dufour E, Hewitt NJ, Jones P, Kirst A, Varlet BL, Macfarlane M, Marrec-Fairley M, Rowland J, Schellauf F, Scheel J. (2009). A tiered approach to the use of Alternatives to Animal Testing for the safety assessment of cosmetics: Eye irritation. Regul Toxicol Pharmacol 54: pp 197–209.

Mishima S. (1981). Clinical pharmacokinetics of the eye. Opthalmol Vis Sci 21: 504–541.

Mutschler J, Gimenez-Arnau E, Foertsch L, Gerberick GF, Lepoittevin JP. (2009). Mechanistic assessment of peptide reactivity assay to predict skin allergens with Kathon CG isothiazolinones. Toxicol In Vitro 23: 439–446.

Natsch A, Emter R. (2008). Skin sensitizers induce antioxidant response element dependent genes: Application to the in vitro testing of the sensitization potential of chemicals. Toxicol Sci 102: 110–119.

Natsch A, Emter R, Ellis G. (2009). Filling the concept with data: Integrating data from different in vitro and in silico assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing. Toxicol Sci 107: 106–121.

NIEHS. (1999). National Institutes of Environmental and Health Sciences. The murine LLNA: A test for assessing the allergic contact dermatitis potential of chemicals/compounds. Fed Register 64:14006–14007.

NRC. (2001). Standing Operating Procedures for Developing Acute Exposure Guideline Levels for Hazardous Chemicals. Washington, DC: National Academy Press.

OECD. (1981a). Test Guideline 401: Acute Oral Toxicity. OECD Guidelines for Testing of Chemicals.

OECD. (1981b). Test Guideline 403: Acute Inhalation Toxicity. OECD Guidelines for the Testing of Chemicals.

OECD. (1992). Test Guideline 406: Skin Sensitisation. OECD Guidelines for the Testing of Chemicals.

OECD. (2000). Guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation. Series on Testing and Assessment.

OECD. (2001). Guidance document on acute oral toxicity testing. Series on Testing and Assessment.

OECD. (2001b). Test Guideline 420: Acute Oral Toxicity—Fixed Dose Procedure. OECD Guidelines for the Testing of Chemicals.

OECD. (2001c). Test Guideline 423: Acute Oral Toxicity—Acute Toxic Class Method. OECD Guidelines for the Testing of Chemicals.

OECD. (2002a). Test Guideline 404: Acute Dermal Irritation/Corrosion. OECD Guidelines for Testing of Chemicals.

OECD. (2002b). Test Guideline 405: Acute Eye Irritation/Corrosion. OECD Guidelines for the Testing of Chemicals.

OECD. (2002c). Test Guideline 429: Skin Sensitisation: Local Lymph Node Assay. OECD.Guidelines for the Testing of Chemicals.

OECD. (2004a). Test Guideline 428: Skin Absorption: In Vitro Method. OECD Guidelines for the Testing of Chemicals.

OECD. (2004b). *Test Guideline* 430: In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER). OECD.Guidelines for the Testing of Chemicals.

OECD. (2004c). Test Guideline 431: In Vitro Skin Corrosion: Human Skin Model Test. OECD Guidelines for the Testing of Chemicals.

OECD. (2006). Test guideline 435: In Vitro Membrane Barrier Test Method for Skin Corrosion. OECD.Guidelines for the Testing of Chemicals.

OECD. (2007). Guidance document on the validation of (quantitative) structure-activity relationship (QSAR) models. Series on Testing and Assessment.

OECD. (2008a). Guidance document on acute inhalation toxicity testing. Series on Testing and Assessment.

OECD. (2008b). Test Guideline 425: Acute Oral Toxicity: Up-and-Down Procedure. OECD Guidelines for the Testing of Chemicals.

OECD. (2009a). Guidance document on acute inhalation toxicity testing. Series on Testing and Assessment.

OECD. (2009b). Test guideline 403: Acute Inhalation Toxicity. OECD Guidelines for the Testing of Chemicals.

OECD. (2009c). Test Guideline 436: Acute Inhalation Toxicity—Acute Toxic Class (ATC) Method. OECD Guidelines for the Testing of Chemicals.

OECD. (2009d). Test Guideline 437: Bovine Corneal Opacity and Permeability (BCOP) Test Method for Identifying Ocular Corrosives and Severe Irritants. OECD Guidelines for the Testing of Chemicals.

OECD. (2009e). Test Guideline 438: Isolated Chicken Eye (ICE) Test Method for Identifying Ocular Corrosives and Severe Irritants. OECD Guidelines for the Testing of Chemicals.

Park SK, Lee WJ, Yang YI. (2007). Organ culture at the air-liquid interface maintains structural and functional integrities of inflammatory and fibrovascular cells of nasal polyps. Am J Rhinol 21: 402–407.

Pedersen F, de Bruijn J, Munn S, van Leeuwen K. (2003). Assessment of additional testing needs under REACH. Effects of (Q)SARs, risk based testing and voluntary industry initiatives. Brussels: European Commission, Joint Research centre. Report EUR 20863 EN.

Python F, Goebel C, Aeby P. (2009). Comparative DNA microarray analysis of human monocyte derived dendritic cells and MUTZ-3 cells exposed to the moderate skin sensitizer cinnamaldehyde. Toxicol Appl Pharmacol 239: 273–283.

Robinson S, Delongeas JL, Donald E, Dreher D, Festag M, Kervyn S, Lampo A, Nahas K, Nogues V, Ockert D, Quinn K, Old S, Pickersgill N, Somers K, Stark C, Stei P, Waterson L, Chapman K. (2008). A European pharmaceutical company initiative challenging the regulatory requirement for acute toxicity studies in pharmaceutical drug development. Regul Toxicol Pharmacol 50: 345–352.

Rovida C, Basketter D, Casati S, de Silva O, Hermans H, Kimber I, Manou I, Weltzien HU, Roggen E. (2007). Management of an integrated project (Sens-it-iv) to develop in vitro tests to assess sensitisation. Altern Lab Anim 35: 317–322.

Ryan CA, Kimber I, Basketter DA, Pallardy M, Gildea LA, Gerberick GF. (2007). Dendritic cells and skin sensitization: Biological roles and uses in hazard identification. Toxicol Appl Pharmacol 221: 384–394.

Schaafsma G, Kroese ED, Tielemans EL, Van de Sandt JJ, Van Leeuwen CJ. (2009). REACH, non-testing approaches and the urgent need for a change in mind set. Regul Toxicol Pharmacol 53: 70–80.

Scott L, Eskes C, Hoffmann S, Adriaens E, Alepee N, Bufo M, Clothier R, Facchini D, Faller C, Guest R, Harbell J, Hartung T, Kamp H, Varlet B. L, Meloni M, McNamee P, Osborne R, Pape W, Pfannenbecker U, Prinsen M, Seaman C, Spielmann H, Stokes W, Trouba K, Berghe CV, Goethem FV, Vassallo M, Vinardell P, Zuang V. (2009). A proposed eye irritation testing strategy to reduce and replace in vivo studies using Bottom-Up and Top-Down approaches. Toxicol In Vitro (In e-publishing: Ahead of print).

Seagrave J, Dunaway S, McDonald JD, Mauderly JL, Hayden P, Stidley C. (2007). Responses of differentiated primary human lung epithelial cells to exposure to diesel exhaust at an air-liquid interface. Exp Lung Res 33: 27–51.

Semple S. (2004). Dermal exposure to chemicals in the workplace: Just how important is skin absorption? Occup Environ Med 61: 376–82.

Stokes W, Casati S, Strickland J, Paris M. (2008). Neutral red uptake cytotoxicity tests for estimating starting doses for acute oral toxicity tests. Curr Protoc Toxicol 36: 20.4.1–20.4.20.

Thomas HD, Dewhurst IC. (2007). What does a dermal acute toxicity study add to the information on a plant protection pesticide? Toxicology 231: 114–115.

Ukelis U, Kramer PJ, Olejniczak K, Mueller SO. (2008). Replacement of in vivo acute oral toxicity studies by in vitro cytotoxicity methods: Opportunities, limits and regulatory status. Regul Toxicol Pharmacol 51: 108–118.

van den Heuvel MJ, Clark DG, Fielder RJ, Koundakjian PP, Oliver GJ, Pelling D, Tomlinson NJ, Walker, AP. (1990). The international validation of a fixed-dose procedure as an alternative to the classical LD_{50} test. Food Chem Toxicol 28: 469–482.

van der Jagt K, Munn S, Torslov J, de Bruijn J. (2004). Alternative approaches can reduce the use of test animals under REACH. Burssels: European Commission, Joint Research Centre. Report EUR 21405 EN.

Walum E. (1998). Acute oral toxicity. Environ Health Perspect 106(Suppl 2): 497–503.

WHO. (2005). The WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification: 2004. Geneva: WHO, International Programme on Chemical Safety.

York M, Griffiths HA, Whittle E, Basketter DA. (1996). Evaluation of a human patch test for the identification and classification of skin irritation potential. Contact Dermatitis 34: 204–212.

York M, Steiling W. (1998). A critical review of the assessment of eye irritation potential using the Draize rabbit eye test. J Appl Toxicol 18: 233–240.

Zbinden G, Flury-Roversi M. (1981). Significance of the LD_{50}-test for the toxicological evaluation of chemical substances. Arch Toxicol 47: 77–99.