Alternatives for Dermal Toxicity Testing

Chantra Eskes
Erwin van Vliet
Howard I. Maibach
Editors

Foreword by
Alan M. Goldberg

Springer

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Editors
Chantra Eskes
SeCAM
Magliaso
Switzerland

Erwin van Vliet
Innovitox Consulting & Services
Houten
The Netherlands

Howard I. Maibach
Department of Dermatology
University of California School
of Medicine
San Francisco
California
USA

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Foreword

This substantial compilation of manuscripts provides an important and comprehensive collection of papers by world-renowned scientists covering the literature on alternatives for dermal toxicity testing.

Historically, dermal testing was initially thought of as one of the more difficult *in vitro* methods. The physiological basis of dermal toxicity is very complex and involves many different cell types and pathways for sensitivity, irritation, and corrosion. Yet surprisingly, dermal toxicity is one of the earliest areas of *in vitro* toxicity to provide useful human cell-based systems.

Initial toxicity assay developments were seen as simple (quick) approaches to commercial human skin systems that were being developed for treating burn patients. A few companies learned the hard way that *in vitro* toxicology was no simpler than using those cultured skin systems as skin grafts. After several years, they all went out of business. Several scientists who understood the complexity, however, focused on developing human skin models for the sole purpose of *in vitro* toxicity. These models, simple at first, became more standardized and more complex and provided a better matrix for testing.

The Johns Hopkins Center for Alternatives to Animal Testing (CAAT) was founded in 1981 specifically to develop *in vitro* methods for hazard evaluation and safety testing of cosmetic products (see [1]). One aspect of the research program, identified as Program Projects, was the coordination of several projects within a selected topic to develop a better understanding of mechanisms responsible for a toxic event.

The Avon Program Project

Avon funded CAAT from the first grant (from the Cosmetic, Toiletry, and Fragrance Association (CTFA)) and then continued independently funding the center. After a few years, Avon, in the person of Yale Gressel, asked if CAAT could take on a larger project—developing an *in vitro* assay to predict skin sensitization.

We approached the problem by inviting about eight laboratories working on various aspects of skin biology to present to their "competitors and colleagues." They were asked how they would approach the issue and what aspects they saw as the most important. At first, the discomfort was obvious: "Will what I share be used by my competitors?" As the day progressed, however, it became clear that each lab

would be focusing on different aspects of the problem. We invited five individuals to submit grant applications with the provision that, if approved, up to three applications would be funded.

The funded project teams would get together twice yearly in a roll-up-your-sleeves discussion about their progress and how to proceed. The attendees at these "lab" meetings were the participants along with other experts from Hopkins, the government, and Avon. And they were wonderful meetings. At almost every meeting a person from one of the sectors would ask a question and the response from another sector would be, "That is a great question—I would have never thought of it." In essence, the corporate and government scientists wanted to know how to use the information generated and the academics wanted to better understand the mechanisms involved.

The project lasted nine years, and the science it generated formed the basis of our understanding of mechanisms of skin sensitization. This project was summarized by Craig Elmets [2].

"By all measures it was a very successful project, characterized by identification of many of the interleukins, cytokine pathways, and the recognition that keratinocytes play an important role in sensitization." (As quoted from [1])

Toxicity Testing in the Twenty-First Century

The NAS report, *Toxicity Testing in the 21st Century: A Vision and a Strategy*, was a seminal moment in the development of *in vitro* assays [3]. This report had undergone external review and I was one of the external reviewers.

The major conclusions of the study included the following:

1. Animal studies are time-consuming and expensive.
2. There is a lack of predictability of animal studies as they relate to humans.
3. We should be using human cells in culture.
4. We should explore systems biology and pathways and mechanisms of toxicity.

This publication was, and is, a major advancement in *in vitro* toxicology, alternatives, and risk assessment. It created major new research approaches and opportunities. It provided an important source of encouragement for the development of alternative toxicological methodologies and stimulated what is now recognized as a scientific revolution.

Human Cell in Culture

As the *in vitro* toxicology field began to develop, animal cells, mainly from rats and mice, were being used, as human cell culture was essentially not available. When CAAT was founded, Leon Golberg (1982) emphasized that human cell cultures would be the key to developing *in vitro* methods for risk assessment that would be accepted for decision making. How correct he was. As a result of this realization, CAAT, from

the very first round of grants, funded research to advance the science of human cell culture. A number of contributors to this volume were funded by CAAT. A summary of many aspects of human cell culture can be found in Bressler et al. [4].

Skin

The skin represents the largest organ of the human body. The ability to understand how drugs and chemicals penetrate the skin and how they may adversely affect the health of skin is important for protecting consumers from undesired effects. Excised human skin sections from cadavers have been used extensively to understand the dermal penetration of drugs and cosmetics. And for more than 30 years, the scientific community has devoted much time developing monolayer cultures of cells and more recently has focused on 3D reconstituted human skin models.

Alternatives for Dermal Toxicity Testing editors Chantra Eskes, Erwin van Vliet, and Howard Maibach have compiled an excellent, important, and comprehensive book that is necessary for anyone in the field—from beginner students to highly acclaimed senior researchers.

The book contains six sections: irritation, corrosion, sensitization, UV-induced effects, genotoxicity, and a concluding section with three papers exploring integrated strategies and high-throughput systems.

I believe that every commercial model is covered, in depth, with adequate information to assist one in identifying the best model for their studies. The volume is an invaluable resource.

The editors should be congratulated for identifying essentially most, if not all, of the contributors in this field and synthesizing a highly readable and important reference publication.

Alan M. Goldberg, PhD
Center for Alternatives to Animal Testing
Departmental of Environmental Health and Engineering
Bloomberg School of Public Health, Global Food Ethics
Johns Hopkins University
Baltimore, MD 21205, USA

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Preface

Dermal toxicity is one of the pioneer areas in which alternative methods to the use of animal testing have gained scientific, industrial, and regulatory acceptance. Over two decades have passed since the publication in 1994 of Mary Ann Liebert's book on *In Vitro* Skin Toxicology (Rougier A., Goldberg A.M., and Maibach H.I. Eds.). Since then, several alternative methods for dermal toxicity have been optimized, scientifically validated, and gained international regulatory acceptance. In some cases it is already possible to fully replace the regulatory animal test, such as for skin irritation and corrosion, by using, e.g., Integrated Approaches to Testing and Assessment (IATAs). In other cases, such as for skin sensitization, it is possible to partially replace the regulatory animal test with *in chemico* and *in vitro* test methods that address key events of the adverse outcome pathway (AOP) leading to allergic contact dermatitis. Furthermore, the use of human *in vitro* models in the area of skin irritation and the use of defined approaches (DA) for skin sensitization testing (i.e., which combine, e.g., *in chemico* and *in vitro* test methods) have shown comparable if not better correlations to human data than the regulatory animal tests.

In view of the considerable progress made, this book aims at providing up-to--date comprehensive information on the most advanced alternative test methods available for the assessment of dermal toxicity with particular emphasis on the areas of skin irritation, skin corrosion, skin sensitization, UV-induced effects, and skin genotoxicity. For each test method, a description of the currently available protocol is given including highlights of its critical steps, applicability, limitations, potential role, and use within testing approaches and correlation with the traditional animal data and, when available, also human data. Furthermore, the book addresses exploratory areas that may be of relevance for the future of dermal toxicity safety testing, including the use of human progenitor skin cells, integration of *in vitro* and clinical methodologies, and application of high-throughput screening techniques.

The editors warmly acknowledge all authors that contributed to make the project of this book a reality and Springer for their great support and belief in the project. Albeit attempting to be comprehensive, new and/or additional methods and authors

that could not be involved in this book will be invited to contribute to the next editions to come, for which any comments and/or suggestions are welcomed.

Magliaso, Switzerland — Chantra Eskes
Houten, The Netherlands — Erwin van Vliet
San Francisco, CA, USA — Howard Maibach

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Contributors

Pierre Aeby Consultant, Marly, Switzerland

Shaheda Ahmed Alcyomics, Ltd., Newcastle-upon-Tyne, UK

Haematological Sciences, Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, UK

Nathalie Alépée L'Oréal Research and Innovation, Aulnay-sous-Bois Cedex, France

Lee Ann Applegate Department of Musculoskeletal Medicine, Plastic, Reconstructive and Hand Surgery, Unit of Regenerative Therapy, University Hospital of Lausanne, Epalinges, Switzerland

Takao Ashikaga Shiseido, Co., Ltd., Tokyo, Japan

Claude Auriault ImmunoSearch, Grasse, France

Marcela Contador Baptista R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

David A. Basketter DABMEB Consultancy Ltd, Sharnbrook, UK

Caroline Bauch Cyprotex, Kalamazoo, MI, USA

Carolina Gomes Benevenuto Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, São Paulo, Brazil

Elodie Boitel ImmunoSearch, Grasse, France

Carl Borrebaeck Department of Immunotechnology, Lund University, Lund, Sweden

Rico Brendtke Department Tissue Engineering and Regenerative Medicine (TERM), University Hospital Wuerzburg, Wuerzburg, Germany

Senetics Healthcare Group GmbH & Co. KG, Erlangen, Germany

Carla Abdo Brohem R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

Petra Budde Proteome Sciences PLC, Cobham, UK

Anthony de Buys Roessingh Department of Pediatric Surgery, University Hospital of Lausanne, Lausanne, Switzerland

Andrezza di Pietro Micali Canavez R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

Emanuela Corsini Laboratory of Toxicology, Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy

José Cotovio L'Oréal Research and Innovation, Aulnay-sous-Bois Cedex, France

Françoise Cottrez ImmunoSearch, Grasse, France

Maria Teresa Cruz Centre for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal

Faculty of Pharmacy, Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Anne Dickinson Alcyomics, Ltd., Newcastle-upon-Tyne, UK

Haematological Sciences, Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, UK

Solène Dreyfuss L'Oréal Research & Innovation, Aulnay-sous-Bois Cedex, France

Oliver Engelking CellSystems GmbH, Troisdorf, Germany

Chantra Eskes SeCAM Services & Consultation on Alternative Methods, Magliaso, Switzerland

Philipp R. Esser Allergy Research Group, Department of Dermatology, Faculty of Medicine, University of Freiburg, Freiburg, Germany

Isabel Ferreira Centre for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Leslie M. Foertsch The Procter & Gamble Company, Mason Business Center, Cincinnati, OH, USA

Horst W. Fuchs CellSystems GmbH, Troisdorf, Germany

Valentina Galbiati Laboratory of Toxicology, Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milan, Italy

Lorena Rigo Gaspar Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, São Paulo, Brazil

G. Frank Gerberick The Procter and Gamble Company, Central Product Safety, Mason, OH, USA

Susan Gibbs Department of Dermatology, VU University Medical Centre, Amsterdam, The Netherlands

Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam (ACTA), Amsterdam, The Netherlands

Marie-Hélène Grandidier L'Oréal Research and Innovation, Aulnay-sous-Bois Cedex, France

Florian Groeber Translational Center Wuerzburg 'Regenerative Therapies in Oncology and Musculoskeletal Diseases', Fraunhofer Institute for Interfacial Engineering and Biotechnology (IGB), Wuerzburg, Germany

Hervé Groux ImmunoSearch, Grasse, France

Robert Guest Envigo Research Limited, Shardlow, Derbyshire, UK

Jan Hansmann Department Tissue Engineering and Regenerative Medicine (TERM), University Hospital Wuerzburg, Wuerzburg, Germany

Translational Center Wuerzburg 'Regenerative Therapies in Oncology and Musculoskeletal Diseases', Fraunhofer Institute for Interfacial Engineering and Biotechnology (IGB), Wuerzburg, Germany

Rosette Van Den Heuvel Unit of Environmental Risk and Health, VITO NV, Antwerp, Belgium

Nathalie Hirt-Burri Department of Musculoskeletal Medicine, Plastic, Reconstructive and Hand Surgery, Unit of Regenerative Therapy, University Hospital of Lausanne, Epalinges, Switzerland

Markus Hofmann Swiss Federal Office of Public Health, Bern, Switzerland

Jef Hooyberghs Unit of Environmental Risk and Health, VITO NV, Antwerp, Belgium

Theoretical Physics, Hasselt University, Hasselt, Belgium

Kazuhiro Hosoi Research & Development Center, Santen Pharmaceutical, Co., Ltd., Osaka, Japan

Paris Jafari Department of Musculoskeletal Medicine, Plastic, Reconstructive and Hand Surgery, Unit of Regenerative Therapy, University Hospital of Lausanne, Epalinges, Switzerland

Henrik Johansson SenzaGen AB, Lund, Sweden

Helena Kandarova MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia

Masakazu Katoh Japan Tissue Engineering Co. Ltd., Gamagori City, Aichi, Japan

Camila Martins Kawakami Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, São Paulo, Brazil

Donald Keller Cyprotex, Kalamazoo, MI, USA

Petra S. Kern The Procter & Gamble Company, Beijing Innovation Center, Beijing, China

Ian Kimber Faculty of Life Sciences, University of Manchester, Manchester, UK

Hajime Kojima National Institute of Health Sciences (NIHS), Tokyo, Japan

Odivania Kruger R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

Jochen Kühnl Beiersdorf AG, Hamburg, Germany

Nathalie Lambrechts Unit of Environmental Risk and Health, VITO NV, Antwerp, Belgium

Robert Landsiedel BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany

Manfred Liebsch "Centre of Alternative Methods to Animal Experiments—ZEBET" at the BfR, Berlin, Germany

Malin Lindstedt Department of Immunotechnology, Lund University, Lund, Sweden

Marcio Lorencini R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

Stefan F. Martin Allergy Research Group, Department of Dermatology, Faculty of Medicine, University of Freiburg, Freiburg, Germany

João Demétrio Martins Centre for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal

Annette Mehling BASF Personal Care and Nutrition GmbH, Düsseldorf, Germany

Laurent Nardelli L'Oréal Research and Innovation, Aulnay-sous-Bois Cedex, France

Andreas Natch Givaudan Suisse SA, Duebendorf, Switzerland

Inge Nelissen Unit of Environmental Risk and Health, VITO NV, Antwerp, Belgium

Caroline Radoski Neumann R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

Bruno Miguel Neves Department of Chemistry, Mass Spectrometry Centre, QOPNA, University of Aveiro, Aveiro, Portugal

Centre for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal

Satomi Onoue The School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka Prefecture, Shizuoka, Japan

Prakash Patel Cyprotex, Kalamazoo, MI, USA

Stefan Pfuhler Procter and Gamble, Mason Business Centre, Mason, OH, USA

Ian Pike Proteome Sciences PLC, Cobham, UK

Cécile Piroird L'Oréal Research and Innovation, Aulnay-sous-Bois Cedex, France

Wassim Raffoul Department of Musculoskeletal Medicine, Plastic, Reconstructive and Hand Surgery, Unit of Regenerative Therapy, University Hospital of Lausanne, Epalinges, Switzerland

Tzutzuy Ramirez BASF SE, Ludwigshafen, Germany

Kerstin Reisinger Henkel Beauty Care, Düsseldorf, Germany

Hendrik Reuter Beiersdorf AG, Hamburg, Germany

Erwin L. Roggen 3Rs Management and Consulting ApS, Kongens Lyngby, Denmark

Hitoshi Sakaguchi Kao Corporation, Tokyo, Japan

Corinne Scaletta Department of Musculoskeletal Medicine, Plastic, Reconstructive and Hand Surgery, Unit of Regenerative Therapy, University Hospital of Lausanne, Epalinges, Switzerland

Andreas Schepky Beiersdorf AG, Hamburg, Germany

Freia Schmid Translational Center Wuerzburg 'Regenerative Therapies in Oncology and Musculoskeletal Diseases', Fraunhofer Institute for Interfacial Engineering and Biotechnology (IGB), Wuerzburg, Germany

Greet Schoeters Unit of Environmental Risk and Health, VITO NV, Antwerp, Belgium

Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium

Dept of Environmental Medicine, Institute of Public Health, University of Southern Denmark, Odense, Denmark

Desirée Cigaran Schuck R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

Ana Silva Centre for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Talita M.T.P. Silveira R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

Sander W. Spiekstra Department of Dermatology, VU University Medical Centre, Amsterdam, The Netherlands

Carine Tornier EPISKIN™, 4 rue Alexander Fleming, 69366 Lyon, France

John A. Troutman The Procter & Gamble Company, Mason Business Center, Cincinnati, OH, USA

Rich Ulmer InVitro International, Placentia, CA, USA

Natalia de Albuquerque Vita R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

Heike Walles Department Tissue Engineering and Regenerative Medicine (TERM), University Hospital Wuerzburg, Wuerzburg, Germany

Translational Center Wuerzburg 'Regenerative Therapies in Oncology and Musculoskeletal Diseases', Fraunhofer Institute for Interfacial Engineering and Biotechnology (IGB), Wuerzburg, Germany

Amy Wang InVitro International, Placentia, CA, USA

Xiao Nong Wang Haematological Sciences, Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, UK

Ana Cristina Weihermann R&D Department, Grupo Boticário, São José dos Pinhais, PR, Brazil

Dirk Weisensee CellSystems GmbH, Troisdorf, Germany

Bart De Wever ATERA SAS, Nice, France

Hilda Witters Unit of Environmental Risk and Health, VITO NV, Antwerp, Belgium

Kathrin Stephanie Zeller Department of Immunotechnology, Lund University, Lund, Sweden

Hans-Dieter Zucht Proteome Sciences PLC, Cobham, UK

Part I

Skin Irritation

1 Overview on Current Status of Alternative Methods and Testing Approaches for Skin Irritation Testing

Chantra Eskes and Markus Hofmann

1.1 Background

If the animal *in vivo* study has been originally used to classify for potential skin corrosion and skin irritation hazard effects (such as the OECD Test Guideline 404 [1] originally adopted in 1981), the area of skin corrosion and irritation represents one of the pioneering areas in which a number of alternative methods have been validated and internationally adopted since 2000 (and 2004) for skin corrosion and since 2009 (and 2010) for skin irritation by the EU (and by the OECD respectively).

In order to replace or minimize to the extent possible the use of *in vivo* animal testing, current internationally agreed approaches (UN, OECD and EU) recommend the use of integrated approaches and strategies for the assessment of skin irritation and corrosion effects, such as the Integrated Approach for Testing and Assessment (IATA) endorsed by OECD member countries [2]. These approaches recommend considering all existing information sources, and conducting a weigh-of-evidence evaluation before performing prospective testing first on alternative test methods, and only as a last resort on animals. Depending upon regulatory requirements, some geographical regions already allow the use of alternative methods for skin irritation and corrosion testing as full replacement of the animal testing, as it is the case in the European Union (EU).

In the EU, a number of legislations indeed call for the use of alternative methods to animal toxicological testing. The EU Cosmetics Regulation [3] prohibits animal testing of finished products since 2004 and of cosmetic ingredients since 2009, reinforced by a marketing ban of cosmetics finished products tested on animals since 2004 and for cosmetics containing ingredients tested on animals since 2013 [3].

C. Eskes (✉)
SeCAM Services & Consultation on Alternative Methods, Magliaso, Switzerland
e-mail: chantra.eskes@secam-ce.eu

M. Hofmann
Swiss Federal Office of Public Health, Bern, Switzerland

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Furthermore, the EU regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; [4, 5]), requires that *in vitro* testing is conducted by OECD member countries for skin corrosion and irritation unless the test chemical falls outside of the applicability domain of the available *in vitro* methods or the results obtained from such methods do not allow a conclusive decision on (non-)classification and risk assessment. The EU regulation on Classification, Labelling and Packaging of substances and mixtures (EU CLP; [6, 7]), which implemented the Globally Harmonized System for classification and labelling of substances and mixtures in the European Union, encourages the use of tiered weight-of-evidence evaluations, and makes use of information from *in vitro* testing in its tiered classification approach for skin corrosion and irritation. Finally, the EU Directive on the protection of animals used for scientific purposes [8] states that (article 13(1)) "*Member States shall ensure that a procedure is not carried out if another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognised under the legislation of the Union*".

1.2 Classification for Skin Irritation Hazard

The UN has published in 2003 the Globally Harmonized System (GHS) for classification and labelling to favour harmonized classification of hazards across the world, which is now in its 6th revision [9]. This classification system was still then based on the traditional *in vivo* animal test adopted within the OECD Test Guideline 404 [1] originally developed by Draize and co-workers [10]. Since validation studies on alternative methods for skin irritation testing have used the animal test as the reference test method, a description of this classification system is given here.

Skin irritation is defined *in vivo* as "*the production of reversible damage of the skin following the application of a test substance for up to 4 hours*" [2, 7, 9]. One main irritant category is defined by the UN GHS classification system, i.e., Category 2, as described in Table 1.1. However, an additional optional category for mild irritants (i.e., Category 3) is also defined for those authorities wanting to have more than one skin irritant category.

In the European Union, the UN GHS classification and labelling system has been implemented by means of the EU CLP regulation (1272/2008; [6, 7]). It replaced from December 2010 the EU Dangerous Substances Directive establishing the former EU classification system for substances (EU DSD; [11]), and from 2015 the EU Dangerous Preparation Directive establishing classification criteria for mixtures (EU DPD; [12]). The EU CLP is equivalent to the UN GHS as shown in Table 1.1, but makes use of a single category (Category 2) only, whereas the mild irritant category 3 is not required. Substances falling in the UN GHS category 3, require No Category classification under the EU CLP.

Figure 1.1 provides with a comparison of the criteria applied for skin irritation classification according to the UN GHS, EU CLP and EU DSD classification systems for skin irritation [6, 7, 9, 11]. In addition to the cut-offs shown in Fig. 1.1, the three classification systems also consider a substance irritant if effects persist at the

Table 1.1 UN GHS skin irritation category(ies)

Categories	Criteria[a]
Irritant Category 2	(1) Mean value of ≥ 2.3 and ≤ 4.0 for erythema/eschar or for oedema in at least 2 of 3 tested animals from gradings at 24, 48 and 72 h after patch removal or, if reactions are delayed, from grades on three consecutive days after the onset of skin reactions; or (2) Inflammation that persists to the end of the observation period normally 14 days in at least two animals, particularly taking into account alopecia (limited area), hyperkeratosis, hyperplasia, and scaling; or (3) In some cases where there is pronounced variability of response among animals, with very definite positive effects related to chemical exposure in a single animal but less than the criteria above
Optional mild irritant Category 3	Mean value of ≥1.5 and < 2.3 for erythema/eschar or for oedema from gradings in at least 2 of 3 tested animals from grades at 24, 48 and 72 h or, if reactions are delayed, from grades on three consecutive days after the onset of skin reactions (when not included in the irritant category above)

[a]Grading criteria are understood as described in the OECD Test Guideline 404 [1]

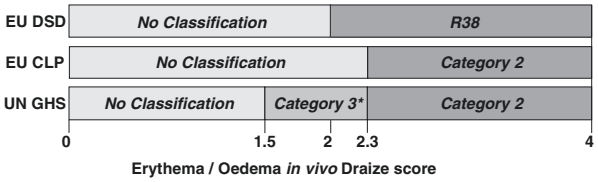

Fig. 1.1 Erythema/oedema Draize score ranges defining EU DSD, EU CLP and UN GHS classification of skin irritation. Scores refer to the mean value from gradings at 24, 48 and 72 h observed in at least two out of three animals (or as required in case of more than three animals). *Category 3 is an optional category available for those authorities wanting to have more than one skin irritant category

end of the observation period (day14) in two or more test animals, and other effects such as hyperplasia, scaling, discoloration, fissures, scabs and alopecia.

1.3 Integrated Approaches for Testing and Assessment (IATA)

Current internationally agreed approaches (OECD, EU and UN) recommend the use of integrated approaches and strategies for the assessment of skin irritation and corrosion effects. In particular, the OECD published in 2014 the first Guidance Document (GD No. 203) on an IATA adopted at an international level by OECD member countries for skin corrosion and irritation [2]. The IATA aims at hazard identification of the skin corrosion or irritation potential of chemicals (or the absence thereof) and to provide adequate information for classification and labelling according to the UN GHS classification system.

The IATA is divided in three major parts including as Part 1 the use of existing information, physico-chemical properties and non-testing methods, as Part 2 a weigh-of-evidence evaluation, and as Part 3 the conduct of prospective testing. The possible individual information sources integrating the IATA have been grouped into eight Modules according to the type of information provided, which can be used in one or more Parts of the IATA as described in Table 1.2. The strengths and limitations as well as the potential role and contribution of each Module and their individual components in the IATA for skin irritation and corrosion are described within the OECD GD 203 [2] with the purpose of minimizing the use of animals to the extent possible, whilst ensuring human safety. Furthermore, a schematic outline of the IATA for skin corrosion and irritation classification and labelling is presented in Fig. 1.2.

Table 1.2 Parts and modules of the IATA for skin corrosion and irritation (extract from [2])

Part[a]	Module	Data
Part 1 (existing information, physico-chemical properties and non-testing methods)	1	Existing human data – Non-standardised human data on local skin effects – Human Patch Test (HPT)
	2	*In vivo* skin irritation and corrosion data (OECD TG 404)
	3	*In vitro* skin corrosion data – OECD TG 430 – OECD TG 431 – OECD TG 435
	4	*In vivo* skin irritation data (OECD TG 439)
	5	Other *in vivo* and *in vitro* data – *In vitro* skin corrosion or irritation data from test methods not adopted by the OECD – Other *in vivo* and *in vitro* dermal toxicity data
	6	Physico-chemical properties (existing, measured or estimated) such as pH, acid/alkaline reserve
	7	Non-testing methods for substances: (Q)SAR, read-across, grouping and prediction systems; for mixtures: bridging principles and theory of additivity
Part 2 (WoE analysis)	8	Phases and elements of Weight of evidence (WoE) approaches
Part 3 (additional testing)	(5b)	Other *in vivo* and/or *in vitro* dermal toxicity testing (if required by other regulations)
	(3)	*In vitro* skin corrosion testing
	(4)	*In vitro* skin irritation testing
	(5a)	*In vitro* skin irritation testing in test method not adopted by the OECD
	(2)	*In vivo* skin irritation and corrosion testing

[a]While the three Parts are considered as a sequence, the order of Modules 1–7 of Part 1 might be arranged as appropriate

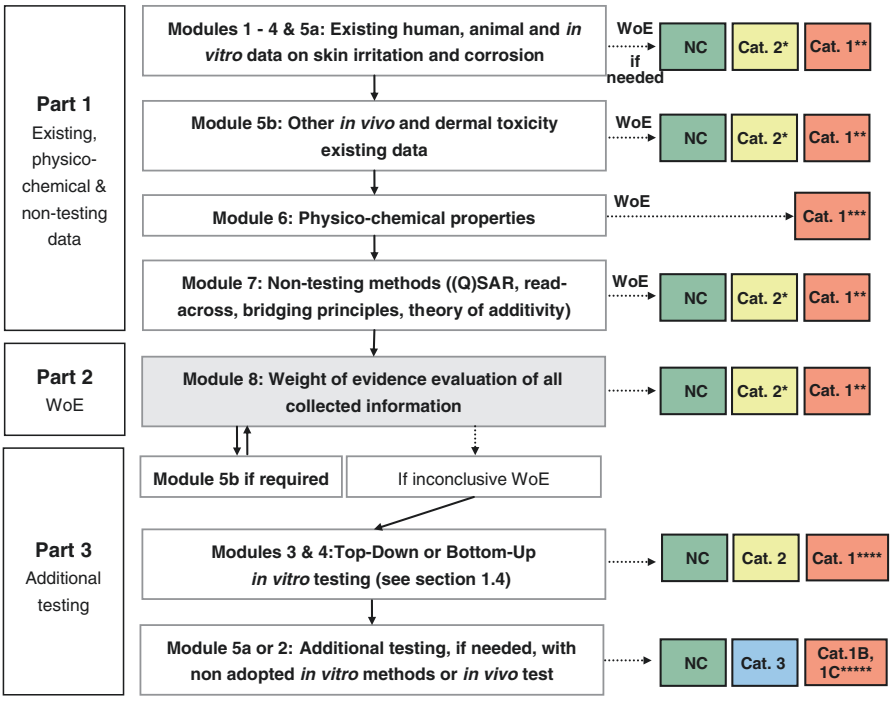

Fig. 1.2 Schematic overview of the IATA for skin irritation and corrosion based on the recommendations from the OECD GD 203 [2]. *Cat. 1* corrosive to skin, *Cat. 2* irritating to skin, *NC* no category. *Including optional Cat. 3, as applicable. **Including corrosive sub-categories 1A, 1B and 1C, as applicable. ***If corrosive sub-categorisation is required an appropriate *in vitro* skin corrosion test needs to be conducted. **** Possibilities to sub-categorise depends on the specific test method used: *OECD TG 435* allows for the discrimination between Sub-cat. 1A, Sub-cat. 1B and Sub-cat. 1C but with a limited applicability domain; *OECD TG 431* allows for the discrimination between Sub-cat. 1A and the combined Sub-cat. 1B-and-1C but does not permit the discrimination between sub-categories 1B and 1C; *OECD TG 430* only allows the identification of corrosives into a single category without sub-categorisation, i.e., Cat. 1. *****If outside of the applicability domain of OECD TG 435

While the three Parts are considered as a sequence, Modules 1–7 of Part 1 might be arranged as appropriate. Ideally, the IATA should be universally applicable to ensure human safety, whilst making maximum use of existing data, being resource efficient and minimising or eliminating the requirement for animal experiments.

Under *Part 1* of the IATA (*existing, physico-chemical & non-testing data*), existing and available information is retrieved from literature and databases and other reliable sources for Modules 1–5, while under Module 6 on physico-chemical properties, primarily the pH and the acidic/alkaline reserve are considered, and under Module 7 non-testing methods are considered. Whilst the retrieval of existing information for Modules 1–5a directly relate to skin corrosion and irritation, Module 5b requires a different search for other *in vitro* and *in vivo* dermal toxicity studies.