EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection
In-Vitro Methods Unit
European Centre for the Validation of Alternative Methods (ECVAM)

5th VMG meeting on retrospective validation of cytotoxicity/cell-function based *in vitro* assays (eye irritation)
14th – 15th October 2008, Ispra, Italy

Minutes

Participants

Horst Spielmann (HSP)	ZEBET (BfR) (Germany)
Pauline McNamee (PMN)	Procter & Gamble (UK)
Laurie Scott (LSC)	Procter & Gamble (Belgium)
Joanne Gartlon (JGA)	University of Nottingham (UK)
Richard Clothier (RCL)	University of Nottingham (UK)
Rodger D. Curren (RDC)	IIVS (USA)
María Pilar Vinardell (MPV)	University of Barcelona (Spain)
Montserrat Mitjans (MMI)	University of Barcelona (Spain)
Raymond Tice (RTI)	NICEATM (USA)
Yasuo Ohno (YOH)	JaCVAM (Japan)
Valérie Zuang (VZU)	ECVAM (Italy)
João Barroso (JBA)	ECVAM (Italy)
André Kleensang (AKL)	ECVAM (Italy)
Thomas Cole (TCO)	ECVAM (Italy)

Excused

Wolfgang Pape (WPA)	Beiersdorf A.G. (Germany)

Meeting Objectives

The 5th VMG meeting was convened to present background review documents (BRDs) respective of four cytotoxicity/cell function based *in vitro* assays for eye irritation potential of chemicals. From comprehensive expert evaluations compiled in the BRDs, the meeting would determine VMG consensus on the regulatory utility of the methods, relevant to integrated testing/screening strategies, weight of evidence assessments, etc.

Key observations (e.g., the merits of individual protocols, range of applicability domain, and predictive capacity for *in vivo* classification) would provide criteria for recommendations (via ESAC peer review) on regulatory implementation by "top down" versus "bottom up" discrimination of severe, mild, non-irritants (e.g., EU R41, R36, no label; GHS cat. 1, cat. 2, no category; EPA I, II, III, IV).

Introduction

Illustrated presentations of the four BRDs were made in turn by the respective contract authors, followed by discussion of assay characteristics, noted here as observations and recommendations.

1) Neutral Red Release (NRR)	University of Nottingham (UK)
2) Fluorescein Leakage (FL)	University of Nottingham (UK)
3) Cytosensor Microphysiometer (CM)	IIVS (USA)
4) Red Blood Cell (RBC) haemolysis	University of Barcelona (Spain)

VMG draft summary (uses and limitations)

a) Observations

1) Neutral Red Release (NRR)

INVITTOX 54

- Based on 29 chemicals and 35 formulations tested among several laboratories [CTFA study (Reader et al. 1990; Gettings et al., 1991 & 1996); BRD pp. 132, 136, 168, 169; BRD summary manuscript Tables 7.3a, 7.3b, 7.4a, 7.4b, 7.5a, 7.5b], the test was considered suitable for a bottom-up approach (discriminating non-irritants from all irritant classes) (false negative rate ~1%[1]), although with high false positive rate (~62%[2]), but not for a top-down approach (discriminating severe irritants from all other classes) due to high false positive rate (~23%[3]). False negative rate of ~4% was obtained in the top-down approach[4].
- Limitations exclude strong acids and bases, fixatives, and highly volatile compounds from the applicability domain.
- INVITTOX 54 is facilitated by use of standard laboratory installations/equipment and requirement of regular training/experience in cell culture practice.
- Refinement of prediction model could be achieved from systematic assay/analysis of an extended range of chemicals.
- Evaluation of between laboratory variability (BLV) would also benefit from additional data, preferably acquired by further testing.

[1] EU: 3.8% (1/26)	GHS: 0% (0/22)	EPA: 8.3% (3/36)
[2] EU: 63.2% (24/38)	GHS: 58.1% (18/31)	EPA: 46.2% (6/13)
[3] EU: 28.6% (6/21)	GHS: 19% (4/21)	EPA: 22.2% (4/18)
[4] EU: 12.5% (1/8)	GHS: 0% (0/7)	EPA: 0% (0/6)

PREDISAFE™

- Based on 23 chemicals and 32 formulations tested in 3 laboratories [COLIPA study (Brantom et al., 1997; Courtellemont et al, 1999); BRD pp. 179-180; BRD summary manuscript Tables 6.1a, 7.3a, 7.3b, 7.4a, 7.4b, 7.5a, 7.5b], the test was considered suitable for a bottom-up approach (discriminating non-irritants from all irritant classes) (false negative rate ~6%[5]), although with significant false positive rate (~36%[6]), but not for a top-down approach (discriminating severe irritants from all other classes) due to high

false positive rate (~32%[7]). False negative rate of ~6% were obtained in the top-down approach[8].

- Limitations exclude strong acids and bases, fixatives, and highly volatile compounds from the applicability domain.
- PREDISAFE™ is facilitated by use of standard laboratory installations/equipment and requirement of regular training/experience in cell culture practice.
- Good agreement of classification (BLV): 47/55 materials (85.5%) with 100% agreement among 3 laboratories [COLIPA study (Brantom et al. 1997); BRD p. 113; BRD summary manuscript Table 6.2].
- Refinement of prediction model could be achieved from systematic assay/analysis of an extended range of chemicals, since mainly surfactants and surfactant based formulations have been tested.
- Further evaluation would benefit from additional data, preferably acquired by further testing.

It is recommended that PREDISAFE™ [based on Méthode Officiel Française (Journal Officiel De La République Française; Dec, 1999)] is selected for further evaluation for the bottom-up approach by testing of an extended range of chemicals. The NRR assay using the PREDISAFE™ protocol appears amenable to a high-throughput testing system. PREDISAFE™ has been demonstrated to be reproducible when used in more than one laboratory, was found to have a lower false positive rate than INVITTOX protocol 54 on a bottom-up approach, and is commercially available.

[5] EU: 4.5% (1/22)	GHS: 7.1% (2/28)	EPA: 16.2% (6/37)
[6] EU: 37.5% (12/32)	GHS: 29.6% (8/27)	EPA: 6.2% (1/16)
[7] EU: 29.7% (11/37)	GHS: 31.6% (12/38)	EPA: 33.3% (13/39)
[8] EU: 5.9% (1/17)	GHS: 5.9% (1/17)	EPA: 7.1% (1/14)

2) <u>Fluorescein Leakage (FL)</u>

INVITTOX 71

- Based on 60 chemicals tested in 4 laboratories [EC/HO study (Balls et al., 1995), BRD pp. 180 & 182; BRD summary manuscript Tables 6.5, 7.5a, 7.5b, 7.6a, 7.6b, 7.7a, 7.7b] the test was considered suitable for a top-down approach (discriminating severe irritants from all other classes) (false positive rate < 10%[9]), although having high false negative rate (~55%[10]), but not for a bottom-up approach (discriminating non-irritants from all irritant classes) due to high false negative (35-40%[11]) and positive (16-36%[12]) rates. These figures refer to analyses without inclusion of recovery data.
- INVITTOX 71 has been applied to a broad range of water soluble chemicals.
- Limitations exclude strong acids and bases, fixatives, and highly volatile compounds from the applicability domain.
- INVITTOX 71 is facilitated by use of standard laboratory installations/equipment for cell culture practice, although specialised training is required.
- Short exposure time (1 min, up to 15 min) followed by immediate measurement, is relevant to regulatory hazard classification. Further evaluation would benefit from additional data on recovery (up to 72 hours), preferably acquired by further testing.

- Evaluation of between laboratory variability (BLV) would also benefit from additional data, preferably acquired by further testing.

[9] EU: 7.1% (7/99)	GHS: 6.8% (7/103)	EPA: 9.1% (9/99)
[10] EU: 54.3% (25/46)	GHS: 56.3% (27/48)	EPA: 53.6% (15/28)
[11] EU: 35.2% (31/88)	GHS: 35.2% (38/108)	EPA: 45.8% (55/120)
[12] EU: 28.1% (16/57)	GHS: 16.3% (7/43)	EPA: 0% (0/7)

INVITTOX 120

- Based on the 11 surfactants and 23 surfactant-based formulations tested in 2 laboratories [COLIPA study (Brantom et al., 1997); BRD pp. 190 & 191; BRD summary manuscript Tables 7.5a, 7.6a, 7.6b, 7.7a, 7.7b] the test was considered suitable for a bottom-up approach (discriminating non-irritants from all irritant classes) (false negative rate, 0-5%[13]), although with a significant false positive rate (~35%[14]), but not for a top-down approach (discriminating severe irritants from all other classes) due to high false positive rate (~32%[15]). However, no false negatives were obtained on the top-down approach[16].
- Predictive Capacity (PC) available only from assay of surfactants and surfactant-based formulations. Insufficient to conclude applicability to a broader chemical domain.
- Further evaluation would benefit from additional data, preferably acquired by further testing.
- Limitations exclude strong acids and bases, fixatives, and highly volatile compounds from the applicability domain.
- INVITTOX 120 is facilitated by use of standard laboratory installations/equipment for cell culture practice, although specialised training is required.
- Short exposure time (15 min) followed by 4 hour incubation prior to measurement, allows assessment of recovery. Further evaluation would benefit from additional data (including recovery up to 72 hours), preferably acquired by further testing.
- Good agreement of classification (BLV): 7/9 materials (77.8%) with 100% agreement among 3 laboratories [Southee (1998); BRD p. 121; BRD summary manuscript Table 6.2]; 26/29 materials (89.7%) with 100% agreement among 2 laboratories [Brantom et al. (1997); BRD p. 137; BRD summary manuscript Table 6.3].

[13] EU: 0% (0/31)	GHS: 5.3% (2/38)	EPA: 8.9% (4/45)
[14] EU: 36.7% (11/30)	GHS: 32.0% (8/25)	EPA: 6.2% (1/16)
[15] EU: no data	GHS: 30.6% (11/36)	EPA: 34.2% (13/38)
[16] EU: no data	GHS: 0% (0/27)	EPA: 0% (0/23)

No preference is given to either protocol 71 or 120 since the purposes and applicability domains are different.

INVITTOX 82 and 86

Due to the inadequacies of the datasets (e.g., lack of comparative *in vitro* and *in vivo* data), INVITTOX protocols 82 and 86 were not evaluated for relevance or reliability.

3) <u>Cytosensor Microphysiometer (CM)</u>

INVITTOX 97 (SM – CTFA study) and 102 modified (CM – EC/HO and COLIPA studies)

- INVITTOX protocol 97 is obsolete (no instruments available to follow protocol).
- INVITTOX protocol 102 requires an update with modified exposure conditions.
- Based on 21 surfactant chemicals and 32 surfactant based formulations tested in 7 laboratories (1 repeated) [CTFA study (Gettings et al., 1996), EC/HO study (Balls et al., 1995) and COLIPA study (Brantom et al., 1997; Harbell et al., 1999); BRD pp. 156-158; BRD summary manuscript Tables VII.a - VII.f] the test was considered suitable for a top-down approach (discriminating severe irritants from all other classes) for water soluble surfactants and surfactant-based formulations (false positive rate, 3-10%[17] with acceptable false negative rate, 9-22%[18]), but not for a bottom-up approach (discriminating non-irritants from all irritant classes) due to high false positive rate (50-69%[19]), although with almost no false negatives (≤ 2%[20]).
    - However, evaluation is limited by deficiency of chemicals with mid-range irritancy potential.
- Based on 29 soluble non-surfactant chemicals tested in 7 laboratories (1 repeated) [CTFA study (Gettings et al., 1996), EC/HO study (Balls et al., 1995) and COLIPA study (Brantom et al., 1997; Harbell et al., 1999); BRD pp. 156-158; BRD summary manuscript Tables VII.a - VII.f] the test was considered suitable for a top-down approach (discriminating severe irritants from all other classes) also for water soluble non-surfactants (false positive rate, 0-6%[21] although with higher false negative rate than for surfactants, 43-55%[22]). A bottom-up approach (discriminating non-irritants from all irritant classes) is again not supported due to high false positive (25-40%[23]) and false negative (24-38%[24]) rates.
- Further evaluation of applicability domain would benefit from additional data, preferably acquired by further testing.
- Instrument used for INVITTOX protocol 102 is no longer commercially available, although it is still in use in a limited number of laboratories. Consumable supplies still commercially available.
- Equipment installation requires committed investment.
- With specialised training, the assay facilitates routine/regular throughput of samples.
- Good agreement of classification (BLV): 15/28 materials (53.6%) with 100% agreement among 4 laboratories and 8/28 materials (28.6%) with agreement in 3/4 laboratories [EC/HO study (Balls et al., 1995)]; 23/26 materials (88.5%) with agreement among 2 laboratories [COLIPA study (Brantom et al. 1997; Harbell et al., 1999)]. The EC/HO and COLIPA studies shared 16 test materials tested in 6 laboratories (1 repeated): 7/14 materials (50%) with 100% agreement among 6 laboratories and 5/14 materials (35.7%) with agreement in 5/6 laboratories. BRD pp. 76, 83, 84, 89; BRD summary manuscript Tables VI.a - VI.h.

[17] EU: 4% (1/27)	GHS: 3% (1/30)	EPA: 10% (3/29)
[18] EU: 19% (5/26)	GHS: 9% (2/23)	EPA: 22% (5/23)
[19] EU: 69% (18/26)	GHS: 68% (17/25)	EPA: 50% (3/6)
[20] EU: 0% (0/27)	GHS: 0% (0/28)	EPA: 2% (1/46)
[21] EU: 6% (1/18)	GHS: 0% (0/18)	EPA: 6% (1/18)
[22] EU: 55% (6/11)	GHS: 45% (5/11)	EPA: 43% (3/7)
[23] EU: 25% (2/8)	GHS: 40% (2/5)	EPA: 25% (1/4)
[24] EU: 24% (5/21)	GHS: 33% (8/24)	EPA: 38% (8/21)

4) <u>Red Blood Cell (RBC) haemolysis</u>

INVITTOX 37 (CTFA) and 99 (COLIPA, EC/HO, Wella-Cosmital)

- Due to discrepancies in specificity and sensitivity apparent among the different studies reviewed (BRD pp. 139-144) a conclusion cannot be drawn on the applicability of the assay for either a top-down (discriminating severe irritants from all other classes) or a bottom-up (discriminating non-irritants from all irritant classes) approach.
- RBC assay protocols may have utility in non-regulatory screening of chemicals for eye irritation potential.
- However if:
    - The data generated from the EC/HO study are not considered due to apparent deviations from protocol and;
    - The data generated from the Wella-Cosmital study are not considered because the prediction model developed in this study did not account for severe irritants (i.e., the prediction model used in the Wella-Cosmital study significantly differs from the PMs developed in the COLIPA and EC/HO studies) and;
    - Only the data generated from the CTFA and COLIPA studies are considered (BRD pp. 115 & 127);

  The test may be suitable for a bottom-up (false negative rate ~11%[25] and false positive rate ~37%[26]) and a top-down approaches (false positive rate ~15%[27] and false negative rate ~21%[28]).
- A more consistent data set would improve confidence on the applicability domain of the RBC assay.

[25] EU: 9.7% (3/31)	GHS: 8.8% (3/34)	EPA: 13.5% (7/52)
[26] EU: 51.2% (21/41)	GHS: 47.4% (18/38)	EPA: 11.1% (2/18)
[27] EU: 15.6% (7/45)	GHS: 14.6% (7/48)	EPA: 14.9% (7/47)
[28] EU: 25.9% (7/27)	GHS: 16.7% (4/24)	EPA: 21.7% (5/23)

b) Recommendations

Potential for use in a top-down approach (discriminating severe irritants from all other classes)

- Cytosensor Microphysiometer (CM)
  - CM considered suitable for a top-down approach (discriminating severe irritants from all other classes) applicable to a broad range of water soluble materials using INVITTOX protocol 102 (modified).
- Fluorescein Leakage (FL)
  - FL considered suitable for a top-down approach (discriminating severe irritants from all other classes) applicable to a broad range of water soluble materials using INVITTOX protocol 71.
- Neutral Red Release (NRR)
  - NRR considered not useful as a screen for ocular corrosives/severe irritants (high false positive rate, including non-irritants classified as corrosive/severe irritants).
- Red Blood Cell (RBC) haemolysis
  - Due to discrepancies in specificity and sensitivity apparent among the different studies reviewed a conclusion cannot be drawn on the applicability of the test for a top-down approach.

Potential for use in a bottom-up approach (discriminating non-irritants from all irritant classes)

- Neutral Red Release (NRR)
  - NRR considered suitable for a bottom-up approach (discriminating non-irritants from all irritant classes) applicable to a broad range of water soluble materials using either INVITTOX protocol 54 or PREDISAFE™.
- Fluorescein Leakage (FL)
  - FL considered suitable for a bottom-up approach (discriminating non-irritants from all irritant classes) applicable to surfactants and surfactant-based formulations using INVITTOX protocol 120.
- Cytosensor Microphysiometer (CM)
  - CM considered not useful as a screen for non-irritants.
- Red Blood Cell (RBC) haemolysis
  - Due to discrepancies in specificity and sensitivity apparent among the different studies reviewed a conclusion cannot be drawn on the applicability of the test for a bottom-up approach.

Follow-up Actions

1. As highest priority, ECVAM will prepare a dossier for distribution among a nominated ESAC peer review panel (PRP) compiling text from the respective BRD summary manuscripts, and including observations and recommendations of this VMG meeting. The dossier will follow conventional format, including module summaries and standard questions for consideration of the PRP.

2. With a view to publication of the four summary manuscripts (to be submitted to ATLA) respective authors (JGA, RCL; RDC; MPV, MMI) should proof read the texts, in particular checking consistency of tabulated data against original references. A fifth publication will be produced by ECVAM for submission to ATLA, consolidating the VMG outcome as a definitive reference. All five manuscripts will be published back-to-back as a coherent series in ATLA.

3. Outcome of the ECVAM review workshop (February 2005) on *in vitro* methods for eye irritation assay, summarised as a manuscript entitled "A Proposed Eye Irritation Testing Strategy to Reduce and Replace *In vivo* Studies Using Bottom-Up and Top-Down Approaches" will be submitted by the corresponding author (VZU) for publication in Toxicology In-Vitro journal, upon receipt of the final version from the principal author (LSC).