シフェラーゼ発現試験
（レセプタープラスミドに作動するエストロゲンレセプター成分-プロモーターのルシフェラーゼ活性）
⑥ERE-βGlob-Luc-SVNeo を安定導入した MCF-7 細胞（ER+）試験
（レセプタープラスミドに作動するエストロゲンレセプター成分-プロモーターのルシフェラーゼ活性）
・受胎能
⑦卵胞バイオアッセイ（マウス）
（卵巣の機能、卵形性、排卵刺激における13日目の極体卵母細胞）
⑧ウシ *in vitro* 成熟試験
（成熟段階中期 II の良好な成果；中期 II への減数分裂の終了）
⑨ウシ *in vitro* 受精試験
（ウシ精子の成熟卵母細胞への透過および雌雄前核のの形成）
⑩マウス胚周囲着床試験
（未分化胚芽細胞段階における 8 日目の生存）
⑪イシカワ細胞試験
（プロゲステロン受容体の mRNA レベル）
・胚発生
⑫全胚培養
（ラット胎仔の成長と形態）
⑬胚性幹細胞試験
（マウス胎児の心筋細胞を拍動させる分化の50%阻害濃度）
⑭ReProGlo 試験
（マウス胎性幹細胞のレセプタープラスミドを作動させる Tcf/Lef プロモーターのルシフェラーゼ活性）

日本では、EST 法の課題を克服する研究が NEDO プロジェクト「高機能簡易型有害性評価手法の開発」として平成 18 年から平成 22 年の 5 カ年で研究が進められた[10)-13)]。本研究により、心筋細胞、神経細胞、筋・骨格系細胞への分化誘導法を確定し、心筋細胞および神経細胞について発光細胞株が樹立され、基本プロトコールが作成された。また、ヒト ES 細胞を用いた検討を行い、マウス ES 細胞で選定したマーカー遺伝子群のヒトにおける有効性も明らかにされた。

さらに、ラット全胚培養法にラット代謝酵素(S-9 mix)を組み合わせ、代謝による被験物質の変化を考慮した催奇形性予測試験法も開発され、24 穴プレートと酸素透過フィルムを用いた培養装置を開発し、培養用の血清を採取する動物数の削減とハイスループット化が達成された。第 24 回日本動物実験代替法学会においても、引続きラット全胚培養法における酸素分圧、代替血液 SALINHES の研究結果も報告された[14),15)]。

OECD テストガイドライン提案に向けてプレバリデーションも計画され、今後のガイドライン化に向けての動きが注目されるところである。

この他、内分泌かく乱物質スクリーニングに関しても試験が進んでいる[16)]。ERα レポーターアッセイ法である HeLa レポーター遺伝子アッセイ（アゴニスト）（STTA 法）は、2009 年 9 月 7 日に OECD ガイドライン 455 として採択された[17)]。一方、HeLa レポーター遺伝子アッセイ（アンタゴニスト）は現在 ECVAM および米国 EPA の共同のもと国内 3 施設、欧州、韓国の 5 施設における国際バリデーション試験中である[18)]。Lumi-cell アッセイに関しては、ICCVAM の評価が終了し、現在ガイドライン案検討の段階である[18)]。また、Cci アッセイは、バリデーションが終了し専門家による第三者評価への準備段階である[18)]。

以上、生殖発生毒性を予測するための *in vitro* 試験法の開発が進められ、一部においては ESAC の承認が得られ、領域毎に有望な方法が開発されつつあるが、生殖発生毒性の検討項目の多さ、複雑さを考えた時、化粧品指令第 7 次改正の期限である 2013 年までに規制に用いることのできる代替法の承認は不可能であると思われる。

C-6-8 経皮吸収性

経皮吸収試験は化粧品、医薬部外品及び医薬品等の皮膚への適用による角質、表皮及び真皮への透過ならびに全身的曝露を評価するために行われる。経皮吸収試験代替法については、実験動物を用いた *in vivo* 試験法（TG 427）[1)]と同時に、動物（主にラット及びブタ）又はヒト摘出皮膚を用いた透過拡散セルによる *in vitro* 試験法（TG 428）[2)]が標準化されている。現在、このガイドラインが経皮吸収試験代替法の中心的な役割を担っている。2006 年 3 月アップデートされた SCCP の“化粧品成分の皮膚吸収における *in vitro* 評価基準”においても、原則的に TG 428 の遵守が求められている[3)]。TG 428 を含めた *in vitro* 試験法において、皮膚の選定、難溶性物質のレセプター相の選択、試験物質の物性など、幾つかの考慮すべき点も報告されている[4)]。

さらに「経皮吸収に関するガイダンス注記」[5)]が 2011 年 8 月 18 日公表されており、*in vivo/in vitro* 実験データ評価における課題解説、*in vivo/in vitro* 動物、*in vivo* ヒトデータの組み合わせ（トリプルパック）アプローチの推奨、実験データ不在の場合の経皮吸収予測に関する解説が示されている。

SCCS は、2010 年 6 月の第 7 回 SCCS 総会で「化粧品成分の皮膚吸収の *in vitro* 評価のための基本的規準」を採択した[6)]。内容として、経皮吸収

に用いる皮膚のサンプル必要数、皮膚質量、経皮吸収係数の相対標準偏差に関する項目などが記載されている。

TG 428 の改良として、代替材料については、再構築ヒト皮膚モデルが最も研究されている。6種の再構築皮膚モデルを用いた透過性が比較され、再構築皮膚モデルの透過性はそれぞれ異なっている事[7]、バリア寄与率や皮内エステラーゼ活性が異なっている事が報告されている[8]。また、再構築ヒト皮膚モデルは有用であるが、その限界についても報告されている[9]。

その他、角層モデルとしての高分子人工膜、PMDS/PEG（ポリジメチルシロキサン/ポリエチレングリコール）共重合体含浸膜を用いたモデルがヒト皮膚との透過係数が近く、代替物としての可能性が検討されている[10,11,12]。

シミュレータによる検討も近年なされており、*in silico* 皮膚中濃度測定方法の有用性[13]、QSARモデルについても報告されている[14]。

また Reduction を考慮した透過性試験における多検体評価用セルについても検討されている[16]。

反復投与による経皮吸収試験については限られた試験期間以内の反復投与でデザインされたいくつかの試験法はあるが、現在、標準化された反復局所投与に利用できる *in vitro* 試験法はなく、代謝を組み込んだ皮膚吸収/透過に利用できる *in vitro* 試験法もない。また、ヒトボランティアによる皮膚吸収試験は、化粧品原料や化粧品製品の低い毒性の場合において行うことができるが、利用できるヒトのデータはほとんどないのが実情である[17]。

C-6-9 小括

本年度の代替法の開発と評価に関する状況を安全性評価項目ごとに取りまとめた。その結果、本年度の特筆すべき動きは、反復投与毒性試験の代替法開発において、EU で SEURAT-1(Safety Evaluation Ultimately Replacing Animal Testing)と呼ばれる巨大プロジェクトが開始されたことが挙げられる。さらに、感作性試験代替法である DPRA、h-CLAT、MUSST などが試験法の評価へ向けて着実に進行していることなど考え合わせると、開発の潮流は、2013 年に禁止される難易度の高い試験の代替へと移ってきていると考えられる。

一方で、光毒性試験代替法・感作性試験代替法では、日本で組織された「ガイダンス検討会」において行政試験法としての受け入れを目指した作業が進められた。また、皮膚一次刺激性試験代替法では24時間曝露による皮膚一次刺激性試験代替法開発へ向けた動きが認められた。このように、代替法が完成したと思われている試験法でも検討が必要な事項が残っていることを示す動きが認められた。

D. 結論

本年度の代替法の開発と評価に関する進展を概観すると、化粧品業界への影響の観点では、動物実験を行った原料を配合する化粧品の販売が 2013 年に EU で禁止される予定の 3 試験項目（反復投与毒性、生殖毒性、薬物動態）について、禁止時期を延期するか否かの検討が行われたことである。予定されていた 2011 年内には、最終結論は公表されなかった。

国際協力の観点では、2011 年 3 月に韓国が代替試験法協力国際会議（ICATM）へ加わったことが挙げられる。代替法の検証研究、評価、ガイドラインの開発等について更なる国際協力が期待される。

代替法開発の観点では、EU において反復投与毒性の開発の促進をめざした巨大プロジェクト Safety Evaluation Ultimately Replacing Animal Testing (SEURAT)-1 が開始されたことが挙げられる。開発の潮流は、2013 年に禁止される難易度の高い試験の代替へと移ってきている。日本においては、*in vitro* を補完する位置づけとして in silico による評価を議論する目的で「in silico ワーキンググループ」が粧工連の動物実験代替専門委員会内に設立された。

行政的な受け入れの観点からは、日本において、2011 年 2 月 4 日に厚労省から事務連絡「医薬部外品の承認申請資料作成時における動物実験代替法の利用と JaVCAM の活用について」が通知されたことを受けて、JaCVAM において行政試験法としての代替法受け入れに配慮した評価法提案のために「ガイダンス検討会」が組織されたことが挙げられる。光毒性試験代替法（3T3 NRU PT）や感作性試験代替法（LLNA）についてガイダンス案が作成された。

代替法の開発と評価は、非常に長い年月を要するためガイドラインとして文書化された場合は別として、単年度では明確にその全貌を捉えることは困難である。したがって、本邦における動物実験代替法の開発と評価を推進し、さらには今後の国際協調への参考情報とするためには、関連する国際情勢の調査と解析を継続して実施し、積み重ねていく必要があると考える。

E. 健康危険情報

なし

F. 参考文献

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なし

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H. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑誌

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Stefan Pfuhler,Mick Fellows, Jafn van Benthem, Raffaella Corvi, Rodger Curren, Kerry Dearfield, Paul Fowler, Roland Frötschl,Azeddine Elhajouji, Ludovic Le Hégarat, Toshio Kasamatsu Hajime Kojima,Gladys Ouédraogo,Andrew Scott and Günter Speit	*In vitro* genotoxicity test approaches with better predictivity : Summary of an IWGT workshop	Mutat Res.	723	101-7	2011
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In vitro genotoxicity test approaches with better predictivity: Summary of an IWGT workshop

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ABSTRACT

Improving current *in vitro* genotoxicity tests is an ongoing task for genetic toxicologists. Further, the question on how to deal with positive *in vitro* results that are demonstrated to not predict genotoxicity or carcinogenicity potential in rodents or humans is a challenge. These two aspects were addressed at the 5th International Workshop on Genotoxicity Testing (IWGT) held in Basel, Switzerland, on August 17–19, 2009. The objectives of the working group (WG) were to make recommendations on the use of cell types or lines, if possible, and to provide evaluations of promising new approaches. Results obtained in rodent cell lines with impaired *p53* function (L5178Y, V79, CHL and CHO cells) and human *p53*-competent cells (peripheral blood lymphocytes, TK6 and HepG2 cells) suggest that a reduction in the percentage of non-relevant positive results for carcinogenicity prediction can be achieved by careful selection of cells used without decreasing the sensitivity of the assays. Therefore, the WG suggested using *p53*- competent – preferably human – cells in *in vitro* micronucleus or chromosomal aberration tests. The use of the hepatoma cell line HepaRG for genotoxicity testing was considered promising since these cells possess better phase I and II metabolizing potential compared to cell lines commonly used in this area and may overcome the need for the addition of S9. For dermally applied compounds, the WG agreed that *in vitro* reconstructed skin models, once validated, will be useful to follow up on positive results from standard *in vitro* assays as they resemble the properties of human skin (barrier function, metabolism). While the reconstructed skin micronucleus assay has been shown to be further advanced, there was also consensus that the Comet assay should be further evaluated due to its independence from cell proliferation and coverage of a wider spectrum of DNA damage.

1. Introduction

When examining the ability of the current standard *in vitro* genotoxicity test batteries to discriminate rodent non-carcinogens from carcinogens [1,2], it becomes clear that improving current *in vitro* tests to help carcinogenicity prediction presents a major challenge for genetic toxicologists. Positive *in vitro* results that are not predictive of the genotoxic/carcinogenic potential in rodents or humans can trigger unnecessary *in vivo* follow up testing and require extensive time and personnel within regulatory agencies as well as industry. Because of the resources involved in clarifying positive results from standard *in vitro* testing, companies often

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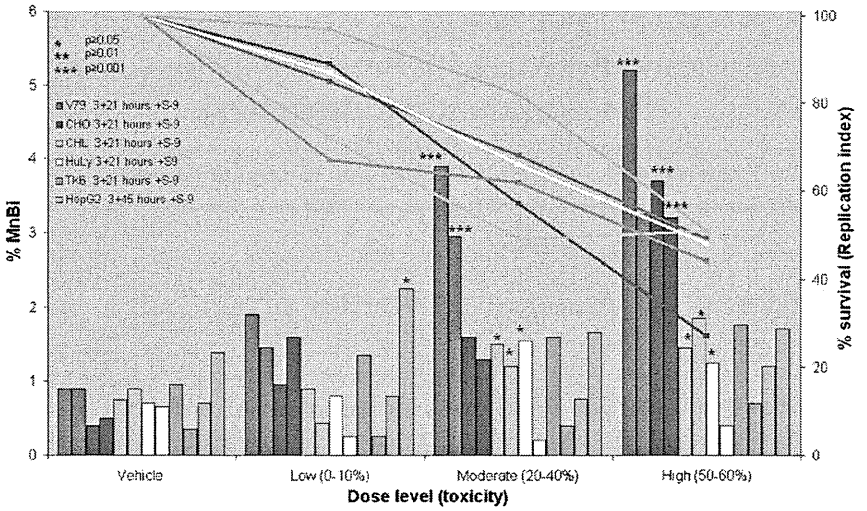

Fig. 1. Micronucleus response in V79, CHO, CHL, TK6, HuLy and HepG2 cells after treatment with 2,4-dichlorophenol for 3 h in the presence of S9. Experiments were performed on two occasions, plotted separately.

eliminate such ingredients from use, thereby losing potentially safe and useful ingredients [3]. The complexity of risk assessments for compounds positive in standard *in vitro* genotoxicity assays is illustrated by the many recent publications, *e.g.* [4,5], external workgroups (ILSI/HESI – [6]), and meetings devoted to this topic (ECVAM – [7]). Efforts towards improving standard genotoxicity test battery approaches are also driven by regulations enforced within the European Union (EU) such as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) [8] and the 7th Amendment to the Cosmetics Directive [9]. The 7th Amendment prohibited any acute *in vivo* genotoxicity tests for cosmetic ingredients as of March 2009, thereby triggering searches for innovative hazard and risk assessment concepts [3]. The requirements of the REACH Integrated Testing Strategy (ITS) (Guidance on information requirements and chemical safety assessment [10]) ask for follow-up of any positive outcome in the *in vitro* standard battery with appropriate *in vivo* tests, regardless of the tonnage level of the chemical which may lead to unnecessary *in vivo* studies in situations where carcinogenicity predictivity is low.

The low specificity for carcinogenicity prediction observed in mammalian cell *in vitro* tests and the question how this can be improved was discussed at the 5th International Workshop on Genotoxicity Testing (IWGT) held in Basel, Switzerland, on August 17–19, 2009. The WG "*In vitro* Genotoxicity Test Approaches with Better Predictivity" consisted of fifteen genotoxicity experts from academia, regulatory authorities and industry representing different geographies. The first objective of the WG was to review data on the response of the cell lines commonly used for mammalian cell tests, and to consider whether it was possible to make recommendations or restrictions on the use of any particular cell line. The second part of the workshop focused on new approaches and reviewed the status of development/validation of a few pre-selected promising new methods, *i.e.* genotoxicity assays performed with 3D human skin equivalents and use of HepaRG cells. It was recognized that other promising methods are under development which were however not discussed due to time constraints. Although the impact of employing different measures of cytotoxicity was discussed during the workshop, it will not be discussed in this paper since it was addressed in a recent report of the WG on toxicity measures and top concentration for *in vitro* cytogenetics assays [11].

2. Summaries of the presentations given at the workshop

2.1. Cell selection

Results were presented from an initiative by the European Cosmetics Industry Association (COLIPA) for improvement of *in vitro* mammalian cell assays. In this project Chinese hamster cell lines with impaired *p53* function (V79, CHL and CHO cells) have been compared with *p53*-competent human peripheral blood lymphocytes (HuLy), TK6 human lymphoblastoid cells, and the human liver cell line HepG2 in terms of *in vitro* MN induction following treatment with a selection of 19 compounds that were accepted as producing "false" positive results for carcinogen prediction in *in vitro* mammalian cell assays [12]. The detailed results generated for the first six chemicals tested (curcumin, urea, resorcinol (1,3-dihydroxybenzene), anthranilic acid, ethyl acrylate and menthol) were presented at the workshop, and are described, along with data from the other 13 chemicals, in Fowler *et al.* [13]. The results clearly demonstrate that the established rodent cell lines were more susceptible to both cytotoxicity and micronucleus (MN) induction than *p53*-competent cells, and are therefore more prone to give non-relevant positive results (Fig. 1). These data suggest that a reduction in the percentage of non-relevant positive results for carcinogenicity prediction can be achieved by careful selection of the mammalian cell type for genotoxicity testing.

Data from the same project were also presented which highlighted the genetic stability of several commonly used cell lines with over 50 passages in continuous culture. Chromosome counts were made at intervals of 3 passages up to a maximum of 50 passages. TK6 cells maintained a stable number of chromosomes whereas the modal chromosome number for CHL decreased by 2 and for CHO increased by 1 (Figs. 2 and 3), thus illustrating that these established and commonly used rodent cell lines are more prone to genomic instability as time in culture increases. Further experiments were performed investigating the differences between two isolates of the same cell line. CHL cells sourced from low passage stocks in Japan (Deposited by Ishidate, Japan Cell Bank) and cells that have been used at Covance (UK) for a number of years, originally derived from the same stock were compared after treatment with resorcinol in the same experiment, from the same formulation of chemical (Fig. 4). The data highlighted significant differences in response with the magnitude of MN induction being

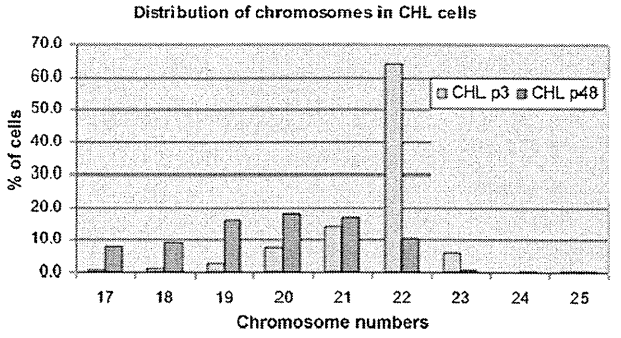

Fig. 2. Distribution of chromosome numbers in early and late passage CHL cells.

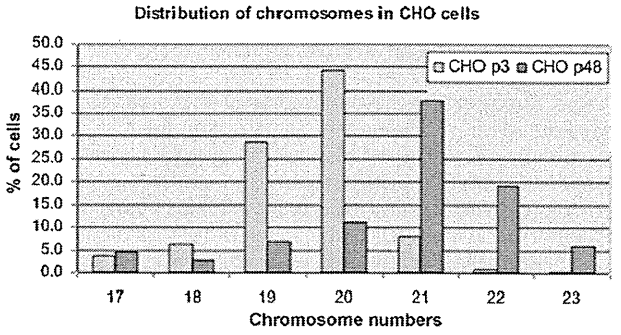

Fig. 3. Distribution of chromosome numbers in early and late passage CHO cells.

much greater in the Covance cell stocks although both isolates ultimately gave a positive MN outcome. These data reinforce the observation that longer periods in culture and continued passaging of cells can lead to greater levels of instability and may therefore affect the reliability of the results. As many laboratories have their own stocks of these and other cell types, it was recommended that laboratories using cell lines *in vitro* adhere to good cell practice, characterize all new cells, check regularly for genetic drift and work from low passage stocks.

Data generated in 10 different laboratories (Sanofi-Aventis, AstraZeneca, HLS, Servier, Roche, Novartis, Institut Pasteur, Covance, Swansea University, BAT) in conjunction with the OECD *in vitro* MN guideline (TG487) finalization were presented [14]. In this multi-laboratory exercise, 11 chemicals were evaluated in five different cell lines (CHL, V79, CHO, L5178Y, TK6). The 11 chemicals

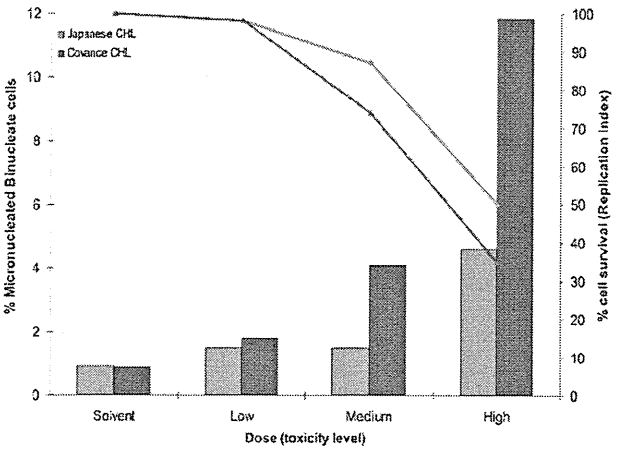

Fig. 4. Micronucleus assay data from two isolates of CHL cells after treatment with resorcinol.

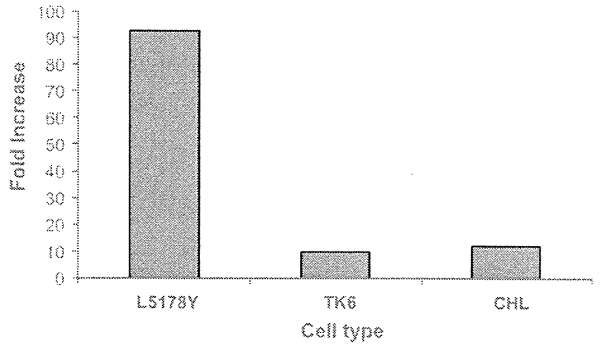

Fig. 5. Fold increase in the number of micronucleated mononuclear cells at approximately 50% RPD for L5178Y, TK6 and CHL cell lines following 3 h MMC treatment and 24 h recovery.

investigated were relevant *in vivo* genotoxic carcinogens, namely cytosine arabinoside, mitomycin C (MMC), benzo[a]pyrene (B[a]P), cyclophosphamide (CPA), colchicine, vinblastine, 5-fluorouracil (5FU), diethylstilboestrol, 2-aminoanthracene (2-AA), etoposide and cadmium chloride. With one exception (2-AA in CHO cells in one lab), all chemicals were, as expected, positive in the *in vitro* MN test, in all cell lines, in all laboratories at concentrations inducing approximately 50% toxicity as measured by relative population doublings. 5FU, 2-AA and the aneugens were weakly positive, but similar responses were seen for all cell lines used. For some of the potent mutagens tested (MMC, CPA, etoposide and cytosine arabinoside), the response in L5178Y cells was often higher than the other cell lines, while the response in TK6 cells was often lower (Fig. 5). There was discussion as to whether this was due to the cell lines' relative *p53* status. However, other factors such as the relative doubling times of the cell lines could also have contributed to the different responses (TK6 cells were far slower growing than L5178Y). While there was some discussion about the impact of the *p53* status on the magnitude of the effects it is important to emphasize that the ability to correctly detect *in vivo* genotoxic carcinogens was not impacted by *p53* status as only quantitative but no qualitative differences were observed.

Another comparison of several cell lines was also presented. Different cell types (V79, L5178Y and TK6) have been used historically at Novartis for *in vitro* MN screening, and therefore can be compared with results from human primary lymphocytes (peripheral blood) for *in vitro* MN induction or the regulatory chromosome aberration assay. The comparison for 65 compounds (V79 cells), 51 compounds (L5178Y cells) and 80 compounds (TK6 cells) were shown and the sensitivity, specificity and concordance were noted in relation to the MN test or the chromosomal aberration (CA) test in human lymphocytes (Tables 1–3). Notably all cell lines were good at detecting the positives from the primary human lymphocyte studies, but the rodent cell lines were expressing a low specificity (around 60%), i.e. gave positive results with up to 40% of chemicals that were negative in human lymphocytes. Not surprisingly, the *p53*-proficient TK6 cells had the best overall concordance (81%) with a specificity of 80%. Therefore it was speculated that the *p53*-

Table 1
Comparison results for the V79 cell line.

Reference test system	V79 Chinese hamster cells		
Chromosome aberration test (human lymphocytes and/or V79 cells) or MN test in human lymphocytes	Sensitivity	100%	(14/14)
	Specificity	61%	(31/51)
	Concordance	69%	(45/65)

Table 2
Comparison results for the L5178Y cell line.

Reference test system	Mouse lymphoma (L5178Y) cells		
Chromosome aberration test in human lymphocytes	Sensitivity	100%	(6/6)
	Specificity	72%	(18/25)
	Concordance	77%	(24/31)
Chromosome aberration test or MN test in human lymphocytes	Sensitivity	100%	(9/9)
	Specificity	60%	(25/42)
	Concordance	67%	(34/51)

Table 3
Comparison results for the TK6 cell line.

Reference test system	Human lymphoblastoid TK6 cells		
Chromosome aberration test in human lymphocytes	Sensitivity	90%	(9/10
	Specificity	85%	(28/33)
	Concordance	86%	(37/43)
Chromosome aberration test or MN test in human lymphocytes	Sensitivity	90%	(18/20)
	Specificity	78%	(47/60)
	Concordance	81%	(65/80)

status might explain the overall predictivity of the different cell lines in detecting the *in vitro* MN induction or chromosome aberration induction in the *p53*-proficient primary human lymphocytes.

2.2. Promising new methods

Data generated with HepaRG cells using a series of chemical genotoxic carcinogens that require metabolic activation to form their ultimate genotoxic metabolite were presented. The human hepatoma cell line HepaRG shows promise for use in the area of genotoxicity testing due to its excellent metabolic competency. The cells were isolated from a differentiated liver tumour, exhibit limited karyotypic alterations, and have the property of transdifferentiation. Differentiated HepaRG cells express wild type P53 protein and various cytochromes P450 phase II enzymes, transporters and nuclear factors, at levels close to those found in primary human hepatocyte cultures [15–18]. A series of genotoxic carcinogens that require metabolic activation to form their ultimate genotoxic metabolite have been tested using HepaRG. At the workshop, results obtained with the Comet assay and the cytokinesis-block MN assay (24 h treatment for both) were presented and are depicted qualitatively in Table 4 (for details see [19,20]). The promutagens aflatoxin B1, B[a]P, acrylamide, N-nitrosodimethylamine, CPA and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) lead to a relevant increase in DNA damage as measured by the alkaline Comet assay while all except PhIP and acrylamide also increased the frequency of micronuclei in HepaRG cells. In contrast, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQx) were not detected by either assay. Pyrene, which was included as a negative control, did give the expected negative result in both assays. The direct mutagens glycidamide and methylmethanesulfonate gave the expected positive responses.

Table 4
Results from selected indirect mutagens in Comet and micronucleus assays in HepaRG cells.

Compounds	Comet assay	Micronucleus assay
Aflatoxin B1	+	+
B[a]P	+	+
Cyclophosphamide	+	+
PhIP	+	–
IQ	–	–
MeIQx	–	–
Acrylamide	+	–
N-dimethylnitrosamine	+/–	ND
Glycidamide[1]	+	+
Methylmethanesulfonate[1]	+	+
Pyrene[2]	–	ND

1: direct mutagens; 2: negative control; ND = not determined.

Also promising is the application of standard genotoxicity endpoints with reconstructed human skin models. These 3-dimensional reconstructed tissues are prepared from primary human cells and are anticipated to have normal DNA repair and cell cycle control. They are also expected to exhibit a human metabolic capability that is more relevant than the exogenous rodent metabolizing enzymes currently used in standard *in vitro* genotoxicity assays [3]. Normal, intact skin is an effective barrier to many chemicals [21] which means that the penetration of many compounds applied to the skin is blocked by the stratum corneum barrier preventing the materials from entering the systemic circulation. The skin is also metabolically active [22,23], chemicals which do penetrate it can be detoxified (or activated) during their passage. Based on this, assays using 3D human reconstructed skin models offer the potential for a more physiologically relevant approach to test dermal exposure.

Micronucleus data generated in human 3-dimensional skin models were presented. The *in vitro* reconstructed skin micronucleus (RSMN) assay in the EpiDerm™ 3D human skin model [24,25] has been recently shown to produce comparable data when utilized in three different laboratories in the United States [26]. As part of a project sponsored by COLIPA, with a contribution from the European Centre for the Validation of Alternative Methods (ECVAM), international prevalidation studies of the RSMN assay have been initiated.

The COLIPA prevalidation project consists of three phases: in phase I the protocol was optimized and the transferability of the method to laboratories with no previous experience was demonstrated, phase II addressed intra- and inter-laboratory reproducibility, and phase III is designed to demonstrate how well the RSMN assay performs in terms of sensitivity, specificity and overall predictivity with respect to detecting genotoxic (rodent) carcinogens. Thirty chemicals were selected for this effort in a similar fashion to the previously described COLIPA project aiming at improving the specificity of standard mammalian cell tests [12]. Ten chemicals each were selected to represent three different groups of chemicals, i.e. *in vivo* genotoxic rodent carcinogens, non-genotoxic non-carcinogens and *in vitro* genotoxic but *in vivo* non-genotoxic rodent non-carcinogens that have been shown to

Table 5
Overview of the tissues models used in the reconstructed skin Comet assay.

Tissue tradename	Model	Cell origin	Donors	Medium
Epiderm™	Reconstructed human epidermis	Newborn foreskin	Single donor	Defined medium
Phenion® FT SM	Reconstructed full thickness skin	Newborn foreskin	Single donor	Defined medium
RealSkin	Reconstructed full thickness skin	Adult female breast	Keratinocytes: 3–4 (pooled donors) fibroblasts: single donor	Serum containing medium

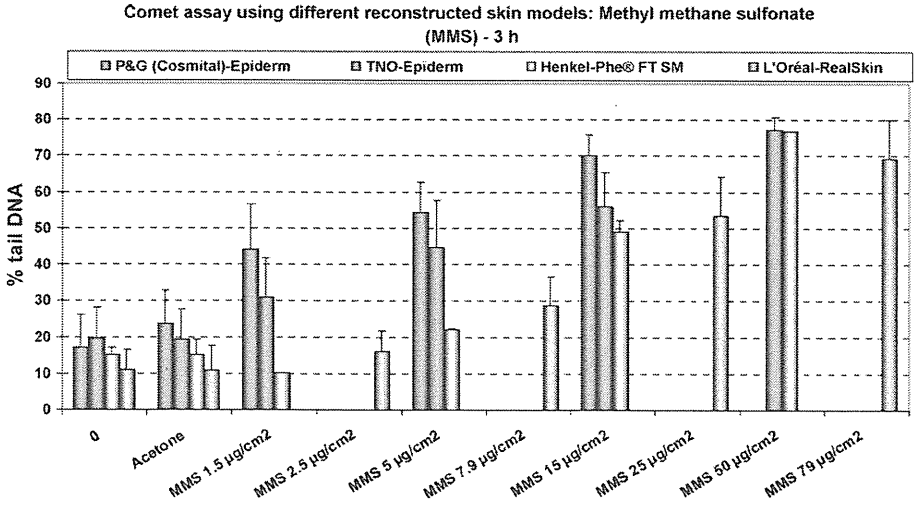

Fig. 6. Results from four different labs with MMS-treated (3 h topical treatment, solvent = acetone) different reconstructed skin models in the Comet assay. The presented data are the mean values from 16 (P&G), 6 (TNO), 2 (Henkel) and 13 (L'Oreal) individual experiments ± SD, performed with 2–4 tissues per concentration.

produce non-predictive positive results in *in vitro* assays. In phase I the assay was optimized by the Institute for In Vitro Sciences (IIVS) and Procter and Gamble (P&G) and then transferred to a laboratory in Europe (L'Oreal, France), where dose-dependent, reproducibly positive results similar to those seen at P&G and IIVS were obtained for MMC and vinblastine sulfate. In phase II, further intra- and inter-laboratory reproducibility of the RSMN assay was established by blind testing of N-ethyl-N-nitrosourea (ENU), cyclohexanone, and MMC in three independent experiments in every laboratory. ENU and MMC, both known genotoxins, were identified by all laboratories in every individual experiment as positive, while cyclohexanone, a known non-genotoxin, was identified as negative by all laboratories [27].

As the MN assay is able to efficiently capture clastogenic and aneugenic effects but is not very effective at detecting DNA damage leading to gene mutation, the other objective of the COLIPA 3D skin project utilized the alkaline Comet assay which detects primary DNA damage caused by a broad spectrum of genotoxic activity [28]. Data generated with this assay using the three different RS models (MatTek EpiDerm™, L'Oreal RealSkin and Henkel Phenion FT SM) were presented. The skin models (Table 5) were topically exposed to the test compounds for 3 h, followed by cell isolation and preparation of slides for the Comet assay. The assay was successfully transferred from P&G and L'Oreal to Henkel, Germany, and TNO, The Netherlands. Good inter-laboratory reproducibility of the 3D skin Comet assay was demonstrated for the direct-acting mutagens methylmethanesulfonate (MMS) (Fig. 6) and 4-nitroquinoline 1-oxide (4NQO) (data not shown). Cytotoxicity was evaluated either by the trypan blue assay or other cell viability assay. In all results shown here, cytotoxicity was ≤20%. All three skin models used showed reproducible background for untreated and solvent (acetone) treated tissues (Table 6) and a significant increase with the two model mutagens. Phase II of the project is ongoing and investigates the inter- and intra-laboratory variability using 3 coded chemicals.

Data from a Comet assay generated at JaCVAM using a 3-dimensional human epidermal model (EPI-MODEL, LabCyte, Japan) were presented. After initial experiments to establish a protocol, a series of well-characterized genotoxic agents were investigated, i.e. CPA, MMC, MMS, nitrosodimethylamine and 4NQO. All of these compounds except CPA clearly increased the background DNA damage, see Figs. 7 and 8 for representative data from treatment with 4-NQO. In parallel with the genotoxic activity, cytotoxicity

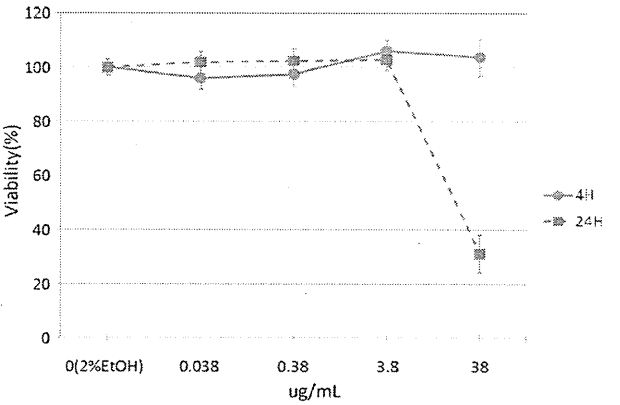

Fig. 7. Cytotoxicity of 4NQO on MTT assay using LabCyte EPI-MODEL. Closed circles show the 4 h treatment data while the closed squares show the 24 h data. 4-NQO was applied onto two models per dose after dissolving in 2% ethanol solution in water.

was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, measured directly after the 4 h treatment, and after 24 h. The decrease in viability indicated by the MTT measurement after 24 h showed a correlation with the increase in DNA damage while the measurement after 4 h showed an effect for high doses of 4-NQO only (Fig. 8).

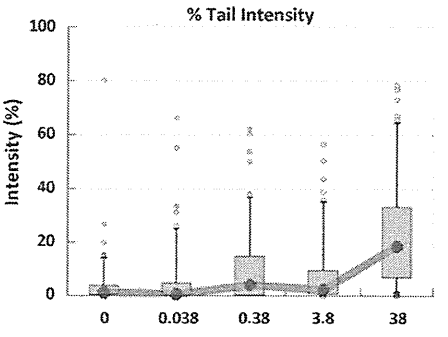

Fig. 8. Percent tail intensity measured in the Comet assay after 4NQO treatment using LabCyte EPI-MODEL. Dots show the median values of 2 × 50 cells measured from two individual tissues per dose after 4 h treatment. 4-NQO (ug/ml) was applied onto models after dissolving in 1/50 ethanol/water mixture.

Table 6
DNA damage background levels for untreated (negative control) and solvent control (3 h treatment) in the reconstructed skin Comet assay.

Skin model	Epiderm™			Phe® FT SM	RealSkin
Laboratory	P&G (Cosmital)	TNO	Henkel	Henkel	L'Oréal
Number of experiments	$n=16$	$n=8$	$n=3$	$n=2$	$n=13$
Negative control	17.0 ± 9.3	19.6 ± 8.5	34.5 ± 3.2	15.3 ± 1.7	11.0 ± 5.7
Solvent control (acetone)	23.8 ± 9.1	19.3 ± 8.2	26.4 ± 3.0	15.1 ± 4.3	10.7 ± 7.0

3. Discussion and consensus statements

3.1. Selection of cells for chromosomal aberration or micronucleus tests

The results from an initiative of COLIPA which looked at a series of chemicals that are accepted to give non-relevant positive results for carcinogenicity prediction indicated that the established rodent cell lines were more susceptible to both cytotoxicity and MN induction than *p53*-competent cells. It was concluded that rodent cell lines therefore are more prone to give possible non-relevant positive results for carcinogenicity prediction. The data suggest that an increase in specificity can be achieved by careful selection of the cell type for genotoxicity testing. The question was discussed whether such an increase of specificity would come at the cost of decreased sensitivity of the assays. To this end, multiple laboratories tested 11 *in vivo* genotoxic carcinogens in 5 cell lines in conjunction with the finalization of the OECD *in vitro* MN guideline (TG487). All of those chemicals were positive in the *in vitro* MN test, in all cell lines and all laboratories at concentrations inducing approximately 50% toxicity as measured by relative population doublings. These results demonstrate that the *p53*-status did not impact the ability of the cell lines to correctly detect *in vivo* genotoxic carcinogens. Data from Novartis supported the above findings. While all cell lines used were good at detecting the positive results from studies performed with primary human lymphocytes, the rodent cell lines were not as good at predicting negative results (around 60% specificity). The *p53*-proficient TK6 cells showed the best overall concordance (81%), with a specificity of 80%, compared to the results with primary human lymphocytes. These findings are also supported by a recent paper showing that in HepG2 cells *p53* was activated by a series of mutagens and promutagens [29].

The workshop WG concluded that the presented data indicate that *p53*-compromised rodent cell lines over-estimate genotoxic potential in the MN test. There was also agreement that it is biologically plausible that this statement is also applicable to the CA assay based on the similarity of both assays. Therefore, the IWGT WG suggested using *p53*-competent cells for *in vitro* MN- or CA-tests.

Another topic that was discussed at the workshop was the impact of the cell lines' genetic stability on the outcome of genotoxicity assays. Data from a COLIPA project highlighted the genetic stability of TK6 cells which maintained a stable number of chromosomes whereas the modal chromosome number for CHL and CHO cells decreased by 2 or increased by 1, respectively. Also a comparison of CHL cells between a low passage stock and an isolate that was used at a contract research organization (CRO) for several years showed differences in the magnitude of response to treatment with an *in vivo* non-genotoxic non-carcinogen. Taken together, it is suggested that longer periods in culture and continued passage of cells can lead to greater levels of instability and will therefore likely negatively impact the reliability of the results. The WG concluded that it has been demonstrated that cell line stability and source can affect the outcome of genotoxicity assays. Therefore, the IWGT WG recommended adherence to good cell culture practice, characterization of all new cells, checking regularly for genetic drift, and working from low passage stocks. It was emphasized that a common cell bank with fully characterized stocks of all cells commonly used for genotoxicity testing would be very useful.

The above agreement already led to an ILSI/HESI initiative that initiated the generation of such a cell bank, a depot for all cell lines commonly used in genotoxicity testing. The idea is to collect, store and provide to the scientific community the most original version of the cell lines which will be tested for stability and functionality.

3.2. Promising new methods

The human hepatoma cell line HepaRG was of interest to the workshop as it expresses wild type *p53* protein and various cytochrome P450 phase II enzymes at levels close to those found in primary human hepatocyte cultures. The data generated with HepaRG cells with a series of chemical carcinogens that require metabolic activation showed that HepaRG is a promising model and the workshop especially acknowledged the better phase I and II metabolizing potential of these cells compared to cell lines commonly used in this area. It was, however, agreed that further data and evaluation will be required to confirm the value of this model for genotoxicity testing.

The workshop acknowledged the advantages of 3-dimensional human skin models in that they resemble the properties of human skin (barrier function, metabolism), making them relevant for dermally applied compounds. A COLIPA prevalidation project is ongoing for the reconstructed skin in MN and Comet assays. Results were presented at the workshop that demonstrate the successful completion of the first phase of the project (optimization and demonstration of the transferability of the method to labs with no previous experience) for both assays. Phase II which addresses the intra- and inter-laboratory reproducibility of coded compounds was successfully completed for the RSMN with two known genotoxins and one non-genotoxin. All were correctly identified by all laboratories in every individual experiment [29].

The WG concluded that the data presented show that the RSMN is further advanced than the RS Comet assay since for the RSMN inter and intra-lab reproducibility has been successfully demonstrated. The IWGT WG however agreed that the Comet assay should be further evaluated as it is seen as a valuable addition to the MN assay due to its independence from cell proliferation and coverage of a wider spectrum of DNA damage. The WG did recommend evaluating further the metabolic capacity of the reconstructed skin models, and acknowledged the fact that this is work in progress by COLIPA, but data from this project were not presented at the workshop. Furthermore it was agreed that it would be valuable to capture the kinetics of penetration and toxicity in order to establish the ideal sampling time(s) for the Comet assay and that the use of appropriate vehicles should be further investigated. In the ongoing project a total of 30 chemicals will be investigated. This however may not be sufficient to establish the applicability domain of the assay and it was noted that more chemicals may have to be tested to do so once the prevalidation is completed. The WG agreed that 3D genotoxicity models, once validated, will be useful to follow up on positive results from standard *in vitro* assays for dermally applied compounds.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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