Table 6. 3T3-NRU test: comparison of *in vitro* and *in vivo* classifications of phototoxicity based on MPE

		In vivo classification			
		phototoxic	non-phototoxic	unclear	total
In vitro classification	phototoxic	189	7	1	197
	non-phototoxic	14	38	7	59
	total	203	45	8	256
		table statistics for the 2 × 2 table in the box			
	sensitivity:	93%	*prevalence:*		4.51
	specificity:	84%			
	positive predicitivity:	96%			
	negative predicitivity:	73%			
	accuracy:	**92%**			
	χ^2:	**129.03**	(> 3.8)		

could not be used in the analysis of interlaboratory variability for chemicals no. 7 and no. 18, since they were not produced according to the SOP (see Tables 3 and 5). The examples given are representative, to illustrate the interlaboratory variability of data that is due to differences in the use of solvents and concentration ranges used for testing as outlined below and shown in Tables 7 and 8.

Interlaboratory variability of the *in vitro* classification

The effect of interlaboratory variability of the PIF and MPE values derived from the concentration–response curves of two independent runs was assessed by computing the classification variability (CV) for each test chemical. According to Table 3, the CV was on an average smaller than 10% (range 0–18.8%) for phototoxic chemicals when using PIF values for classification, and 6% (range 0–16%) for the five non-phototoxic chemicals. When the MPE was used for classification, the CV was 4% (range 0–16.8%) for phototoxic chemicals and 10% (range 0–20%) for the non-phototoxic ones.

These results indicate that the classifications obtained in Tables 3 and 5 would be confirmed in more than 90% of all repeat experiments, provided that the data produced in these experiments would have the same distribution and extent of errors as the data generated in this study. The statistical analysis demonstrates that, despite large deviations among the PIF and MPE values obtained with some of the chemicals, the classification of phototoxic potential derived from PIF or MPE values in the 3T3 NRU PT test is very robust.

Technical factors affecting the determination of PIF and MPE

As the 3T3 NRU PT test is a cellular *in vitro* method, test chemicals have to be added to the aqueous culture medium. Thus, solubility in water may limit the spectrum of chemicals that can be tested in this way. Table 2 shows that eight of the test chemicals are reported in the literature to be "practically insoluble in water" (solubility class 7). Testing of these chemicals in an aqueous cellular system therefore requires the use of appropriate solvents. The effective concentration of insoluble chemicals in the incubation medium and the test results will depend on the solvent used and the maximum concentration reached.

Solubility in water

Two solubility categories were designated for the test chemicals, namely, category I, "poorly soluble in water" (H_2O solubility classes 6 or 7 in Table 2) and category II, "soluble in water" (H_2O solubility classes 1–5 in Table 2), and the *in vitro/in vivo* discordances observed within the two categories were compared. Statistical analysis showed that there was no significant influence of the water solubility properties of the test chemicals on the rate of discordance. The discordances observed with some of the chemicals in the PIF calculations and in the MPE approach cannot be accounted for simply by their poor solubility in water and any experimental problems related to it.

Solvents used

The solvents used by individual laboratories are given in Table 7. Despite the guidance provided by the SOP, the use of solvents varied considerably among the laboratories for a given chemical. According to Table 7, one laboratory (No. 2) never used a solvent at all, while another laboratory (No. 11) used solvents other than EBSS in all of the experiments. To add to the problem, some of the laboratories used different solvents in the two independent test runs.

Table 7. 3T3 NRU PT test: solvents used

Chemical	sol. class	LAB 1	LAB 2	LAB 3	LAB 4	LAB 6	LAB 8	LAB 9	LAB 10	LAB 11
2-hydroxy-4-methoxybenzophenone	7	DMSO	●	DMSO	ETOH	ETOH	DMSO	DMSO	ETOH	DMSO
Sodium lauryl sulfate	3	●	●	●	●	●	●	●	●	PBS
Hexachlorophene	6/7	● / DMSO	●	DMSO	ETOH	ETOH	DMSO	DMSO	●	ETOH
p-Aminobenzoic acid (PABA)	5	●	●	DMSO	ETOH	●	DMSO	●	ETOH	DMSO
Penicilin G	2/3	●	●	●	●	●	●	●	●	PBS
Chlorhexidine dihydrochloride	4/6	DMSO	●	●	●	●	DMSO	DMSO	●	DMSO
Furosemide	5/7	not tested	●	●	ETOH	●	DMSO	●	DMSO	ETOH
Bergamot oil	?	●	●	DMSO	ETOH	● / ETOH	DMSO	DMSO	ETOH	DMSO
Amiodarone	6	● / DMSO	●	PME	DMSO	●	DMSO	DMSO	DMSO	DMSO
Nalidixic acid–free acid	6	●	●	DMSO	ETOH	ETOH	ETOH	DMSO	●	DMSO
Nalidixic acid–sodium salt	?	●	●	DMSO	ETOH	DMSO	●	●	●	ETOH
Ofloxacin	?/5	●	●	●	●	●	●	●	ETOH	PBS
Anthracene	7	●	●	●	DMSO	●	ETOH	DMSO	DMSO	DMSO
Fenofibrate	7	●	●	DMSO	ETOH	ETOH	DMSO	DMSO	DMSO	ETOH
Musk ambrette	7	●	●	DMSO	ETOH	ETOH	DMSO	DMSO	DMSO	DMSO
Ketoprofen	7	●	●	DMSO	ETOH	●	DMSO	●	ETOH	DMSO
Bithionol	7	DMSO	●	●	ETOH	●	DMSO	DMSO	●	DMSO
Chlorpromazine	1	●	●	●	●	●	●	●	DMSO	DMSO
Promethazine	1	●	●	●	ETOH	ETOH	●	●	DMSO	PBS
Rose bengal	6	●	●	DMSO	●	●	●	●	DMSO	PBS
Protoporphyrin IX–free acid	?	●	●	●	●	ETOH	●	●	●	DMSO
6-methylcoumarin	7	●	●	DMSO	ETOH	ETOH	ETOH	DMSO	DMSO	DMSO
Protoporphyrin IX–disodium salt	?	●	●	●	●	●	●	●	●	DMSO
Tiaprofenic acid	5	●	●	●	ETOH	ETOH	DMSO	●	ETOH	ETOH
Demeclocycline	3/4	●	●	●	●	ETOH	DMSO	●	DMSO	PBS
Acridine–hydrochloride	3	●	●	●	ETOH	●	DMSO	DMSO	DMSO	DMSO
Acridine–free base	?/5	●	●	●	ETOH	DMSO	ETOH	DMSO	●	DMSO
Norfloxacin	4/5	●	●	●	DMSO	●	DMSO	●	DMSO	DMSO
Neutral red	3	●	●	●	●	●	ETOH	●	●	PBS
5-methoxypsoralene (5-MOP)	7	●	●	●	DMSO	DMSO	DMSO	DMSO	ETOH	DMSO

● = no solvent used (tested in EBSS).
●/ DMSO = tested without solvent in one experiment and with DMSO in the other.
●/ ETOH = tested without solvent in one experiment and with ETOH in the other.

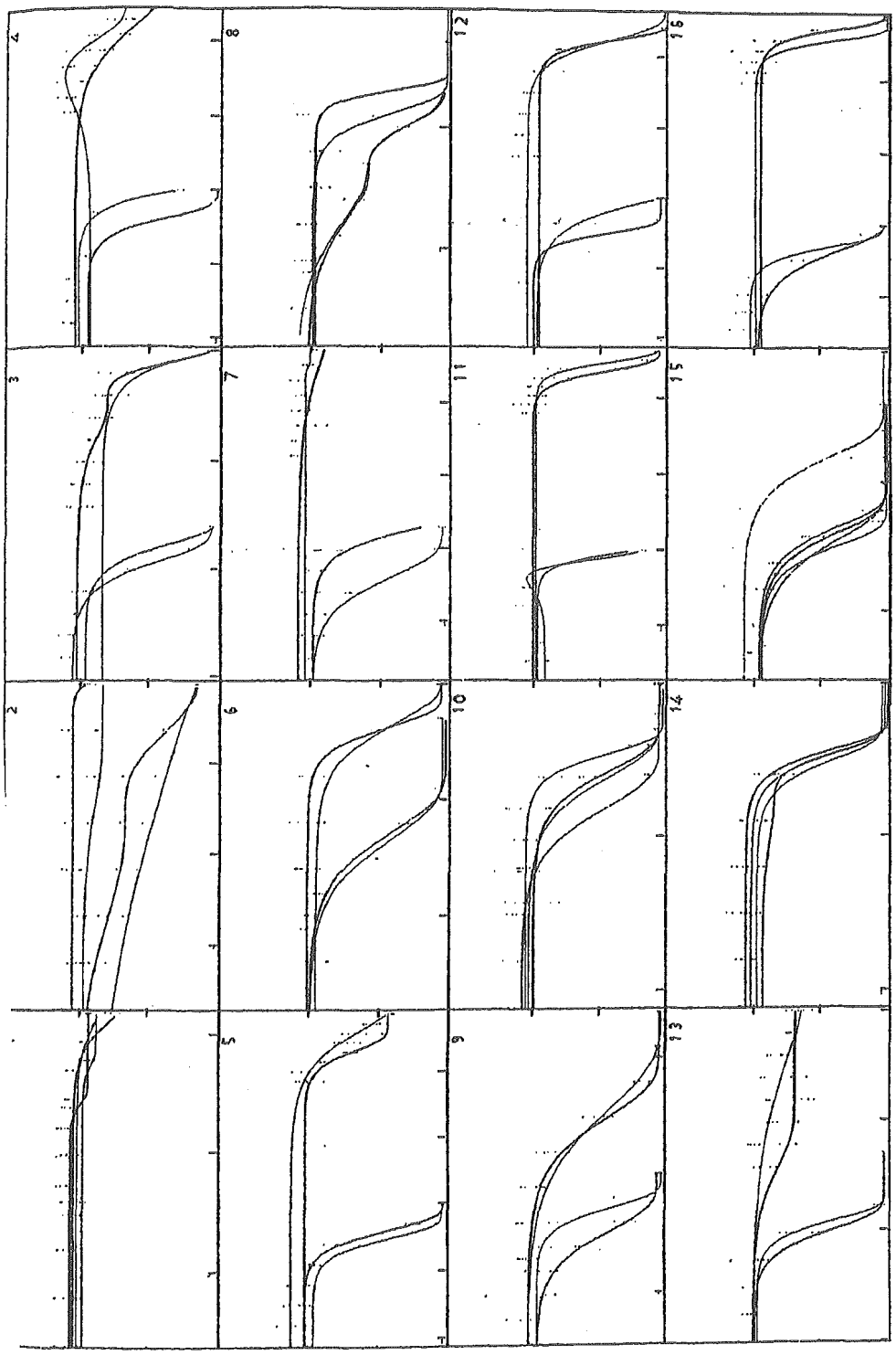

Fig. 4. Intralaboratory variability of the 3T3 NRU PT test. An example is given for two independent determinations with chemicals 1–16 in laboratory 9, to illustrate the intralaboratory reproducibility of the test and differences in the shapes of –UV/ + UV concentration–response curves. The "miniplots" were generated by using the mathematical concentration–response model FITGRAPH (Holzhütter and Quedenau, 1995). The chemicals were tested at different concentrations ranging from μg/ml to mg/ml. Therefore, no units are given on the x-axis. Moreover, to illustrate differences in the –UV/ + UV concentration–response curves, for some of the chemicals a log scale was used in the "miniplots" and for others a linear scale.

Table 8. 3T3 NRU PT test: highest concentrations tested in μg/ml (or % saturated solution if noted)

Chemical	solub. class	LAB 1	LAB 2	LAB 3	LAB 4	LAB 6	LAB 8	LAB 9	LAB 10	LAB 11
2-hydroxy-4-methoxybenzophenone	7	1800	10000	1000	100	250	1000	1500	215	12
Sodium lauryl sulfate	3	43.2	8000	100	100	100	56.2	200	100	50
Hexachlorophene	6/7	10	100	19.3	10	50	17.8	40	100%	12
p-Aminobenzoic acid (PABA)	5	10360	6000	10000	2150	5000	1000	10000	1230	4000
Penicilin G	2/3	100000	10000	10000	100000	50000	100000	10000	100000	100
Chlorhexidine dihydrochloride	4/6	80	10000	100%	100	5000	31.6	100	100%	15
Furosemide	5/7	●	10000	10000	100	5000	1000	10000	1780	1500
Bergamot oil	?	1500	5000	562	100	10	100	1000	7740	12
Amiodarone	6	100	1000	1000	68.1	100	100	100	63.1	3
Nalidixic acid–free acid	6	●	10000	1000	100	1000	1000	1000	100%	150
Nalidixic acid–sodium salt	?	10000	5000	10000	3000	20000	10000	10000	100%	50
Ofloxacin	?/5	●	100000	100000	100000	840000	100000	10000	251000	200
Anthracene	7	●	10000	100%	10	100	1000	50	10	0.75
Fenofibrate	7	●	10000	1000	100	100	1000	1000	562	6
Musk ambrette	7	●	10000	1000	100	1000	178	1500	215	6
Ketoprofen	7	●	5000	10000	1000	10	1000	10000	5010	1500
Bithionol	7	10	1000	100%	21.5	25	10	25	100%	5.1
Chlorpromazine	1	20	50	68.1	178	50	100	40	100	50
Promethazine	1	50	50	100	100	100	100	200	100	150
Rose bengal	6	3.47	5	10	10	1	3.16	10	10	1
Protoporphyrin IX–free acid	?	30	100	100%	10	50	5.62	5000	3160	50
6-methylcoumarin	7	●	10000	10000	400	1000	1000	1000	1470	1125
Protoporphyrin IX–disodium salt	?	10000	1000	100%	10	500	562	5000	2510	3
Tiaprofenic acid	5	8000	10000	10000	1000	200	1000	5000	1000	2000
Demeclocycline	3/4	2500	1000	10000	1000	1000	1000	5000	1780	300
Acridine–hydrochloride	3	●	100	100%	100	1000	1000	300	316	99
Acridine–free base	?/5	●	100	100%	10	1000	1000	750	100%	35
Norfloxacin	4/5	●	1000	100%	100	1000	1000	5000	251	300
Neutral red	3	●	1000	1000	100	25	1000	32	100	300
5-methoxypsoralene (5-MOP)	7	80	1000	100%	10	100000	1000	75	0.251	30

● = no information provided.

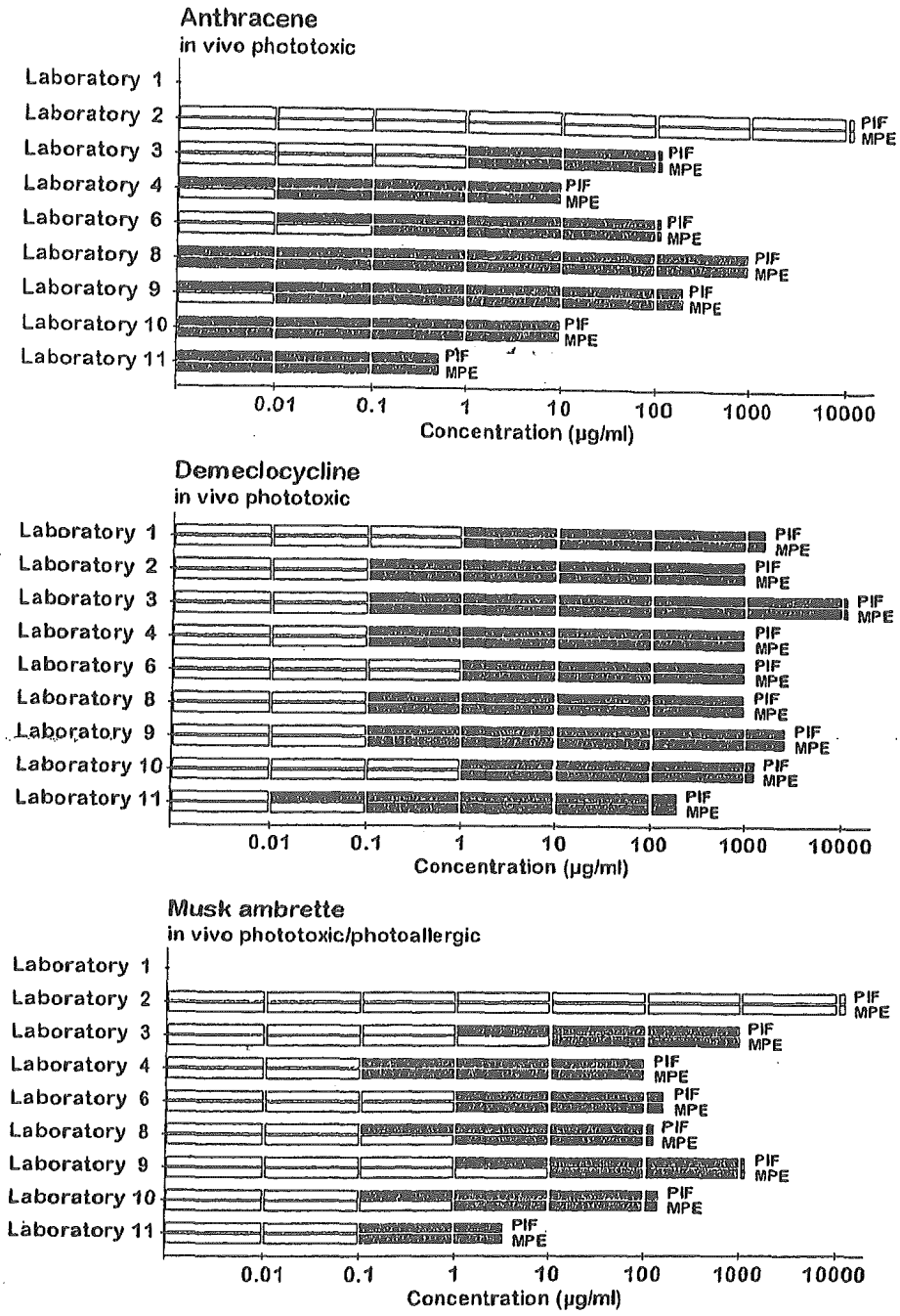

Fig. 5. Effect of test concentration ranges on interlaboratory variability of PIF and MPE for three test chemicals. PIF and MPE age given for three phototoxic chemicals anthracene (no. 8), demeclocycline (no. 12) and musk ambrette (no. 18) to illustrate the dependence of the determination of the phototoxic potential on test concentrations used. Test concentrations are given on the x-axis in µg/ml. Dark bars indicate positive classification according to the prediction model for both PIF and MPE.

No statistically significant correlations were nd between the discordances of *in vitro/in vivo* a and the solvents used. In classifications ived from PIF values, the number of cases ht) in which the classification of chemicals rly soluble in water was wrong, when a solvent used, was only slightly smaller than the number discordances (10) observed when no solvents e used.

Concentration range

The maximum concentrations of chemicals used in individual laboratories for each of the test chemicals is given in Table 8, together with the solubility classes. Although guidance was provided in the SOP for selecting the optimum concentrations for testing, Table 8 shows that one of the laboratories (No. 11) used much lower concentrations than all

of the others. At the same time, data in Tables 3 and 5 prove that the results obtained in this laboratory were better than in most of the others, most of which tested at the highest possible concentrations rather than at the optimum concentrations.

To evaluate whether the concentration ranges chosen by individual laboratories had an effect on *in vitro*/*in vivo* discordance, the test chemicals were grouped into two categories related to test concentrations:

category I – max concn in the(–)UV experiment $< 1000\ \mu g/ml$

category II – max concn in the (–)UV experiment $> 1000\ \mu g/ml$

No significant differences were found between the rates of *in vitro*/*in vivo* discordance between chemicals tested at low (category I) or high (category II) concentrations.

DISCUSSION

Biostatistical analysis

The two versions of the prediction model proposed for the 3T3-NRU PT test, PIF and MPE, provided an accurate prediction of *in vivo* phototoxicity for almost all the test chemicals. Only one chemical (no. 14) could not be correctly classified by the majority of the participating laboratories. As furosemide was incorrectly classified as "non-phototoxic" by the majority of laboratories, the *in vivo* data should to be carefully evaluated again. There was no indication that the remaining *in vitro*/*in vivo* discordances could be accounted for by solubility problems.

The effect of data variability on *in vitro* classification was small. Taking into account *in vitro* classifications derived from all available data for a given chemical, CV was computed. The analysis revealed that only for 10% of the test chemicals (three of 30) would repeated testing of the chemicals result in a different classification in more than 10% of the tests, if MPE values were used for classification. The classification variability was slightly higher when PIF values were used. In general, however, the classification of phototoxicity derived from MPE and PIF values in the 3T3 NRU PT test was very robust.

To compare the predictive power of a variety of *in vitro* phototoxicity tests, in phase I of the study, we tested a more even distribution of phototoxic (11) and non-phototoxic (nine) test chemicals (Spielmann *et al.*, 1994a,b and 1995). As all the non-phototoxic chemicals were correctly identified in phase I, we focused in phase II on the technical limitations of the *in vitro* assays by testing chemicals from different chemical classes, as well as chemicals with differences in solubility in water. Thus, only five non-phototoxic chemicals were tested in the present study, which resulted in a prevalence of 4.5 of phototoxic chemicals *v.* non-phototoxic chemicals (Tables 3 and 5). This resulted in a high positive predictivity (MPE: 95%) and a low negative predictivity (MPE: 72%). An even distribution of phototoxic and non-phototoxic test chemicals would have allowed the predictivity assessment to be less biased.

Determination of PIF v. MPE

The data in Tables 3 and 5 show that, from a biostatistical point of view, using the MPE to express the performance of the 3T3 NRU PT test was a major improvement, since an MPE value could be determined for all of the chemicals, while PIF values could not be determined for some of the chemicals. As an MPE value can be determined even when only a limited section of the concentration response curve has been measured, in instances where solubility was a limiting factor for determining the PIF, phototoxic potential could be assessed by the MPE approach. Therefore, the number of "false negatives" could significantly be reduced (Tables 3 and 5). This is evident for bergamot oil (no. 8), nalidixic acid, both in the free acid (no. 20) and sodium salt (no. 19) forms, and ofloxacin (no. 23). In particular, MPE values for bergamot oil, which is not water soluble, and ofloxacin, which was distributed as a 2.5% solution, gave highly positive results in all of the laboratories. The results of the present study suggest that the MPE approach, devised by Holzhütter (1997), will be very useful in the development and validation of new *in vitro* phototoxicity tests in the future.

Influence of solubility

To test whether the aqueous solubility of chemicals might limit the predictive power of the 3T3 NRU PT assay, three phototoxic chemicals were tested in phase II in the form of free acids or bases and also as salts (acridine, nalidixic acid and protoporphyrin IX). The data obtained with both the PIF and the MPE approaches revealed that the three chemicals were correctly identified to be phototoxic, irrespective of their solubility. We therefore conclude that the test is able to identify phototoxic chemicals of the classes tested, irrespective of their aqueous solubility, which was not expected at the beginning of the study.

Chemicals not identified correctly in the 3T3 NRU PT test

Some of the test chemicals were not classified correctly in this 3T3 NRU PT blind trial test due to technical problems that can clearly be identified. Some examples are as follows:

Furosemide (no. 14)

Furosemide, classified as "phototoxic" *in vivo* by the chemicals TF and the MT, was identified as "non-phototoxic" in the 3T3 NRU PT test. A critical review of the literature shows that in most of the human data, including standardized patch testing (Spielmann *et al.*, 1994c), furosemide was reported not to be photoirritant, except in a few case reports. This drug also gave negative results in animal tests (see Table 2), and, not only in the 3T3 NRU PT test, but also in all the other *in vitro* assays tested under blind conditions in phase II of the present study (*data to be published in Part 2 of the validation study report*).

Photoallergens

Ketoprofen (no. 16) and musk ambrette (no. 18) are photoallergens with some weak phototoxic properties, while **PABA** (no. 24) has no phototoxic properties within the range tested. Although the 3T3 NRU PT test was not designed to identify photoallergens, the test might pick up photoactivation potential that may eventually lead to photoallergy. Therefore, the positive results obtained with two of the three photoallergens in the 3T3 NRU PT test are not unexpected and are, in fact, to be welcomed.

Errors due to blind testing

Blind testing may also have contributed to misclassification. **Ofloxacin** (no. 23) is a good example, since it was distributed as a 2.5% solution in saline. However, no information was provided to the laboratories, in order to maintain absolutely blind testing conditions. Analysis of the raw data and laboratory protocols shows that, in all of the laboratories, the 2.5% **ofloxacin** solution was further diluted, as the laboratories assumed from the general information provided by the MT that liquids contained 100% of the active test chemical. Ofloxacin is a good example to prove that the 3T3 NRU PT test is very robust, as the MPE method provided positive data for ofloxacin in all of the laboratories, irrespective of the dilution chosen. To avoid some of the potential technical problems resulting from blind testing, in a special blind trial on UV-filter chemicals in the 3T3 NRU PT test that is currently under way, an appropriate solvent is recommended for each test chemical, and 10 mg/ml is suggested as the highest concentration for testing.

Deviations from the SOP

Deviations from the SOP (INVITTOX, 1994) nay also have resulted in misclassification. In ssence, any deviation from the SOP should disqua-fy such data from being included in the final nalysis of the performance of the 3T3 NRU PT :st. For this particular reason, data from one lab-oratory for 14 chemicals were exclud overall analysis of the performance NRU PT test, since the –UV/+UV co response curves were not produced con was required by the SOP. Quite often, is very difficult during a blind trial to ic ations from the SOP. By accident, the after the study was finished that one of tories had used a SOL 3 lamp instead 500 lamp from the same company. H SOL 3 and SOL 500 lamps gave sim because their spectra were very similar.

Solubilizing the test chemicals appr another problem that may not have bee sufficiently in the SOP, which suggested be used, and if necessary, other so instance, ethanol. Our analysis of the which is compiled in Table 7, reveale laboratory had tested all the chemical (EBSS) and had never used any othe whereas other laboratories had used o vents with almost all the chemicals. D lack of precision in the SOP, the result prisingly good, irrespective of the solvent a few exceptions, as demonstrated, for Fig. 5 and discussed above for laborato consequence, the SOP has now been a give better guidance for the selection of priate solvent.

Another problem arose from differen concentrations used in individual la (Table 8; Fig. 5). The statistical analysis revealed that the concentration range testing had an influence on the measurem phototoxic potential. However, the phot tential of all chemicals could be identi appropritately high test concentrations we Therefore, the SOP has now been amend vide better guidance on the selection of mum concentrations of chemicals tested i NRU PT assay.

Application of the 3T3 NRU PT test to t identification process

The 3T3 NRU PT test was developed the phototoxic potential of chemicals, ir of the route of administration. Therefor assay, mouse fibroblasts are exposed to concentrations of test chemicals to –UV/+UV concentration–response cur concentrations that induce an acute p effect in 3T3 mouse fibroblasts may cor exceed the concentrations effective at th level in the *in vivo* situation in humans. some of the test chemicals may not be able trate through the stratum corneum and/or tinocyte layer of the epidermis into the Three-dimensional human skin analogues better information on this important aspe hazard identification. This assumption is s

by results obtained with a commercially distributed human skin testing kit (*the data will be published in Part 2 of the validation study report*). Moreover, while it seems unlikely that cells of the mouse 3T3 fibroblast cell line are able to metabolize xenobiotics in the same way as the human body after systemic application or as human cells *in vivo* after systemic application or as the human skin, in particular, all of the chemicals known to be phototoxic in humans also proved phototoxic in the 3T3 NRU PT test. However, the data in the present validation study do not indicate that IC_{50} values obtained with the 3T3 NRU PT test are significantly correlated to the phototoxic potency of chemicals *in vivo* in humans.

Despite this limitation, the 3T3 NRU PT test is well suited to identifying the phototoxic potential of chemicals. This information is essential for hazard assessment in the field of phototoxicity. For safety evaluation in humans, however, additional information may be required on the metabolism and skin penetration of chemicals.

Conclusions and recommendations

The MT and the participating laboratories of phase II of the ECVAM/COLIPA validation study on *in vitro* phototoxicity tests conclude that the 3T3 NRU PT test is a validated, robust, *in vitro* phototoxicity test according to the criteria laid down by the ECVAM *Workshop on practical aspects of the validation of toxicity test procedures* (Balls *et al.*, 1995). Biostatistical analysis employing the determination of either a PIF or a MPE revealed that this *in vitro* test is characterized by high sensitivity, high specificity and high predictivity.

Owing to the convincing performance of the 3T3 NRU PT test in phase I and phase II of the present study, the test is now established and in use, even in industry laboratories which did not participate in the study. We therefore recommend the implementation of the 3T3 NRU PT test for regulatory purposes. In a special study supported by ECVAM, 10 UV-filter chemicals, which are regulated under the provisions of the Cosmetics Directive of the EU, will be tested in the 3T3 NRU PT test, at the request of the Scientific Committee on Cosmetology (SCC), which advises the European Commission on all matters related to the safety of cosmetic products. As an additional part of this ECVAM contract, an OECD-style guideline for *in vitro* phototoxicity testing will be drafted for the 3T3 NRU PT test, for submission to the OECD test guidelines programme.

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（添付資料）
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A Study on UV Filter Chemicals from Annex VII of European Union *Directive 76/768/EEC*, in the *In Vitro* 3T3 NRU Phototoxicity Test

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Summary — In 1996, the Scientific Committee on Cosmetology of DGXXIV of the European Commission asked the European Centre for the Validation of Alternative Methods to test eight UV filter chemicals from the 1995 edition of Annex VII of *Directive 76/768/EEC* in a blind trial in the *in vitro* 3T3 cell neutral red uptake phototoxicity (3T3 NRU PT) test, which had been scientifically validated between 1992 and 1996. Since all the UV filter chemicals on the positive list of EU *Directive 76/768/EEC* have been shown not to be phototoxic *in vivo* in humans under use conditions, only negative effects would be expected in the 3T3 NRU PT test. To balance the number of positive and negative chemicals, ten phototoxic and ten non-phototoxic chemicals were tested under blind conditions in four laboratories. Moreover, to assess the optimum concentration range for testing, information was provided on appropriate solvents and on the solubility of the coded chemicals. In this study, the phototoxic potential of test chemicals was evaluated in a prediction model in which either the Photoirritation Factor (PIF) or the Mean Photo Effect (MPE) were determined. The results obtained with both PIF and MPE were highly reproducible in the four laboratories, and the correlation between *in vitro* and *in vivo* data was almost perfect. All the phototoxic test chemicals provided a positive result at concentrations of 1μg/ml, while nine of the ten non-phototoxic chemicals gave clear negative results, even at the highest test concentrations. One of the UV filter chemicals gave positive results in three of the four laboratories only at concentrations greater than 100μg/ml; the other laboratory correctly identified all 20 of the test chemicals. An analysis of the impact that exposure concentrations had on the performance of the test revealed that the optimum concentration range in the 3T3 NRU PT test for determining the phototoxic potential of chemicals is between 0.1μg/ml and 10μg/ml, and that false positive results can be obtained at concentrations greater than 100μg/ml. Therefore, the positive results obtained with some of the UV filter chemicals only at concentrations greater than 100μg/ml do not indicate a phototoxic potential *in vivo*. When this information was taken into account during calculation of the overall predictivity of the 3T3 NRU PT test in the present study, an almost perfect correlation of *in vitro* versus *in vivo* results was obtained (between 95% and 100%), when either PIF or MPE were used to predict the phototoxic potential. The management team and participants therefore conclude that the 3T3 NRU PT test is a valid test for correctly assessing the phototoxic potential of UV filter chemicals, if the defined concentration limits are taken into account.

Keywords: in vitro toxicology, phototoxicity, 3T3 NRU PT test, UV filter chemicals, EU Directive 76/768/EEC.

Introduction

A joint European Union/European Cosmetic, Toiletry and Perfumery Association (EU/COLIPA) validation study on *in vitro* phototoxicity tests has recently shown that the *in vitro* 3T3 cell neutral red uptake phototoxicity (3T3 NRU PT) test is a scientifically validated, sensitive and robust, *in vitro* phototoxicity test according to the criteria laid down by the European Centre for the Validation of Alternative Methods (ECVAM) for the validation of toxicity test procedures (1). In the 3T3 NRU PT test, the phototoxic potential of chemicals is assessed by determining the Photoirritation Factor (PIF) or the Mean Photo Effect (MPE) from concentration–response curves in the presence or absence of UV light (2). In the EU/COLIPA validation study, the 3T3 NRU PT test underwent prevalidation in Phase I (3), and a formal validation study under blind conditions with 30 coded chemicals in Phase II (1). The biostatistical analysis revealed that the 3T3 NRU PT test is an *in vitro* test that is characterised by high sensitivity, specificity and predictivity. The ECVAM Scientific Advisory Committee (ESAC) has concluded that the 3T3 NRU PT test is a scientifically validated test, which is ready to be considered for regulatory purposes (4). Therefore, the ESAC and ECVAM, as well as DGIII and DGXI of the European Commission have recommended the adoption of the 3T3 NRU PT test for regulatory purposes, and the submission to the OECD test guidelines programme of a draft guideline for *in vitro* phototoxicity testing based on the 3T3 NRU PT test. Due to its convincing performance in Phase I and Phase II of the validation study, the 3T3 NRU PT test is already widely established in laboratories of the pharmaceutical and cosmetic industries.

The formal validation study was aimed at evaluating the limitations of the 3T3 NRU PT test and, in particular, assessing whether the phototoxic potential of test chemicals could be correctly assessed irrespective of solubility and the solvents used. Thus, the selection of test chemicals was intentionally unbalanced by the inclusion of 25 phototoxic and five non-phototoxic chemicals, most of which were pharmaceuticals, rather than chemicals used by the cosmetic industry. To evaluate whether or not the 3T3 NRU PT test would permit assessment of the phototoxic potential of UV filter chemicals, the Scientific Committee on Cosmetology (SCC, now the Scientific Committee on Cosmetology and Non-Food Products [SCCNFP]), which advises the European Commission on all matters related to the safety of cosmetic ingredients, asked ECVAM in 1996 to sponsor a study on eight to ten UV filter chemicals in the 3T3 NRU PT test. Since UV filter chemicals currently registered in Annex VII of EU *Directive 76/768/EEC* have been shown not to be phototoxic under use conditions in humans, negative effects were expected in the 3T3 NRU PT test.

We have previously reported that, when no information was provided with the coded chemicals in Phase I and Phase II of the EU/COLIPA validation study, the laboratories used a wide spectrum of solvents and a broad range of test concentrations (1). Statistical analysis indicated that classification of phototoxic potential was robust and not affected by these factors. Nevertheless, two of the least soluble materials (anthracene and musk ambrette) demonstrated variability attributed to solvent use and the concentration range applied (1). Information on physicochemical properties would be available under routine testing conditions in a toxicology laboratory. Therefore, in this study, guidance was provided on the most appropriate solvent and on the maximum soluble concentration for each coded chemical.

Materials and Methods

Management and funding

This study was conducted according to the recommendations of ECVAM on validation (5), as a blind trial involving four laboratories in Europe and the USA. A subgroup of the COLIPA Task Force on Photoirritation was established to review the phototoxicity, safety data and physicochemical characteristics of suitable test chemicals. ZEBET did not participate in the phototoxicity testing in the study, but was responsible for refining the Standard Operating Procedures (SOPs), for the selection of appropriate solvents and test concentrations, and for the distribution and coding of the test chemicals. Special software for data collection was developed by Hermann-Georg Holzhütter (Humboldt University, Berlin), who also conducted the data

analysis according to the guidelines of the ECVAM Task Force on Biostatistics (6). COLIPA and ZEBET provided data to establish the phototoxicity database for selecting the UV filter chemicals, as well as for the other test chemicals. The participating laboratories and the names of all those who actively contributed to the study are listed in Table I. ECVAM awarded a contract to ZEBET to manage the study. In addition, ECVAM's funding covered the purchase, coding and shipping of the test chemicals, and, via subcontracts, the biostatistical analysis and the expenses of the laboratories conducting the 3T3 NRU PT test.

Selection of test chemicals

To provide a balanced representation of positive and negative chemicals in the study, as well as a sufficient number of test chemicals for biostatistical evaluation, the management team decided to test ten phototoxic and ten non-phototoxic chemicals (Table II). A subgroup of the COLIPA Task Force evaluated the safety data of the UV filter chemicals listed in Annex VII of *Directive 76/768/EEC*. From the UV filter chemicals listed in Annex VII, only eight were backed by safety data of acceptable quality from testing in humans or animals (Table II). These UV filter chemicals' data met the quality criteria recommended by ECVAM (5), the US Interagency Coordinating Committee on Validation of Alternative Methods (ICCVAM; 7), and the OECD (8). The additional two non-phototoxic and ten phototoxic test chemicals were selected from the list of chemicals that had been tested in Phase I and Phase II of the EU/COLIPA validation study, which is supported by *in vivo* data from clinical testing in humans (1, 3, 9).

Irradiation, design of the 3T3 NRU PT test, and the prediction model

The 3T3 NRU PT test was carried out according to a SOP, as described in the report on Phase II of the EU/COLIPA validation study (1, *INVITTOX* protocol number 78[1]). For the present study, the SOP was refined to include more-stringent guidance on the use of solvents and on the most appropriate concentration of test chemicals. This information was derived from experiments carried out at the ZEBET laboratory. Furthermore, the phototoxic potential of test chemicals was assessed in the 3T3 NRU PT test, applying two versions of the prediction model from Phase II of the EU/COLIPA validation study, which were based on the PIF or the MPE (1, 2).

Prediction model

Original version, based on the Photoirritation Factor (PIF)

The prediction model described in the SOP used in this validation study was derived from an analysis of data obtained in the preceding EU/COLIPA prevalidation study (3). It is based on a comparison of two equally effective cytotoxic chemical concentrations (EC50 values) obtained in concurrently performed experiments in the presence (+UV) and absence (–UV) of UVA irradiation. The EC50 values obtained in the light and dark experiments were compared by calculating the PIF:

$$PIF = \frac{EC50\ (-UV)}{EC50\ (+UV)}$$

The PIF is low for non-phototoxic chemicals and high for phototoxins. Discriminant analysis was conducted on the results obtained in the prevalidation study (3). This biostatistical procedure revealed that, for predicting the phototoxic potential of chemicals, PIF = 5 is the best cut-off value for discriminating between phototoxic (PIF > 5) and non-phototoxic (PIF < 5) chemicals. If a chemical is cytotoxic only in the presence of UV light, the PIF cannot be calculated, although this is a result which indicates phototoxic potential. In such cases, a "> PIF" can be calculated, provided that the -UV cytotoxicity test is performed up to the highest test concentration (C_{max}), and this value is used for calculation of the "> PIF" value:

$$> PIF = \frac{C_{max}\ (-UV)}{EC50\ (+UV)}$$

If only a "> PIF" value can be obtained, any value above 1 predicts a phototoxic potential. If neither EC50 (–UV) nor EC50 (+UV) can be calculated, because a chemical exhibits no

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Table I: Institutions and scientists that participated in the study

Institutions	Contributing scientists
ZEBET, BgVV Berlin, Germany (Management)	Horst Spielmann,[a] Manfred Liebsch, Christa Barrabas, Dieter Traue
COLIPA Brussels, Belgium (Sponsor)	Jack Dupuis[a]
European Commission — ECVAM Ispra, Italy (Sponsor)	Michael Balls[a]
Beiersdorf AG Hamburg, Germany[b]	Wolfgang J.W. Pape,[a] Uwe Pfannenbecker
Humboldt-Universität zu Berlin (Charité) Berlin, Germany (Biostatistics)	Hermann-Georg Holzhütter
L'Oréal Aulnay-sous-Bois, France	Odile de Silva[a]
Subcontract to: Bioalternatives Gençay, France[b]	Alain Deguercy, François Xavier Bernard
The Procter & Gamble Company Cincinnati, OH, USA[b]	G. Frank Gerberick, Lynn W. Cruse
Unilever Environmental Safety Laboratory Sharnbrook, UK[b]	Will W. Lovell, Penny Jones

[a]*Management team members.*

[b]*The four laboratories which performed the 3T3 NRU PT test.*

cytotoxicity up to the highest test concentration, this indicates no phototoxic potential. In such cases, a formal "PIF = *1" is used to characterise the result:

$$PIF = *1 = \frac{C_{max}\,(-UV)}{C_{max}\,(+UV)}$$

If only a "PIF = *1" can be obtained, this indicates no phototoxic potential.

Refined version, based on the Mean Photo Effect (MPE)

To overcome the limitations of the PIF-based prediction model, a novel measure of phototoxic potential, the MPE, has recently been proposed (2). It is based on a comparison of the +UV and -UV concentration–response curves on a grid of concentrations, c_i (i = 1 . . . N), chosen from the common concentration range of the dark and light experiments. The photo effect (PE_i) at concentration c_i is calculated as a product of the concentration effect (CE_i) and the response effect (RE_i). The MPE is obtained by averaging across all PE_i values. Analogous to PIF, the MPE can be used in the prediction model for the phototoxic potential of chemicals by comparing it with a cut-off value, MPE_c = 0.1, which was derived from data obtained

Table II: The 20 test chemicals used in the study

Number	COLIPA number	Chemical	CAS number
1.	S13	Octyl salicylate	118-60-5
2.	S28	Octyl methoxycinnamate	5466-77-3
3.	S59	Benzylidene camphor sulphonic acid and its salts	56039-58-8
4.	S60	4-Methylbenzylidene camphor	36861-47-9 38102-62-4 95342-41-9
5.	S61	3-Benzylidene camphor	15087-24-8
6.	S71	Terephthalidene dicamphor sulphonic acid and its salts	90457-82-2
7.	S72	Polyacrylamidomethyl benzylidene camphor	113783-61-2
8.	S40	Benzophenone-4[a]	4065-45-6
9.		L-Histidine free base[a]	7006-35-1
10.		Sodium lauryl sulphate[a]	151-21-3
11.		Protoporphyrine IX, disodium	50865-01-5
12.		Chlorpromazine hydrochloride	69-09-0
13.		Anthracene	120-12-7
14.		Acridine hydrochloride	17784-47-3
15.		Ketoprofen	22071-15-4
16.		Promethazine hydrochloride	58-33-3
17.		Amiodarone hydrochloride	1951-25-3
18.		Demeclocycline hydrochloride	64-73-3
19.		Bithionol	97-18-7
20.		Musk ambrette	83-66-9

[a]*Used as a non-phototoxic test chemical in Phase I or Phase II of the EU/COLIPA in vitro phototoxicity validation study (1, 3).*

In vivo data for UV filter chemicals 1–7 were obtained in human patch tests according to the protocol of Kaidbey & Kligman (11) and in animal tests according to the protocol of Guillot et al. (12).

Chemicals 8–20 were evaluated in Phase I and Phase II of the EU/COLIPA in vitro phototoxicity validation study (1, 3).

Chemicals 1–8 are UV filter chemicals (sunscreens) identified by the letter "S" in the COLIPA number.

Chemicals 1–10 are non-phototoxic in vivo. Chemicals 11–20 are phototoxic in vivo.

with keratinocytes in Phase II of the EU/COLIPA study (2).

Both the MPE and PIF calculations are based on a comparison of two concentration–response curves obtained concurrently for a chemical +UV and –UV. However, the two versions of the prediction model use different algorithms.

Biostatistical analysis

The statistical data analysis focused on the assessment of the quality of the prediction

models developed during Phase II of the EU/COLIPA study (1). The values of both endpoints were determined by the participating laboratories in a unique fashion, by employing the NRU-PIT2 software package, which was designed as part of the SOP of this assay. This software converts the discrete raw data (optical densities at various concentrations of the test chemical) into a concentration–response relationship, which is then fitted to a mathematical model to yield a continuous concentration–response curve. The computation of PIF and MPE was performed by comparing pairs of concentration–response curves obtained in the absence (-UV) and presence (+UV) of UV light. In this study, values of PIF and MPE were determined for two independent runs, and the mean of these two values was used to classify chemicals and to determine the variability of data within and between laboratories (intralaboratory and interlaboratory variability).

Comparison of in vitro data and prediction of phototoxic potential in vivo

Concordance between predictions of phototoxic potential derived from *in vitro* data (based on either PIF or MPE) and the *in vivo* properties of the test chemicals was determined according to the recommendations of the ECVAM Task Force on Biostatistics (6) by using a 2 × 2 contingency table to calculate sensitivity, specificity, positive predictivity, negative predictivity, accuracy and significance.

Analysis of intralaboratory and interlaboratory data variability

The influence of the three factors, replicate, run and laboratory, on the variability of PIF and MPE values was analysed by breaking down the overall sum of deviation squares (SDS) for a given chemical tested in all laboratories into three contributions:

$$SDS = SDS_{inter} + SDS_{exp} + SDS_{rep}$$
$$= SDS_{inter} + SDS_{intra}$$

These represent the mean sum of deviation squares due to interlaboratory variability (SDS_{inter}), variability among independent experiments (SDS_{exp}), and variability among replicates (SDS_{rep}). The latter two contributions can be put together to measure the mean intralaboratory variability (SDS_{intra}). The computation of the mean square sums in the right-hand side of this equation is based on the corresponding square sums for each laboratory.

Analysis of the effect of the highest test concentration on the correct prediction of phototoxic potential

In order to investigate how the prediction of phototoxic potential from *in vitro* data is affected by the concentration interval chosen for testing in the 3T3 NRU PT test, the values for PIF and MPE and the associated classifications of phototoxicity were calculated by continuously increasing the concentration interval from 0.01μg/ml up to the highest achievable concentration, as shown in Figure 1. The curves were evaluated by using PIF or MPE, and the results are shown below the curves in Figure 1. Grey-shadowed bars indicate the classification "non-phototoxic", while black bars indicate "phototoxic", when the upper concentration limit for curve evaluation was the value indicated by the arrows. The highest testable concentration recommended by ZEBET is indicated by a dashed vertical line in the concentration–response diagram. The results of this analysis are shown in Figure 2 as bar diagrams for the two endpoints, PIF and MPE.

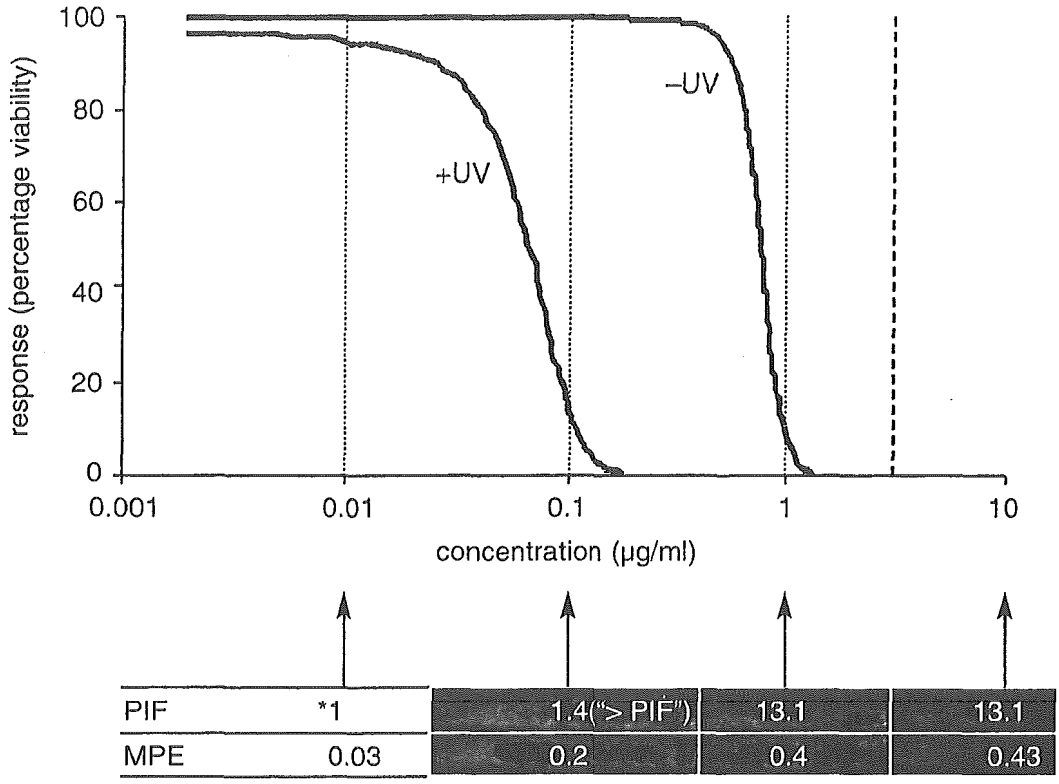
Results

Prediction of phototoxic potential from in vitro data according to the prediction model and comparison with in vivo data

For each test chemical, the PIF values from two independent runs and the resulting means are shown separately for each laboratory in Table III, with the predicted phototoxic potential according to PIF. Two MPE values and the corresponding means obtained from the same concentration–response curves as the PIF values are given for each test chemical and laboratory in Table IV, which also shows the predicted phototoxic potential. The overall concordance between *in vitro* data and the predicted phototoxic potential *in vivo*, made by using PIF or MPE, is summarised for all four laboratories in Table V.

One of the four laboratories (laboratory 4) obtained 100% correct *in vitro* classifications

Figure 1: Analysis of the effect of the highest test concentration on correct prediction of phototoxic potential



The values for Photoinhibition Factor (PIF) and Mean Photo Effect (MPE) and the predicted phototoxic potential were calculated from the two concentration–response curves by continuously increasing the concentration interval between the lowest concentration value (0.01µg/ml) up to the highest concentration achieved in the experiment, in a stepwise fashion.

= non-phototoxic chemicals and ■ *= phototoxic chemicals, when the upper concentration limit for curve evaluation was the value indicated by the arrows.*

------ *= highest test concentration recommended by ZEBET.*

The results of this analysis are shown in Figure 2.

with the 20 test chemicals, irrespective of whether PIF or MPE was used (Tables III and IV). This reflects a positive and negative predictivity of 100%, and also a sensitivity, specificity and accuracy of 100% in this laboratory. One of the UV filter chemicals (chemical 6), was predicted to be positive when tested at concentrations greater than 100µg/ml by the remaining three laboratories, when either PIF or MPE was applied (Tables III and IV). In addition, were predicted in one laboratory, two UV filter chemicals to be positive, chemical 8 according to both PIF and MPE at concentrations greater than 100µg/ml, and chemical 2 by MPE only (Tables III and IV, Figure 2). Two false negative results were obtained in the study, both in the same laboratory (laboratory 3), with chemicals 17 and 18, when the prediction was based on PIF (Table III). However, when the MPE method was applied to the same data set, the two chemicals were both cor-

Table III: Photoirritation Factors (PIFs) of two different experiments, mean values and predictions of phototoxicity

No.	Chemical (COLIPA number)	*In vivo* toxicity	Run	Laboratory 1 PIF	Laboratory 1 Mean	Laboratory 1 Prediction	Laboratory 2 PIF	Laboratory 2 Mean	Laboratory 2 Prediction	Laboratory 3 PIF	Laboratory 3 Mean	Laboratory 3 Prediction	Laboratory 4 PIF	Laboratory 4 Mean	Laboratory 4 Prediction
1.	Octyl salicylate (S13)	npt	1	*1	*1	npt	*1	*1	npt	*1	*1	npt	1.09	1.1485	npt
			2	*1			*1			*1			1.207		
2.	Octyl methoxycinnamate (S28)	npt	1	*1	*1	npt	*1	*1	npt	*1	*1	npt	0.94	0.94	npt
			2	*1			1			*1			*1		
3.	Benzylidene camphor sulphonic acid (S59)	npt	1	1.569	1.4235	npt	1.531	1.546	npt	1.069	1.08	npt	0.907	1.0495	npt
			2	1.278			1.561			1.091			1.192		
4.	4-Methylbenzylidene camphor (S60)	npt	1	1.505	1.3805	npt	1.282	1.3625	npt	0.902	0.964	npt	1.279	1.1695	npt
			2	1.256			1.443			1.025			1.06		
5.	3-Benzylidene camphor (S61)	npt	1	1.345	1.214	npt	*1	*1	npt	*1	*1	npt	1.091	1.091	npt
			2	1.083			*1			*1			*1		
6.	Terephthalidene dicamphor sulphonic acid (S71)	npt	1	1.55	2.1065	**pt**	1.289	1.6335	**pt**	1.039	1.163	**pt**	1.038	1.128	npt
			2	2.663	[a]		1.978	[a]		1.287	[a]		1.218		
7.	Polyacrylamidomethyl benzylidene camphor (S72)	npt	1	*1	*1	npt	*1	*1	npt	*1	*1	npt	*1	*1	npt
			2	*1			*1			*1			*1		
8.	Benzophenone-4 (S40)	npt	1	*1	*1	npt	3.784	3.028	**pt**	*1	*1	npt	1.115	1.06	npt
			2	*1			2.272	[a]		*1			1.005		
9.	L-Histidine free base	npt	1	*1	*1	npt	*1	*1	npt	*1	*1	npt	*1	*1	npt
			2	*1			*1			*1			*1		
10.	Sodium lauryl sulphate	npt	1	1.836	1.7795	npt	1.189	1.2185	npt	0.929	0.929	npt	0.994	0.9765	npt
			2	1.723			1.248						0.959		

[a] *Mean PIF value comprises at least one non-numerical PIF value of ">" type indicating that no IC50 (–UV) could be calculated. Thus the chemical was classified as phototoxic, irrespective of the mean PIF value.*

pt = phototoxic; npt = non-phototoxic. Inaccurate predictions are shown in bold type.

Table III: continued

No.	Chemical (COLIPA number)	*In vivo* toxicity	Run	Laboratory 1 PIF	Laboratory 1 Mean	Laboratory 1 Prediction	Laboratory 2 PIF	Laboratory 2 Mean	Laboratory 2 Prediction	Laboratory 3 PIF	Laboratory 3 Mean	Laboratory 3 Prediction	Laboratory 4 PIF	Laboratory 4 Mean	Laboratory 4 Prediction
11.	Protoporphyrin IX, disodium	pt	1	666.667	388.128	pt	41.867	45.97	pt	105.263	206.478	pt	30000	16500.8	pt
			2	109.589	[a]		50.073	[a]		307.692	[a]		3001.64		
12.	Chlorpromazine hydrochloride	pt	1	70.2	65.2925	pt	35.264	38.4025	pt	25.589	24.931	pt	22.207	19.0215	pt
			2	60.385			41.541			24.273			15.836		
13.	Anthracene	pt	1	95.238	100.251	pt	632.849	546.31	pt	19.01	44.348	pt	55.096	71.8195	pt
			2	105.263	[a]		459.77	[a]		69.686	[a]		88.543	[a]	
14.	Acridine hydrochloride	pt	1	619	642.834	pt	124.224	125.277	pt	547.945	583.57	pt	587.248	629.921	pt
			2	666.667	[a]		126.33	[a]		619.195	[a]		672.594	[a]	
15.	Ketoprofen	pt	1	307.692	307.692	pt	550.964	559.472	pt	330.579	285.989	pt	528.751	495.762	pt
			2	307.692	[a]		567.98	[a]		241.4	[a]		462.772	[a]	
16.	Promethazine hydrochloride	pt	1	101.206	72.714	pt	207.27	196.27	pt	42.446	29.181	pt	31.125	27.3915	pt
			2	44.222			185.269			15.916			23.658		
17.	Amiodarone hydrochloride	pt	1	13.233	11.3665	pt	14.465	12.6135	pt	2.896	3.08	**npt**	6.67	7.0665	pt
			2	9.5			10.762			3.265			7.463		
18.	Demeclocycline hydrochloride	pt	1	57.333	225.167	pt	1097.98	854.361	pt	*1	*1	**npt**	3.049	2.5135	pt
			2	393			610.739			*1			1.978	[a]	
19.	Bithionol	pt	1	27.222	24.5275	pt	18.941	17.9705	pt	16.08	11.982	pt	10.653	11.135	pt
			2	21.833			17			7.885			11.617		
20.	Musk ambrette	pt	1	27.918	24.6295	pt	118.343	111.187	pt	8.039	6.868	pt	8.853	8.229	pt
			2	21.341			104.031	[a]		5.696			7.605		

[a] *Mean PIF value comprises at least one non-numerical PIF value of ">" type indicating that no IC50 (–UV) could be calculated. Thus the chemical was classified as phototoxic, irrespective of the mean PIF value.*

pt = phototoxic; npt = non-phototoxic. Inaccurate predictions are shown in bold type.

Table IV: Mean Photo Effect (MPE) of two independent experiments, mean values and predictions of phototoxicity

No.	Chemical (COLIPA number)	*In vivo* toxicity	Run	Laboratory 1 MPE	Laboratory 1 Mean	Laboratory 1 Prediction	Laboratory 2 MPE	Laboratory 2 Mean	Laboratory 2 Prediction	Laboratory 3 MPE	Laboratory 3 Mean	Laboratory 3 Prediction	Laboratory 4 MPE	Laboratory 4 Mean	Laboratory 4 Prediction
1.	Octyl salicylate (S13)	npt	1	0.036	-0.0315	npt	0.022	0.011	npt	0.025	0.017	npt	0.023	0.0145	npt
			2	-0.099			0			0.009			0.006		
2.	Octyl methoxycinnamate (S28)	npt	1	0.058	0.0315	npt	0.123	0.1315	**pt**	-0.064	-0.046	npt	0.027	0.023	npt
			2	0.005			0.14			-0.028			0.019		
3.	Benzylidene camphor sulphonic acid (S59)	npt	1	0.059	0.0335	npt	0.035	0.0335	npt	0.141	0.0745	npt	0.002	0.0035	npt
			2	0.008			0.032			0.008			0.005		
4.	4-Methylbenzylidene camphor (S60)	npt	1	0.023	0.0205	npt	0.01	0.0285	npt	0.002	0.003	npt	0.009	0.0055	npt
			2	0.018			0.047			0.004			0.002		
5.	3-Benzylidene camphor (S61)	npt	1	0.054	0.0285	npt	-0.001	0.018	npt	0.096	0.061	npt	0.008	0.0515	npt
			2	0.003			0.037			0.026			0.095		
6.	Terephthalidene dicamphor sulphonic acid (S71)	npt	1	0.126	0.108	**pt**	0.091	0.236	**pt**	0.075	0.1005	**pt**	0.007	0.0065	npt
			2	0.09			0.381			0.126			0.006		
7.	Polyacrylamidomethyl benzylidene camphor (S72)	npt	1	0.036	0.018	npt	-0.011	-0.016	npt	-0.218	-0.11	npt	-0.003	-0.003	npt
			2	0			-0.021			-0.002			-0.003		
8.	Benzophenone-4 (S40)	npt	1	0.015	0.0095	npt	0.527	0.443	**pt**	-0.006	-0.067	npt	0.012	0.006	npt
			2	0.004			0.359			-0.128			0		
9.	L-Histidine free base	npt	1	0.01	0.0055	npt	-0.281	-0.3305	npt	-0.01	-0.265	npt	0.243	0.097	npt
			2	0.001			-0.38			-0.52			-0.049		
10.	Sodium lauryl sulphate	npt	1	0.124	0.0975	npt	0.004	0.034	npt	-0.001	-0.001	npt	0.006	0.003	npt
			2	0.071			0.064			n.d.			0		

npt = non-phototoxic; pt = phototoxic; nd = not determined. Inaccurate predictions are shown in bold type.