AN INTEGRATED APPROACH TO PHOTOTOXICITY TESTING: THE IN VITRO 3T3 NRU PHOTOIRRITANCY ASSAY AND THE ALBINO HAIRLESS MOUSE MODEL (CRL:SKH1-HR) WITH INTRACUTANEOUS ADMINISTRATION AND A HIGH DOSE OF ULTRAVIOLET A RADIATION

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Christopher P. Sambuco[1]; Douglas B. Learn[1]; Sergey Ksenzenko[1]; Tamika Coston[1]; Mark Schwartz[1]; Marvin Arocena[1]; Hillary Beasley[1]; Alan M. Hoberman[1]; Trudy Grossman [2].

[1]Charles River, Horsham, PA USA, [2]Tetraphase Pharmaceuticals, Inc., Watertown, MA, United States

SOT, Salt Lake City, UT

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www.criver.com

ABSTRACT

In assessing the potential phototoxicity of drugs and chemicals, the *in vitro* 3T3 NRU Photoirritancy assay and other *in vitro* phototoxicity models tend to produce results indicative of phototoxic potential for a relatively large number of compounds. Surveys from laboratories that conduct the 3T3 assay indicate that 35% to 60% of tested compounds result in findings suggestive of phototoxic potential. Once a compound has been found to be positive in this *in vitro* assay, a reasonable approach is to conduct an *in vivo* phototoxicity test. However, certain compounds that are considered phototoxic in man are not readily phototoxic in standard animal tests (e.g., chlorpromazine and tetracyclines), but do show activity in the 3T3 assay. To address the apparent lack of sensitivity of the animal models and correlation with clinical experience, we modified the albino hairless mouse model to maximize sensitivity for cutaneous phototoxicity by the use of intracutaneous administration of formulations and exposure to a relatively high dose of ultraviolet A (315-400 nm) radiation. This approach enabled us to readily detect phototoxicity in mice for compounds that are considered phototoxic clinically, but not easily demonstrated to be phototoxic in more standard animal models. This maximized test reveals excellent concordance for compounds that are positive in the 3T3 assay and are considered phototoxic clinically. Additionally, some compounds that elicit positive findings in the 3T3 assay do not elicit phototoxicity in the maximized mouse test. Use of this integrated *in vitro* and *in vivo* approach should help address some difficulties associated with preclinical phototoxicity testing. Further work is needed to define the underlying mechanisms that account for these findings.

INTRODUCTION

Phototoxicity testing can be problematic:

The *in vitro* 3T3 Neutral Red Uptake Photo-Irritancy assay has been found to produce results indicating that large numbers of compounds have phototoxic potential.

Some compounds that are known to elicit cutaneous phototoxicity in the clinic, do not elicit skin reactions indicative of phototoxicity when tested under standard preclinical protocols (e.g., chlorpromazine and doxycycline).

Therefore, the *in vitro* assay appears to be too sensitive while the preclinical assays appear to lack sensitivity, making human risk assessment difficult. In order to address these problems, we have integrated the *in vitro* and *in vivo* preclinical assays.

For the *in vitro* 3T3 Neutral Red Uptake Photo-Irritancy assay we have used either the standard method as described in the guidelines or a screening method.

For the *in vivo* method we maximized the standard hairless mouse method by injecting compounds directly into the skin (intracutaneous) and administering a relatively high dose of UVA (315 – 400nm).

The results suggest that:

Compounds that are known to be phototoxic in the clinic elicit responses indicative of having phototoxic potential in both the *in vitro* and the maximized *in vivo* assays.

Compounds not yet evaluated clinically elicited positive findings in both the *in vitro* and the maximized *in vivo* assays; suggesting a high phototoxic risk clinically.

Compounds not yet evaluated clinically elicited positive findings in the *in vitro* assay, but negative findings in the maximized *in vivo* assay, suggesting a low phototoxic risk clinically.

METHODOLOGY IN VIVO

Test Articles:

Tested compounds are included in Table 1. Compounds were typically tested at concentrations of 3.75 and 0.375 mg/mL and formulations were adjusted to pH 6.0-6.5 using NaOH. Administration volume was 0.05mL/skin site (2 sites/mouse). Route of administration was intracutaneous and the injection sites were on the mid-dorsum. The vehicle was 0.9% sodium chloride injection USP. All formulations were administered within 90 minutes of preparation.

Test System:

Female Crl:SKH1-hr hairless mice, 7-8 weeks of age (Charles River Laboratories)

UVR Source and Exposure:

Ultraviolet A (UVA) Radiation Source: Fluorescent fixture (KBD, Inc) containing 12 Philips Natural™ fluorescent tanning bed lamps filtered with window glass (~4 mm thick) to minimize UVB exposure (Figures 1 and 2). Exposure began no later than 20 minutes after test article administration. UVA dose was 20 – 21 J/cm² at an intensity of 5 mW/cm², monitored using a calibrated Solar Light™ PMA 2100 meter and UVA detector. Duration of exposure was approximately 60 minutes.

Procedures and Observations:

After formulation administration, mice were lightly anesthetized as soon as possible using chloral hydrate, and a mask with a 1.3 cm round hole was placed on each mouse. The hole was placed over one of the formulation administration sites on the mid-dorsum to allow for UVA exposure. Viability was recorded at least twice daily. Skin reactions and clinical observations were recorded before UVR exposure, approximately 60 minutes, 4 hours and 1, 2 and 3 days after UVR exposure. Body weights were recorded daily.

Animal use was approved by the Institutional Animal Care and Use Committee.

METHODOLOGY IN VITRO

Guideline:

OECD GUIDELINE FOR TESTING OF CHEMICALS. In vitro 3T3 NRU phototoxicity test, No. 432 Section 4 Health Effects (Pink pages); adopted April 13, 2004. See Figure 5.

UVR Source and Exposure:

6500 W Xenon Arc Solar Simulator, spectral emission filtered through the plastic 96-well plate lid (Figures 3 and 4). UVR exposure was performed using a custom cooling bed to minimize cell heating and maintain a constant temperature during the exposure. UVA dose was equal to 5 J/cm².

Procedures and Observations:

The *in vitro* assays were conducted either according to the OECD guideline or using a modified screening procedure in which eight concentrations of six compounds were included on each 96-well plate. See Figures 6 and 7. All other aspects of the screening assay were conducted according to the OECD guideline.

FIGURE 1

Figure 1. Fluorescent fixture (KBD, Inc) used in the maximized *in vivo* phototoxicity test to allow for delivery of a relatively high dose of UVA (315 – 400 nm).

FIGURE 2

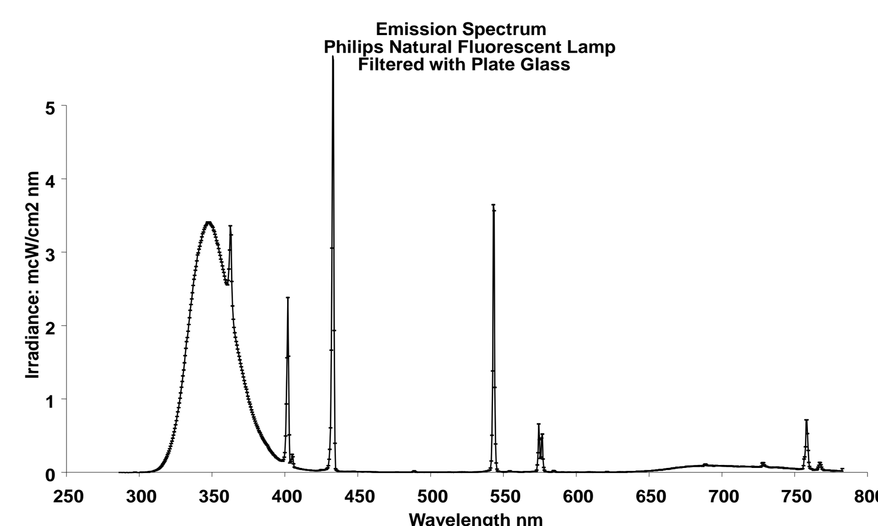

Figure 2. Emission spectrum of the UVA fluorescent fixture (KBD, Inc).

FIGURE 3

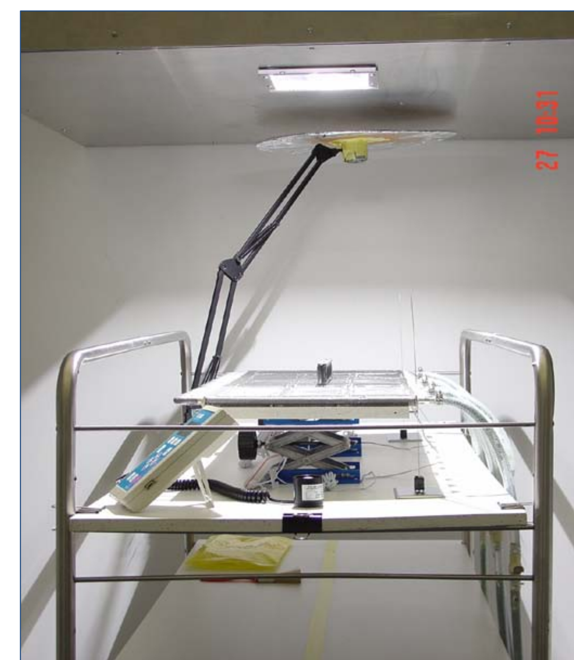

Figure 3. The 6500 W Xenon Arc Solar Simulator used in the *in vitro* phototoxicity assays. Note the custom cooling bed that is used to minimize cell heating and maintain a constant temperature during UVA exposure.

FIGURE 4

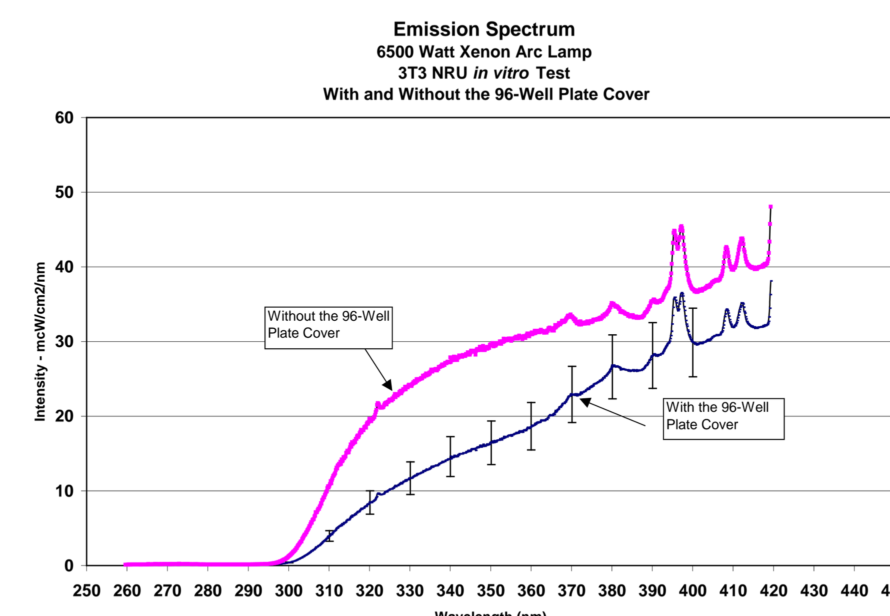

Figure 4. Emission spectrum of the 6500 W Xenon Arc Solar Simulator

FIGURE 5

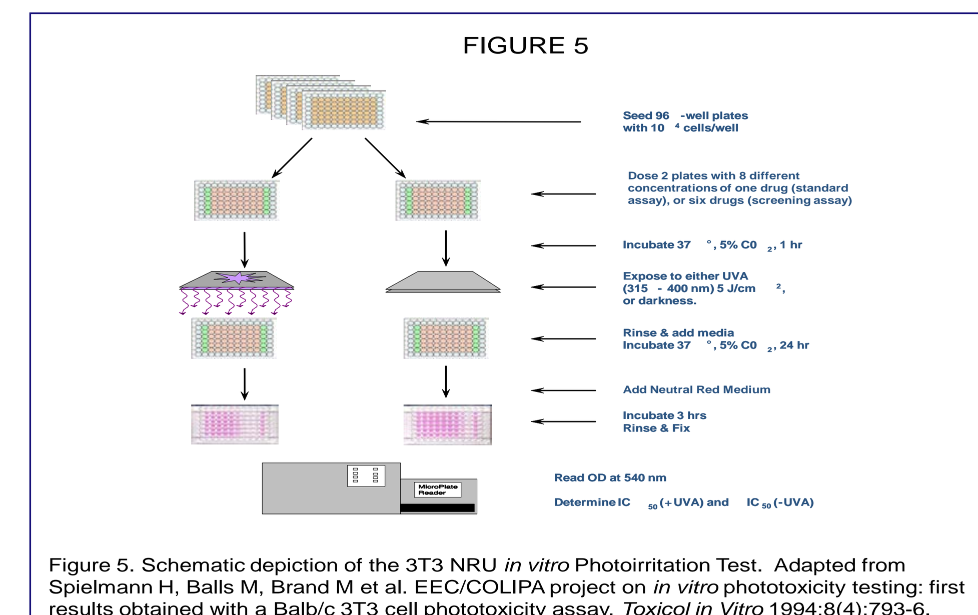

Figure 5. Schematic depiction of the 3T3 NRU *in vitro* Photoirritation Test. Adapted from Spielmann H, Balls M, Brand M et al. EEC/COLIPA project on *in vitro* phototoxicity testing: first results obtained with a Balb/c 3T3 cell phototoxicity assay. *Toxicol in Vitro* 1994;8(4):793-6.

FIGURE 6

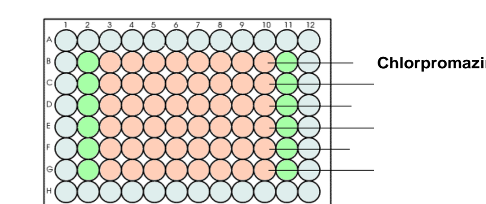

Figure 6. A 96-well plate schematic depicting the arrangement of wells when the 3T3 NRU phototoxicity test is performed according to the OECD guideline. Note that eight concentrations of a single test article are evaluated in six replicates.

FIGURE 7

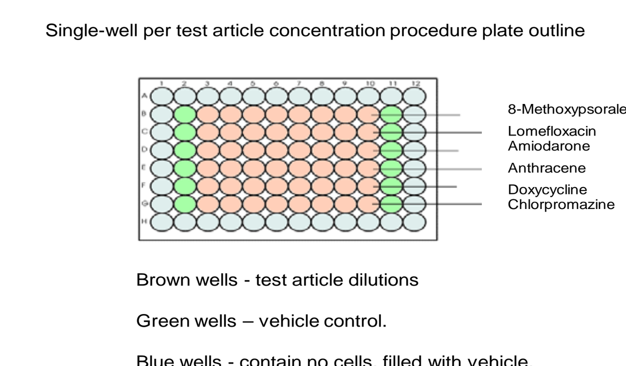

Figure 7. A 96-well plate schematic depicting the arrangement of wells when the 3T3 NRU phototoxicity test is performed according to the screening method. Note that eight concentrations of six test articles are evaluated.

FIGURE 8

Figure 8. Representative photograph of hairless mice administered vehicle (top mouse) or Doxycycline (bottom mouse). Formulations were administered immediately behind the two tattoo marks on the back and the front site was exposed to UVA. At 24 hours after UVA exposure, the bottom mouse shows erythema and edema at the UVA exposure site, while the unexposed site does not. The mouse administered saline shows no skin reaction in either site.

RESULTS AND DISCUSSION

- **Compounds that are known to be phototoxic in the clinic elicited responses indicative of having phototoxic potential in both the *in vitro* and the maximized *in vivo* assays (Figure 8).**
- **Compounds not yet evaluated clinically elicited positive findings in both the *in vitro* and the maximized *in vivo* assays, suggesting a high phototoxic risk clinically.**
- **Compounds not yet evaluated clinically elicited positive findings in the *in vitro* assay, but negative findings in the maximized *in vivo* assay, suggesting a low phototoxic risk clinically.**
- **In the maximized *in vivo* assay, compounds that are considered phototoxic clinically, but not easily demonstrated to be phototoxic in standard preclinical phototoxicity tests, were clearly demonstrated to be phototoxic.**
- **In the *in vitro* 3T3 NRU phototoxicity test, the screening method revealed results comparable to the standard method.**
- **Use of this integrated *in vitro* and *in vivo* approach should help address some difficulties associated with preclinical phototoxicity testing.**
- **Further work is needed to assess concordance among *in vitro*, *in vivo*, preclinical, and clinical data.**
- **Further work is needed to define the underlying mechanisms that account for these findings.**

TABLE 1

Table 1. Results of the *in vitro* 3T3 NRU phototoxicity tests and the preclinical maximized tests in hairless mice, compared with clinical information available for cutaneous phototoxicity.

Formulation	3T3 NRU Photoirritancy: Mean Photo-Effect (MPE)	3T3 NRU Photoirritancy: Photo-Irritancy Factor (PIF)	Preclinical Intracutaneous Photoirritancy	Clinical Phototoxicity
Chlorpromazine	0.562	34.783	Yes	Yes
Doxycycline	0.433	123.169	Yes	Yes
Levofloxacin	0.511	>86.428[a]	Yes	Yes
Minocycline	0.000	0.812	No	Ambiguity
A	0.363	>51.565[a]	Yes	Unknown
B	0.600	38.587	No	Unknown
C	0.248	11.088	No	Unknown
D	0.559	45.200	No	Unknown
E	0.057	2.228	Not Tested	Unknown
F	0.584	66.019	Not Tested	Unknown
G	0.591	19.407	Not Tested	Unknown
H	0.018	1.531	Not Tested	Unknown
I	0.662	56.928	Not Tested	Unknown
J	0.440	60.690	Not Tested	Unknown
K	0.412	60.523	Not Tested	Unknown
L	0.657	16.248	Not Tested	Unknown
M	0.545	45.736	Not Tested	Unknown
N	0.668	61.932	Not Tested	Unknown
O	0.420	18.201	Not Tested	Unknown
P	0.358	27.191	Not Tested	Unknown
Q	0.530	34.782	Not Tested	Unknown
R	0.384	79.660	Not Tested	Unknown
S	0.448	19.020	Not Tested	Unknown
T	0.006	1.656	Not Tested	Unknown
U	0.412	>63.133[a]	Not Tested	Unknown
V	0.377	10.715	Not Tested	Unknown
W	0.652	21.887	Not Tested	Unknown

a. The IC50(-UVA) for the tested article was not achieved and a ">PIF" index was calculated using the highest concentration tested in the assay to estimate the results. According to the ZEBET/ECVAM/COLIPA recommendations, when only >PIF can be obtained, any >PIF value >1 indicates that the test article has phototoxic potential.

CONCLUSIONS

- **The maximized *in vivo* assay using the intracutaneous route of administration in hairless mice and a relatively high UVA dose allows for detection of phototoxicity for compounds that are considered phototoxic clinically, but are not readily demonstrated to be phototoxic in standard preclinical tests.**
- **In the *in vitro* 3T3 NRU phototoxicity test, the screening method appears to be a reasonable alternative to the standard method.**
- **Compounds that are known to be phototoxic in the clinic elicit responses indicative of having phototoxic potential in both the *in vitro* and the maximized *in vivo* assays.**
- **Compounds not yet evaluated clinically elicited positive findings in both the *in vitro* and the maximized *in vivo* assays; suggesting a high phototoxic risk clinically.**
- **Compounds not yet evaluated clinically elicited positive findings in the *in vitro* assay, but negative findings in the maximized *in vivo* assay, suggesting a low phototoxic risk clinically.**
- **Use of this integrated *in vitro* and *in vivo* approach should help address some difficulties associated with preclinical phototoxicity testing.**

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