

## **A 3D Skin Model For Cosmetic, Chemical And Medical Device Phototoxicity Testing**

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### ***ABSTRACT***

We have enhanced our validated in vitro phototoxicity test using human skin models by exploring inflammatory mediator and gene expression endpoints. The Enhanced Phototoxicity Assay in Reconstituted Skin (EPARS) is based upon a 3D skin model that closely parallels human skin morphology. Major advantages of this test system are that test substances can be applied topically, avoiding the problems of (1) difficulty in solubilizing test materials, and (2) indirect application of test materials to cell monolayers via culture media. In addition, the tissues are composed of differentiated layers of primary human keratinocytes, a more relevant model than mouse tumor fibroblasts. Phototoxic effects are determined by measuring the viability of UV irradiated vs. non-irradiated exposed tissues. In order to increase the sensitivity and specificity of the test, we have measured the release of cytokines into the culture media via ELISA. The release of the inflammatory factor PGE<sub>2</sub> was shown to be an early predictor of the toxic effects demonstrated in the viability assay. When compared to human phototoxicity test results and the 3T3 NRU PT validation test material set, EPARS had 100% accuracy, sensitivity and specificity. Microarray analysis of gene expression showed that chlorpromazine treatment with UVA irradiation caused changes in gene expression over time that were not observed without UVA irradiation. These genes include those for keratins, collagens and fibronectins. EPARS is an accurate and sensitive test for detecting phototoxic substances at doses representative of those that cause actual human skin reactions. Thus, EPARS is a highly predictive phototoxicity assay, with endpoints of inflammatory mediator and gene expression that allow for investigation into the mechanisms of photosensitivity in a wide variety of consumer products.

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### **INTRODUCTION AND BACKGROUND**

Photoreactivity is a serious concern in dermatological and pharmaceutical products, as well as reagents used to give taste and smell to consumer products. The “Enhanced Phototoxicity Assay in Reconstituted Skin” (EPARS) test system employs 3-dimensional keratinocyte-based reconstituted skin tissue equivalents, specifically MatTek Corporation’s EpiDerm™ tissue models. The ability of this model to predict irritancy has been highly characterized (Koschier et al., 1997; Faller et al., 2002). The EPARS test overcomes the two greatest initial weaknesses of the 3T3 NRU PT which are: 1) the test agents must be soluble in cell culture medium, and 2) the 3T3 model system consists of non-human fibroblast cell monolayer cultures, which are of questionable applicability to human skin. EPARS, in contrast, employs topical dosing of human keratinocyte-based tissues.

Using a validated MTT viability assay, the cytotoxicity of the agent was determined both with and without UV irradiation. Historically, cytokine release has been used to evaluate the irritant properties of test chemicals in these tissues (Bernhofer et al., 1999). In this study, molecular and mechanistic endpoints relevant to phototoxicity, namely PGE<sub>2</sub> release, and inflammatory cytokine production (IL-1 $\alpha$ ) were evaluated as endpoints to increase the sensitivity and specificity of the test. Cytotoxicity and cytokine data for the following representative chemicals are presented: CPZ, an anti-psychotic; Bergamot oil, a reagent used to give taste and smell to consumer products; 5-MOP, an anti-carcinogen; and Bithionol, an anti-parasitic reagent. The data (MTT uptake, PGE<sub>2</sub> and IL-1 $\alpha$  release) for 26 chemicals is presented in the form of contingency tables.

To determine which genes are expressed in skin during a phototoxic reaction versus a cytotoxic reaction, the anti-psychotic and well-known phototoxin, chlorpromazine, was used to dose tissues at 1, 6, and 20 hours. Half of these tissues were exposed to solar simulated light (+SSL), while the other half were placed in the dark (No SSL). RNA was then isolated from the tissues in each time point and used to probe a gene array of oligos representing greater than 25,000 human genes. Selected results from the top 50 over- and under-expressed genes at each irradiated time point as compared to their dark counterparts are presented.

### **METHODS AND MATERIALS**

#### Test Substances and Dosing

MatTek EpiDerm™ (EPI-200) tissues were equilibrated according to standard protocol and dosed with test substances falling into one of three groups: 1) known phototoxins, 2) irritants (non-phototoxic), and 3) non-irritants (non-phototoxic). Tissues suspended in 0.9 ml assay media were dosed with either vehicle control or 100  $\mu$ l test substance applied directly to tissue surface, and then incubated at 37°C, 5% CO<sub>2</sub> for 18-24 hours.

#### UV Irradiation

Following treatment, a Höppler SOL 500 solar simulator was used to deliver UVA light doses of 6 J/cm<sup>2</sup> (approximately 60 min. at 1.7 mW/cm<sup>2</sup>) to the tissue equivalents. Control tissues were incubated at room temperature in the dark for equivalent time increments. Tissues were rinsed with PBS and incubated an additional 18-24 hours at 37°C, 5% CO<sub>2</sub>.

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### MTT Viability Assay Endpoint

Tissue viability was measured using the MatTek MTT Viability Assay protocol (Phototoxicity Protocol For Use With EpiDerm™ Model). Briefly, tissues were incubated with 1 mg/ml MTT in unsupplemented DMEM for 3 hours at 37°C, 5% CO<sub>2</sub>. Following a wash step, the reduced formazan product was extracted with 95% ethanol overnight at room temperature. Absorbance was measured on a microplate reader at 540 nm with a 690 nm reference wavelength. The percent viability was calculated as follows:

$$\% \text{ Viability} = 100 * (\text{OD}_{\text{sample}} / \text{OD}_{\text{vehicle control}})$$

### Criteria for the Evaluation of Phototoxicity

MTT Viability: The EC<sub>50</sub> of the test substance was determined for the tissues +/- SSL irradiation. The photo-irritancy factor (PIF) was calculated as:

$$\text{PIF} = \text{EC}_{50 \text{ No SSL}} / \text{EC}_{50 \text{ +SSL}}$$

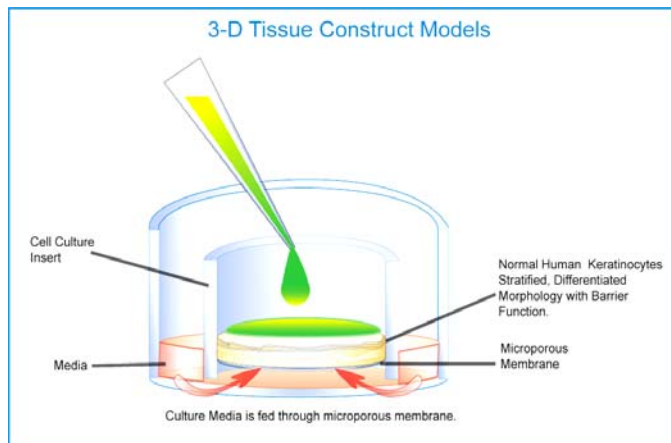
One criterion for the detection of a positive phototoxin is a PIF value >2. This criteria was based on the ECVAM-validated 3T3 NRU Phototoxicity Assay. A second criterion was based on the ZEBET (Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch) validation of the Phototoxicity Protocol for MatTek's EpiDerm™, wherein a test substance for which there is a 30% or greater reduction in cell viability versus correspondingly treated un-irradiated tissues is considered to be a phototoxin. We used both criteria.

### Gene Expression Profiling

EpiDerm tissue discs treated with 0.0003% chlorpromazine, an antipsychotic and known phototoxin, were then harvested at 1,6, and 20 hours after one hour +/- light exposure. RNA was purified, amplified and labeled with fluorescent dye before being hybridized to six individual H25K Human Genome microarrays (Telechem International, Sunnyvale, CA).

Analyses were performed using the GeneSifter Microarray Data Analysis Suite. All experimental conditions were compared against the CPZ 1 hour dark condition and the light condition versus the comparable dark condition.

## TEST SYSTEM



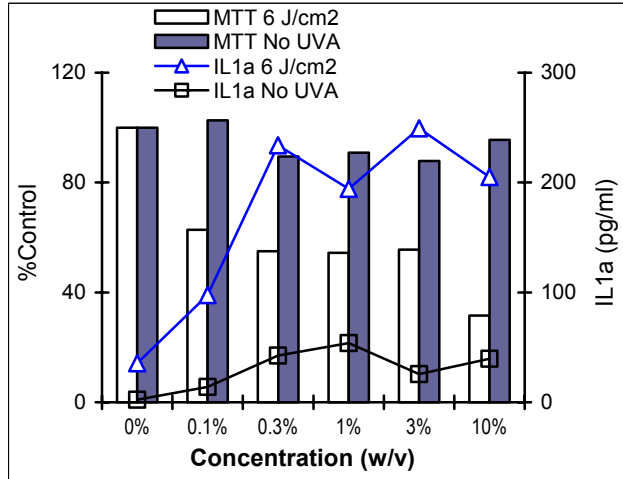
MatTek EpiDerm is an *in vitro* human tissue model, which is constructed of normal, human-derived epidermal keratinocytes (NHEK) that have been cultured to form a multilayered, highly differentiated model that closely parallels human skin.

EpiDerm is organized into basal, spinous, and granular layers along a multi-layered stratum corneum with an air-liquid interface. These tissues also exhibit *in vivo*-like morphological and growth characteristics that allow topical application to the surface of the tissue, which mimics the route of human exposure.

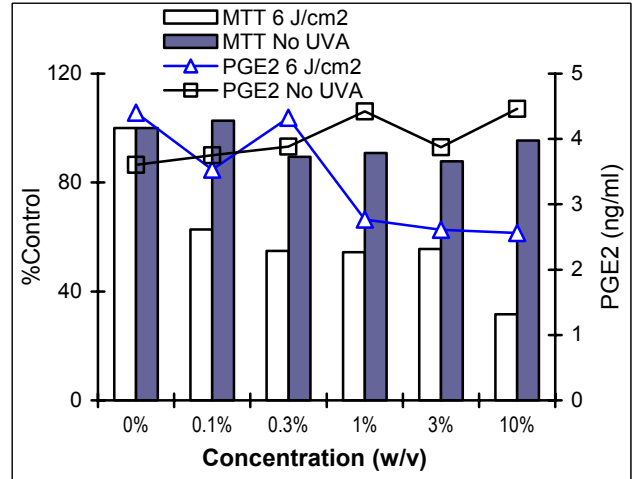
## RESULTS: MTT Viability vs. Cytokine

### Tetracycline

MTT viability vs. IL1a production

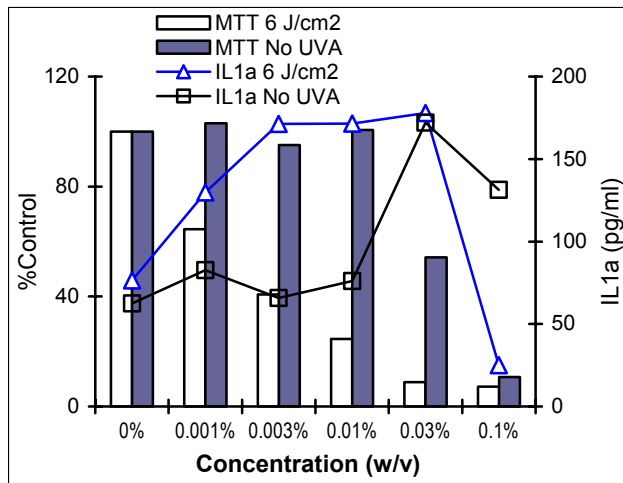


MTT viability vs. PGE2 production

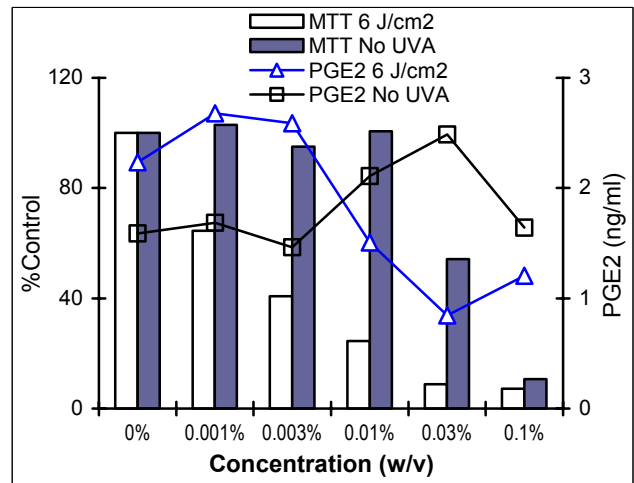


### CPZ

MTT viability vs. IL1a production



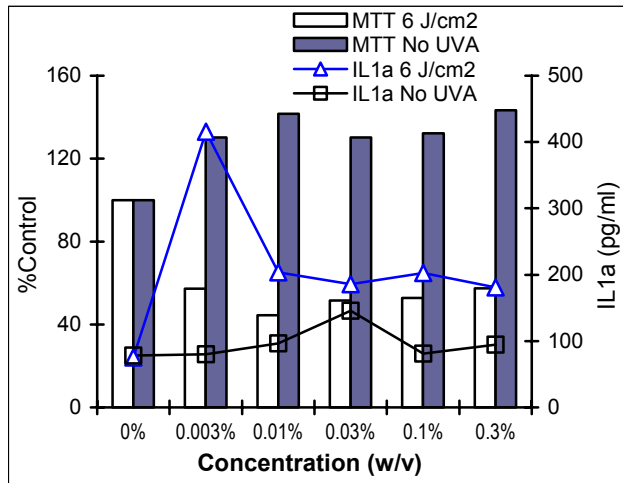
MTT viability vs. PGE2 production



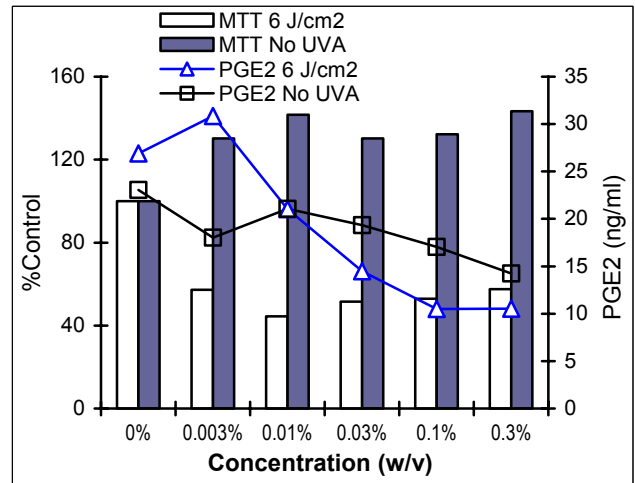
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**5-MOP**

MTT viability vs. IL1a production

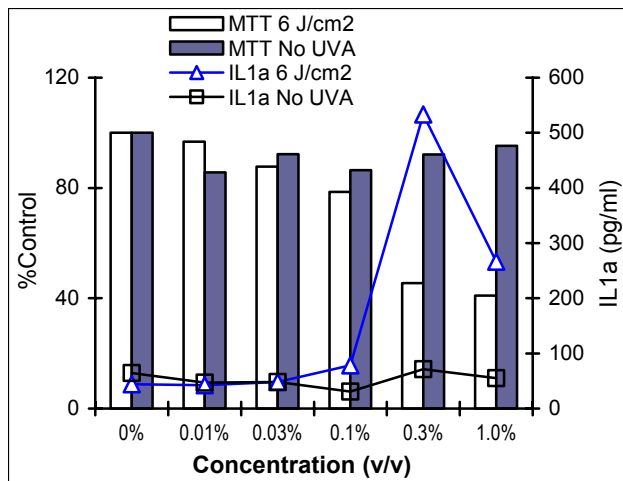


MTT viability vs. PGE2 production

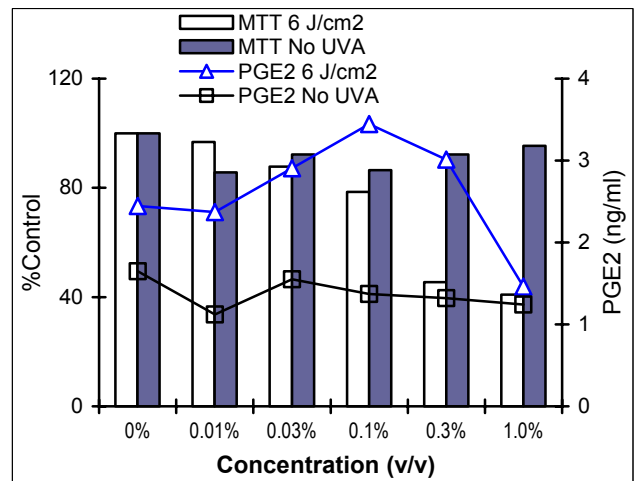


**Bergamot Oil**

MTT viability vs. IL1a production



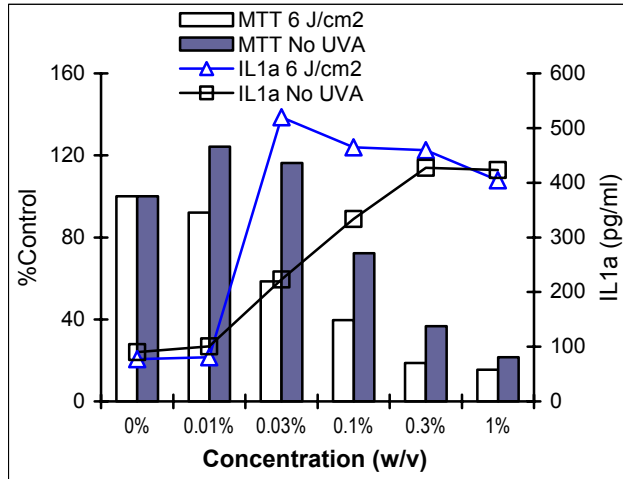
MTT viability vs. PGE2 production



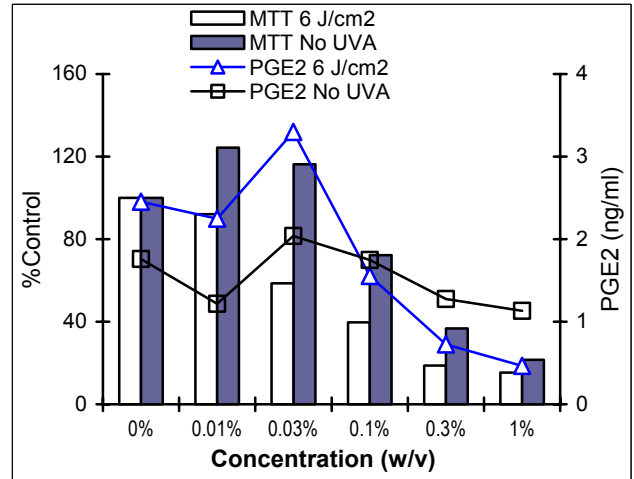
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**Bithionol**

MTT viability vs. IL1a production



MTT viability vs. PGE2 production



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## RESULTS: Gene Chip

This selected gene table compares skin-specific and predominant genes expressed by the UV-exposed CPZ-treated samples vs. the unirradiated CPZ-treated samples at each time point.

Tissues were treated with CPZ overnight, exposed to 1 hour of UV or dark, rinsed, and harvested at 1, 6 or 20 hours after exposure.

<b>DOWN-Regulated</b>			
Rank	Ratio	Identifier	Gene Name
<b>1 hour CPZ Treatment UV vs Dark</b>			
2	10.9	NM_207647	Fibronectin type III
6	6.38	D90279	Collagen, type V, alpha 1
35	3.88	NM_005203	Collagen, type XIII, alpha 1
<b>6 hour CPZ Treatment UV vs Dark</b>			
9	4.21	AB104446	Hornerin
16	3.57	D90279	Collagen, type V, alpha 1
18	3.5	NM_033188	Keratin associated protein 4-5
20	3.42	NM_014357	Late cornified envelope 2B
21	3.37	NM_004948	Desmocollin 1
42	2.95	NM_000421	Keratin 10
<b>20 hour CPZ Treatment UV vs Dark</b>			
10	2.72	NM_021189	Cell adhesion molecule 3
34	2.34	NM_002283	Keratin 85
49	2.25	NM_032488	Cornifelin

<b>UP-Regulated</b>			
Rank	Ratio	Identifier	Gene Name
<b>1 hour CPZ Treatment UV vs Dark</b>			
2	12.27	NM_002176	Interferon, beta 1, fibroblast
5	8.47	NM_005534	Interferon gamma receptor 2
8	7.90	NM_002163	Interferon regulatory factor 8
10	7.27	NM_000874	Interferon ( $\alpha$ , $\beta$ , and $\omega$ ) receptor 2
49	4.68	NM_031854	Keratin associated protein 4-12
<b>6 hour CPZ Treatment UV vs Dark</b>			
1	31.43	NM_002276	Keratin 19
5	18.95	NM_181624	Keratin associated protein 23-1
10	14.85	NM_002278	Keratin 32
28	6.69	NM_181537	Keratin 27
29	6.59	NM_003770	Keratin 37
<b>20 hour CPZ Treatment UV vs Dark</b>			
4	14.51	NM_000423	Keratin 2
5	13.36	NM_021009	Ubiquitin C
16	8.79	NM_175834	Keratin 4
19	8.35	NM_003969	Ubiquitin-conjugating enzyme E2M

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**RESULTS:**

**Evaluation of Phototoxicity by Cell Viability**

**Tissue Viability Endpoint (MTT Uptake)**

Criteria PIF  $\geq$  2

	Known Photo +	Known Photo -	
Tested +	15	0	15
Tested -	3	8	11
	18	8	26

Accuracy	88%	(23/26)
Sensitivity	100%	(15/15)
Specificity	73%	(8/11)
Positive Predictivity	83%	(15/18)
Negative Predictivity	100%	(8/8)

**Tissue Viability Endpoint (MTT Uptake)**

Criteria =  $>30$   $\downarrow$  UV vs. Dark

	Known Photo +	Known Photo -	
Tested +	18	0	18
Tested -	0	8	8
	18	8	26

Accuracy	100%	(26/26)
Sensitivity	100%	(18/18)
Specificity	100%	(8/8)
Positive Predictivity	100%	(18/18)
Negative Predictivity	100%	(8/8)

**Evaluation of Phototoxicity by Inflammatory Mediator Release and Production**

**PGE<sub>2</sub> Release**

Criteria = 4-fold  $\uparrow$  UV vs Dark

	Known Photo +	Known Photo -	
Tested +	14	4	18
Tested -	4	4	8
	18	8	26

Accuracy	69%	(18/26)
Sensitivity	78%	(14/18)
Specificity	50%	(4/8)
Positive Predictivity	78%	(14/18)
Negative Predictivity	50%	(4/8)

**IL-1 $\alpha$  Release**

Criteria = 2-fold  $\uparrow$  UV vs. Dark

	Known Photo +	Known Photo -	
Tested +	14	1	15
Tested -	1	6	7
	15	7	22

Accuracy	91%	(20/22)
Sensitivity	93%	(14/15)
Specificity	86%	(6/7)
Positive Predictivity	93%	(14/15)
Negative Predictivity	86%	(6/7)

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**List of Tested Chemicals and Current Validation Results**

PHOTOTOXINS	Criteria		NON-PHOTOTOXINS	Criteria	
	PIF $\geq 2$	$\geq 30\%$ ↓		PIF $\geq 2$	$\geq 30\%$ ↓
5-Aminolevulinic Acid	+	+	Benzalkonium chloride	-	-
5-Methoxypsoralen	+	+	Dimethyl Sulfoxide	-	-
7-Methoxycoumarin	+	+	Ethanol	-	-
8-Methoxypsoralen	+	+	Eucalyptus Oil	-	-
Acridine	-	+	Hexachlorophene	-	-
Amiodarone	+	+	L-Histidine	-	-
Anthracene	+	+	Penicillin G	-	-
Bergamot Oil	+	+	Sodium Dodecyl Sulfate	-	-
Bithionol	+	+			
Chlorpromazine	+	+			
Lemon Oil	-	+			
Neutral Red	-	+			
Norfloxacin	+	+			
Promethazine	+	+			
Protoporphyrin-IX	+	+			
Rose Bengal	+	+			
TetraChlorSalicylAnilide	+	+			
Tetracycline	+	+			

**CONCLUSIONS:**

EPARS improves on the existing 3T3 phototoxicity test by (1) accommodating the topical application of test articles and (2) using a primary human cell-based tissue with a morphology that approximates the 3-dimensional characteristics of human skin.

- ❖ EPARS determines phototoxicity by MTT uptake, and accurately identified phototoxins and non-phototoxins in the EpiDerm™ model.
- ❖ Measurement of PGE<sub>2</sub> release into the culture media improved the sensitivity of the assay at approximately 3-fold lower concentrations, when compared to the MTT Viability endpoints.
- ❖ Release of IL-1 $\alpha$ , was increased in UVA-irradiated tissues when compared to paired 'dark' treatments. Increases ranging from 200% to 500% over control were typically predictive of a phototoxic response.
- ❖ Upon evaluation of each endpoint for accuracy, specificity and sensitivity, we determined that the most useful endpoints for identifying phototoxins were (1) cytotoxicity, (2) PGE<sub>2</sub>, and (3) IL-1 $\alpha$ . However neither the PGE<sub>2</sub> or IL-1 $\alpha$  endpoints alone can predict phototoxicity 100% of the time.
- ❖ Gene expression was specifically altered by treatment with chlorpromazine +UVA irradiation. However more experiments with light-only and dark-only controls need to be done to assess what gene expression is a drug-effect vs a light and drug effect.

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