KeratinoSens test submission - Attachment 12e_PC-epoxides.

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Structure-activity relationship between the *in vivo* skin sensitizing potency of analogues of phenyl glycidyl ether and the induction of Nrf2-dependent luciferase activity in the KeratinoSens *in vitro* assay

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Running title:

SAR study in the KeratinoSens assay

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Abstract

Due to regulatory constraints and ethical considerations, research on alternatives to animal testing to predict the skin sensitization potential of novel chemicals has gained a high priority. Ideally, these alternatives should predict not only the hazard of novel chemicals, but also rate the potency of skin sensitizers. Currently no method gives reliable potency estimations for a wide range of chemicals in differing structural classes. It has thus been proposed to perform potency estimations within specific structural classes. Detailed structure-activity studies for the *in vivo* sensitization capacity of a series of analogues of phenyl glycidyl ether (PGE) were recently published. These studies are part of an investigation regarding the allergenic activity of epoxy-resin monomers. Here we report data on the same chemicals in the KeratinoSens *in vitro* assay, which is based on a stable transgenic keratinocyte cell line with a luciferase gene under the control of an antioxidant response element (ARE). A strong correlation between the EC3 values in the LLNA and both the luciferase-inducing concentrations and the cytotoxicity in the cell based assay was established for six analogues of PGE. This correlation allowed the potency in the LLNA of two novel structurally closely related derivatives to be predicted by read-across with an error of 1.4- and 2.3-fold. However, the LLNA EC3 values of two structurally different bifunctional monomers were overpredicted based on this dataset, indicating that accurate potency estimation by read-across based on *in vitro* data might be restricted to a relatively narrow applicability domain.

[1]**Abbreviations:** ACD, allergic contact dermatitis, ARE, antioxidant response element; DGEBA, diglycidyl ether of bisphenol A; DGEBF, diglycidyl ether of bisphenol F; DMSO, dimethylsulfoxide; DNCB, 2,4-dinitrochlorobenzene; EC3, extrapolated concentration for 3-fold cellular proliferation in the LLNA; $EC_{KS}1.5$, $EC_{KS}4.5$, extrapolated concentration for luciferase induction above threshold of 1.5 and 4.5 in the KeratinoSens assay; ERS, epoxy resin system, HaCaT, Human adult low Calcium Temperature, IC_{50}, inhibitory concentration for 50% reduction in viability as determined with the MTT assay; Keap1, Kelch-like ECH-associated protein 1; LLNA, local lymph node assay; LOEL, lowest observed effects level; Nrf2, nuclear factor-erythroid 2-related factor 2; PGE, phenyl glycidyl ether.

Introduction

Due to regulatory constraints and ethical considerations, research on alternatives to animal testing to predict the skin sensitization potential of novel chemicals has gained a high priority. Currently, the skin sensitization potential is estimated with the local lymph node assay in mice (LLNA[1]), in which the cellular proliferation in the draining lymph nodes is measured after repeated topical application of the test compound onto the ears. Results are expressed as EC3 values indicating the concentration which induces a threefold enhanced cellular proliferation (*1*). Whereas the LLNA has officially been validated only for classification and labelling, and thus hazard prediction, it is also used by both academia and industry for potency prediction, since a significant correlation between the EC3 values and the *in vivo* sensitization capacity in humans was established (*2*). A number of *in vitro* and *in chemico* assays has been proposed in recent years to detect skin sensitizers, and great progress has been made for hazard identification with several assays giving a predictive accuracy of 85% (*3-5*). Additionally, for several assays a certain correlation to potency was noted (*3, 6*), but a close prediction of the LLNA EC3 result currently is not possible based on global models. This is partly due to the fact that specific classes of compounds have higher or lower relative activities in certain *in vitro* assays and thus are over- or underpredicted if the prediction is based on correlations derived from large databases of structurally very diverse molecules. Alternatively, more accurate predictions might be made, if the *in vitro* and *in vivo* data of specific classes of compounds are used to make predictions by read-across within certain structural classes (i.e. with so called local models).

Skin sensitization is a T-cell mediated immune reaction to small exogenous molecules. In general skin sensitizing compounds are reactive chemicals (or chemicals metabolically transformed into reactive intermediates) which have the potential to react with skin proteins and render them immunogenic (*7*). Cell-based in vitro tests model a certain aspect of the cellular response to skin sensitizers. Most of these assays do not address the specific T-cell response, instead they focus on the unspecific innate (and thus early) response of the skin to sensitizing agents. We have proposed a reporter cell-based approach based on the finding that the majority of the skin sensitizers induce the Nrf2-Keap1-ARE regulatory pathway (*8-11*).

The antioxidant response element (ARE) from the human AKR1C2 gene was inserted in front of a SV40 promotor and placed upstream of a luciferase gene. Stable insertion of the resulting construct in HaCaT keratinocytes resulted in the KeratinoSens reporter cell line. Induction of luciferase in this cell line can be used to screen for skin sensitizers. The predictivity of this approach has been analyzed in detail on a set of 67 reference chemicals (5).

Allergic contact dermatitis (ACD) caused by epoxy resin systems (ERS) is an important occupational health problem comprising extreme sensitizers with an extensive usage (*12*). ERS are used when strong, flexible, light weight construction materials are required. In 2001, 1,100.000 tons of epoxy resins were sold worldwide. The epoxy resin monomers, diglycidyl ethers of bisphenol A (DGEBA) (Figure 1). and bisphenol F (DGEBF) (Figure 1) containing epoxy groups (epoxides) are considered the major allergens. PGE (Figure1) is a reactive diluent in the ERS and known to be a strong sensitizer (*12*).

We have recently published detailed structure-activity studies for the *in vivo* sensitization capacity of epoxy-resin half monomers using PGE as the lead compound *(13, 14)*. Here we report data on the same chemicals in the KeratinoSens *in vitro* assay. A strong correlation between the EC3 values in the LLNA and two luciferase-inducing concentrations and the cytotoxicity in the cell based assay was established for phenyl glycidyl ether (PGE) and six structurally similar analogues. The potential of this correlation for read-across to estimate the LLNA EC3 values of four new compounds based on the *in vitro* results was evaluated.

Experimental Procedures

Test chemicals. Phenyl glycidyl ether (**PGE**), benzyl glycidyl ether (**1**), butyl glycidyl ether (**5**), diglycidyl ether of bisphenol F (DGEBF) (**9**) and diglycidyl ether of bisphenol A (DGEBA) (**10**) were purchased from Aldrich Chemicals (Stockholm, Sweden). 2-(2-Phenoxyethyl)oxirane (**2**), cyclohexyl glycidyl ether (**3**), phenyl glycidyl ether (**4**), 2-butenyl glycidyl ether (**6**), phenyl 2,3-epoxypropyl sulphide (**7**), 1,2-epoxy-4-phenylbutane (**8**) were synthesized as previously described (*13, 14*). The purity of both synthesized and purchased test compounds was >98% (GC/MS) before testing. Structures are shown in Figure 1.

Cell line. The KeratinoSens cell line is derived from the human keratinocyte culture HaCaT (*15*). It contains a stable insertion of a luciferase gene under the control of the ARE-element of the gene AKR1C2. The optimization of this cell line has been described in detail (*5*).

Test procedure. All tests were run according to the previously published standard operating procedure (*5*). Briefly, cells were grown for 24 h in 96-well plates. The medium was then replaced with medium containing the test chemical and a final level of 1% of the solvent DMSO. Each compound was tested at 12 binary dilutions in the range from 0.98 µM to 2000 µM. Each test plate contained 7 test chemicals, 6 wells with the solvent control, 1 well with no cells for background value and 5 wells with the positive control cinnamic aldehyde in five different concentrations (4 - 64 µM). In each repetition, three parallel replicate plates were run with this same set-up and a fourth parallel plate was prepared for cytotoxicity determination. Cells were incubated for 48 h with the test agents, and then luciferase activity and cytotoxicity (with the MTT-assay) were determined. This full procedure was repeated three times for each chemical. The LLNA test results for PGE and the analogues **1 - 6** were known to the laboratory conducting the *in vitro* tests (Givaudan), but this lab was blind to the LLNA results for compounds **7 - 10** when testing the compounds and making the read-across predictions.

Analysis of test compound evaporation. To test the evaporation of volatile compounds from the vehicle used in the LLNA, test compounds were dissolved at 5% in a mixture of acetone and olive oil (AOO, 4:1). This mixture (25 µL as used in the LLNA) was added to 1 cm^2 glass slides and incubated at

32 °C to simulate the skin surface temperature. After fixed intervals (0 min/ 5 min/ 10 min/ 20 min/ 40 min/ 80 min) glass slides were extracted in 1 mL diethyl ether and analyzed with gas chromatography to determine the amount of the remaining test compound.

Instrumentation. Electron-ionization mass spectral analysis (70 eV) was performed on a Hewlett-Packard 5973 mass spectrometer connected to a gas chromatograph (Hewlett-Packard 6890). The GC was equipped with a cool on-column capillary inlet and an HP-5MSi fused silica capillary column (30 m × 0.25 mm, 0.25 µm, Agilent Technologies, Palo Alto, CA). Helium was used as carrier gas, and the flow rate was 1.2 mL/min. The temperature program started at 35 °C for 1 min, increased by 10 °C/min, and ended at 250 °C for 5 min. For mass spectral analysis, the mass spectrometer was used in the scan mode detecting ions with *m*/*z* values ranging from 50 to 1500.

Data analysis and statistical evaluation. For each chemical in each repetition and at each concentration, the gene induction was compared to DMSO controls. For each concentration it was determined whether the gene induction is statistically significant and over the threshold of 1.5-fold (i.e. 50% enhanced gene activity). Furthermore, the maximal fold-induction (I_{max}) and the $EC_{KS}1.5$ and $EC_{KS}4.5$ values (concentration in µM for induction above the threshold of 1.5 or 4.5, based on linear extrapolation as done in the LLNA) were calculated.

Results and Discussion

Results from the KeratinoSens assay. All the tested epoxides significantly induced the luciferase gene in the KeratinoSens assay at non-cytotoxic concentrations. Thus, based on the prediction model which rates chemicals as positive if there is over 50% gene induction at non-cytotoxic concentrations, all the tested chemicals would be correctly predicted as skin sensitizers by the KeratinoSens assay. Thus, hazard identification with this assay is straightforward for this class of chemicals. Interestingly, the dynamic range for this substance class is very high, with the maximal fold-induction (I_{max}) between 40 and 160 for most of the chemicals. Table 1 lists the luciferase induction result as the $EC_{KS}1.5$ values and the I_{max}. Additionally, since these compounds have such a high dynamic range (i.e. high I_{max}), the $EC_{KS}4.5$ values are also given in Table 1. The cytotoxicity data from the parallel assessment with the MTT assay are reported as IC_{50} values. Figure 2 illustrates the dose-response curves for PGE for both luciferase induction and cytotoxicity as an example. It is particularly striking that relatively small changes in structure have dramatic impact on the LLNA EC3 value for PGE and compounds **1** - **6**. Compared to these *in vivo* differences, the *in chemico* differences in terms of reactivity with a test peptide reported before (*13*) were comparatively low. Hence a little difference in reactivity has a pronounced effect on the EC3. Interestingly, a similar effect is now seen when comparing the cell-based *in vitro* data with the *in vivo* sensitization results: Thus the difference in the LLNA EC3 between the strongest and the weakest sensitizer PGE and **5** are 71-fold, whereas the difference in the $EC_{KS}1.5$ and $EC_{KS}4.5$ is only 3.7-fold.

Correlation analysis of the luciferase inducing concentrations with the LLNA EC3 values - The quantitative data from Table 1 were used for correlation analysis, and the resulting parameters are summarized in Table 2. For this correlation analysis only PGE and compounds **1** - **6** were used. There was a significant linear correlation between the inducing concentrations and the logarithmic LLNA EC3 value. Interestingly, this correlation was much better if the $EC_{KS}4.5$ values were used for correlation analysis as compared to the $EC_{KS}1.5$ values. Figure 3A illustrates the correlation analysis of the logEC3 with the $EC_{KS}4.5$. This relationship between the EC3 and the luciferase-inducing concentration is log-linear (Table

2), thus a comparatively small difference in the concentration inducing the Nrf2-dependent gene correlates to large differences in the LLNA value.

In the standard operating procedure of the KeratinoSens assay an $EC_{KS}1.5$ value is calculated as a universal parameter and it is the threshold used to rate whether a chemicals is predicted positive or negative (5). This threshold was selected since a (i) 50% gene induction above background in almost all cases is statistically significant and (ii) since some weak sensitizers give a relatively small dynamic range in this assay with an I_{max} between 1.5 and 2. However, the threshold of 1.5 used for hazard identification must not necessarily be the best parameter for potency prediction. Since the dynamic range of the assay is very high for the tested epoxides, a 1.5-fold induction threshold is at the extremely low end of the dose-response. This may explain why, for these compounds, the $EC_{KS}4.5$ value is more predictive for quantitative evaluation of the sensitization potential as compared to the $EC_{KS}1.5$ value and gives a better correlation to the *in vivo* sensitization data.

Correlation analysis of the cytotoxic concentrations with the LLNA EC3 values - We also noted a strong correlation between the cytotoxicity and the logarithmic LLNA EC3 values (Table 2). Figure 3B illustrates the correlation analysis of the logEC3 with the IC_{50} values. Consequently, although the luciferase induction always clearly starts far below the cytotoxic levels, there is also a very strong correlation between the $EC_{KS}4.5$ values and the cytotoxic concentrations (data not shown). On the other hand, neither the LLNA values nor the inducing concentrations are significantly correlated to the cLogP, indicating that the observed *in vivo* and *in vitro* biological activity is not strongly influenced by this physicochemical parameter.

Electrophiles induce Nrf2-dependent genes by covalent modification of the regulatory protein KeapI, and therefore the luciferase induction is a measure of the electrophilicity as sensed by the test cells. Hence the correlation of the LLNA EC3 values to the inducing concentrations can easily be explained and indeed forms the underlying hypothesis of the current work. At first sight surprising, however, is the equally well (or even slightly better) correlation of the LLNA EC3 values to the cytotoxic nature of the molecules. Does this indicate, that both Nrf2-induction and skin sensitization potency are simply a consequence of

cytotoxicity? We tend to explain the strong link between cytotoxicity and EC3 values differently: Cytotoxicity of chemicals in general may be caused by different modes of action: (i) narcotic toxicity due to the interaction of hydrophobic chemicals with cell membranes, (ii) reactive toxicity due to damage to cellular constituents by reactive chemicals, and (iii) specific toxicity by binding of the toxicants to specific receptors or interaction with specific cellular pathways such as specific action on apoptotic pathways. Narcotic toxicity often correlates with the cLogP. The fact that $EC_{KS}4.5$ is not significantly linked to cLogP indicates that the narcotic effects are not affecting the luciferase induction. The IC_{50} value significantly correlates to cLogP, but this correlation is weaker as compared to the correlation of the IC_{50} to the LLNA EC3. Thus in the case of the tested epoxides the cytotoxicity appears only partly to be explained by the narcotic action of the molecules, and the toxicity may rather be explained as reactive toxicity (*16*). Hence, since cytotoxicity, Nrf-2 induction and skin sensitization are all caused (at least partially) by the reactivity of these molecules there is a correlation of these parameters even if sensitization potency is not directly affected by the cytotoxic nature of the molecules.

Read across for compounds **7 - 10.** The log-linear correlation models in Figure 3 were used to make a prediction of the LLNA EC3 value for compounds **7 - 10**, these values were not known to the persons (RE and AN) performing the experiments and the read across calculations. The results are summarized in Table 3. Both based on the $EC_{KS}4.5$ and the IC_{50} values, the LLNA EC3 values for these compounds could be predicted with a margin of error of 2 – 3 fold. These data are shown in Figure 3 as open squares (predicted value) and open triangles (measured value). As both IC_{50} and $EC_{KS}4.5$ values are correlated to the logarithmic EC3, we also performed a multiple regression including both parameters, although we expect that these two parameters are strongly correlated to each other. The resulting equation is:

$$\text{Log EC3 (LLNA)} = -2.022 + 0.00451 \times EC_{KS}4.5\ (\text{KeratinoSens}) + 0.00174 \times IC_{50}\ (\text{KeratinoSens})$$

$$F = 41.86,\ p = 0.002,\ R^2 = 95.4\%$$

The read across prediction for compounds **7 – 10** from this equation is also included in Table 3.

Considering that the LLNA EC3 values themselves are subject to some variation, this is a good quantitative prediction for compounds **7** and **8**. These chemicals are structurally close analogues to the training set of the seven compounds (similar substitution pattern around the epoxide group). The set of investigated compounds also contained the two compounds **9** and **10**. These bi-functional epoxides are both physicochemically and chemically quite distinct, as they have the potential to react with both epoxide groups and thus theoretically also may crosslink proteins. These two compounds induced luciferase activity at clearly lower concentrations and they were significantly more cytotoxic. If their potency would be predicted based on the log-linear models from Figure 3, their predicted LLNA EC3 values are overpredicted by a factor of 3. However it should be noted that this prediction is based on the extrapolated, not interpolated part of the regression line. Since it is a log-linear relationship (which levels out at low concentrations), this extrapolation is particularly prone to errors and should be used with caution.

The volatility of the test compounds. The two compounds **5** and **6** are the weakest in the *in vitro* assay and in the LLNA, however, their LLNA EC3 values are unexpectedly high. We wondered whether this could be due to a low bioavailability caused by a high volatility of these compounds. The evaporation of PGE and compounds **1 - 8** from the LLNA vehicle placed on an inert surface was thus tested. Indeed, compounds **5** and **6** are quite volatile and within 5 - 10 min, 50% is lost from the LLNA vehicle under the conditions used (Table 4). However, to reflect the skin temperature (32 ºC) also the surrounding temperature was increased which makes the conditions quite different from that on the mouse ear. Furthermore, a glass slide is not readily comparable to the skin of the mouse ear where we can assume that the compound only will reside for a short period of time before it is absorbed. In addition, if the grooming behaviour of the animals is taken into account the effect of a difference in volatility would probably diminish. That the volatility of test compounds might affect the test result in the open application of the LLNA has received relatively little attention until recently. Siegel et al. (*17*) showed the importance of this parameter to explain the structure-activity in the LLNA observed for *n*-bromoalkanes. In addition, the volatile compounds 2-hexenal and ethyl acrylate were shown to be overpredicted by a linear model linking kinetic rate constants for peptide reactivity to the LLNA potency (*18*). The hypothesis that the high EC3 of

5 and **6** are affected by the open application in the LLNA might also explain the fact that for the most volatile compound **5** data from a human repeat insult patch test indicate a lowest observed effect level (LOEL) of 44 µg / cm^2, whereas the LOEL level in the LLNA calculated based on the EC3 is 7737 µg / cm^2 (ICCVAM database) (*19*). The human data are obtained under occluded conditions and the discrepancy in potency between the two tests might partly be explained by the volatility affecting the result for **5** in the open LLNA more than in the occluded human test.

Alternative regression models. The fact that a log-linear model best describes the correlation between the *in vitro* and the *in vivo* data could be due to the particularly high EC3 values of the two most volatile compounds and their high EC3 values which could have a dramatic impact on the read-across predictions. We thus repeated the correlation analysis with and without these two compounds, testing different models, i.e. a linear-linear, log-linear and log-log model. The results are shown in Table 5. For this analysis all the monofunctional monomers were used, including compounds **7** and **8**. Indeed, the log-log model gives the best correlation if the two volatile compounds are excluded (R^2=80.2 for IC_{50} and R^2=71.3% for EC_{KS}4.5), and the log-linear model best fits the data if these compounds are included (R^2=93.2 for IC_{50} and R^2=88.5% for EC_{KS}4.5). However, the difference is relatively small, and the regression equations in Table 2 are not significantly altered if the volatile compounds are excluded (data not shown). Therefore, the use of the log-linear model used initially to predict the *in vivo* response is not significantly influenced by the two volatile test compounds and thus the use of the whole data-set including the two volatiles compounds for read-across is justified, and this also gives the best correlation statistics (Table 5). This is also confirmed as we obtain similar read-across results for **7** and **8** if we perform the prediction with the log-linear model with the volatile compounds excluded from the training set (Table 6). On the other hand, if the LLNA EC3 values of compounds **9** and **10** are predicted by extrapolation based on the log-log model (which better fits the data without the volatile compounds and which does not level out at low concentrations) a more pronounced overprediction is obtained. We would 36-fold overpredict the sensitizing potency of compound **9** and 60-fold overpredict that of compound **10;** thus, they would be predicted to be much too strong sensitizers based on the KeratinoSens result only**.**

In conclusion, these data indicate that *in vitro* based read-across within a defined structural class is possible and the potency of structurally related epoxides in the LLNA can be predicted based on quantitative data from the KeratinoSens assay. Yet it has to be kept in mind that for many structural classes such nice datasets with animal data on such a large number of closely related structural analogues do not exist, and read-across would need to be made just on one or two related molecules. In such cases the potency prediction will be more challenging. In addition the data for the bifunctional compounds indicate that if extrapolations are made to compounds of significantly different structures, the reliance on one single *in vitro* assay for potency prediction is not sufficient. It has been proposed, that potency prediction should be made with an integrated testing strategy (ITS) combining a number of different tests (*20, 21*), giving less weight to the result of a single assay. As illustrated by the results for **9** and **10** this may become more important as soon as local models in narrow applicability domains are not possible.

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Table 1. LLNA and KeratinoSens test results

	LLNA EC3	**KeratinoSens results**			
	(M)	**I_{max} [2]**	**$EC_{KS}1.5$ [3]**	**$EC_{KS}4.5$ [4]**	**IC_{50} [5]**
PGE	0.031	56.3	16.1	63.2	182.1
1	0.15	102.5	27.2	101.4	303.7
2	0.13	67.7	57.1	128.0	276.0
3	0.33	167.5	52.1	162.5	437.6
4	0.083	44.0	41.2	128.4	339.9
5	2.2	107.1	59.4	245.4	726.8
6	1.1	86.2	55.8	206.3	655.9
7	0.035 [1]	18.2	48.1	87.8	186.7
8	0.16 [1]	127.1	54.6	176.4	454.0
9	0.036 [1]	5.0	6.5	12.0	22.9
10	0.036 [1]	12.6	5.2	9.9	21.7

[1] The *in vitro* testing lab was blind to these results at the time of testing
[2] Maximal fold-induction in the tested concentration range (0.98 – 2000 µM)
[3] Extrapolated concentration for 1.5 fold gene induction (µM)
[4] Extrapolated concentration for 4.5 fold gene induction (µM)
[5] Extrapolated concentration for 50% reduction in cellular viability (µM)

Table 2. Results from the correlation analysis on the training set of seven compounds (**PGE**, **1** – **6**)

	a[1]	b	R^2 (%)	F	P
logEC3 vs. $EC_{KS}1.5$	0.030	-1.935	59.0	7.2	0.044
logEC3 vs. $EC_{KS}4.5$	0.010	-2.114	94.0	77.9	<0.0005
logEC3 vs. IC_{50}	0.003	-1.918	94.5	86.1	<0.0005
EC4.5 vs. cLogP	338.28	-122.60	44.8	4.06	0.100
IC_{50} vs. cLogP	1168.8	-483.9	65.6	9.55	0.027

[1] Log-linear correlation logEC3 = a x + b

Table 3. Prediction of compounds 7, **8, 9** and **10** with read across based on the KeratinoSens data

	Predicted EC3 (M)			
	7	**8**	**9**	**10**
Based on $EC_{KS}4.5$	0.059 [1]	0.455	0.010	0.010
Based on IC_{50}	0.045	0.304	0.014	0.014
Based on equation 1	0.050	0.366	0.012	0.011
Measured	0.035	0.160	0.036	0.036

[1] The *in vitro* data of Table 1 were entered into the Log-linear models of Table 2 and into equation1

Table 4. Evaporation of the test compounds from the LLNA vehicle

	Time (min) for 50% evaporation if applied in 5% AOO[1] onto glass surface	**LLNA EC3 (M)**
PGE	>80	0.031
1	>80	0.15
2	>80	0.13
3	51.37	0.33
4	>80	0.083
5	4.72	2.2
6	10.22	1.1
7	>80	0.035
8	73.59	0.16

[1]AOO = Acetone : Olive oil, 4:1

Table 5. Alternative regression models with and without the most volatile compounds (**5** and **6**)

	All monofunctional epoxides except the volatile **5** and **6**		All monofunctional epoxides (**PGE**, **1** – **8**)	
	R^2	*P*	R^2	*p*
EC3 vs $EC_{KS}4.5$	54.1	0.060	70.3	0.005
Log EC3 vs $EC_{KS}4.5$	68.3	0.022	88.5	<0.0005
Log EC3 vs Log $EC_{KS}4.5$	71.3	0.017	82.4	0.001
EC3 vs IC_{50}	63	0.033	77.2	0.002
Log EC3 vs IC_{50}	75	0.012	93.2	<0.0005
Log EC3 vs Log IC_{50}	80.2	0.006	90.4	<0.0005

Table 6. Prediction of the EC3 of compounds **7** and **8** with read-across based on the log-linear model and excluding the volatile compounds

	7	**8**
Based on $EC_{KS}4.5$	0.067	0.294
Based on IC_{50}	0.036	0.312
Measured	0.035	0.160

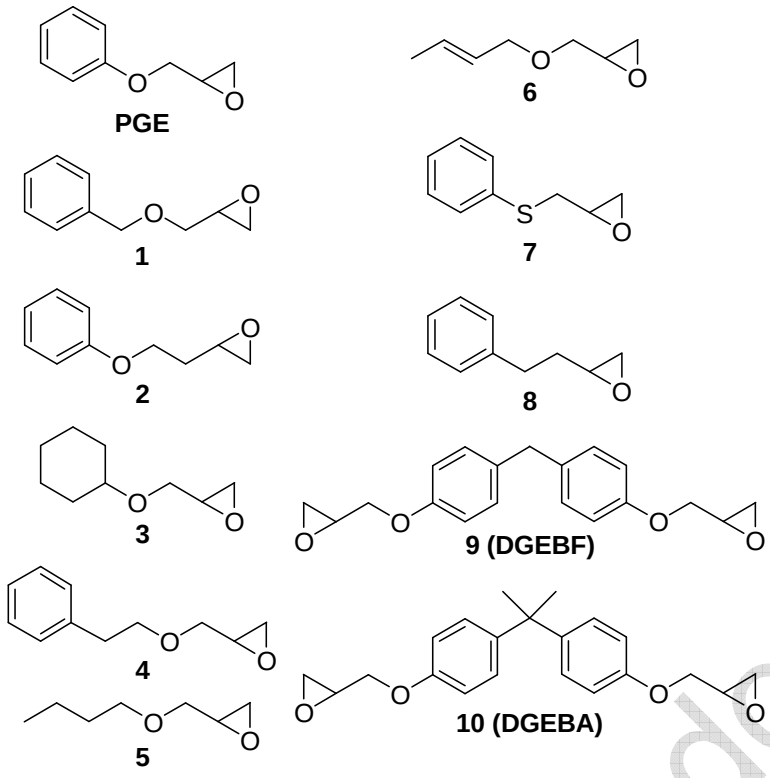

Figure 1. Compounds tested in this study. PGE and compounds **1** - **6** were used in the correlation analysis while compounds **7** – **10** were used for read across predictions.

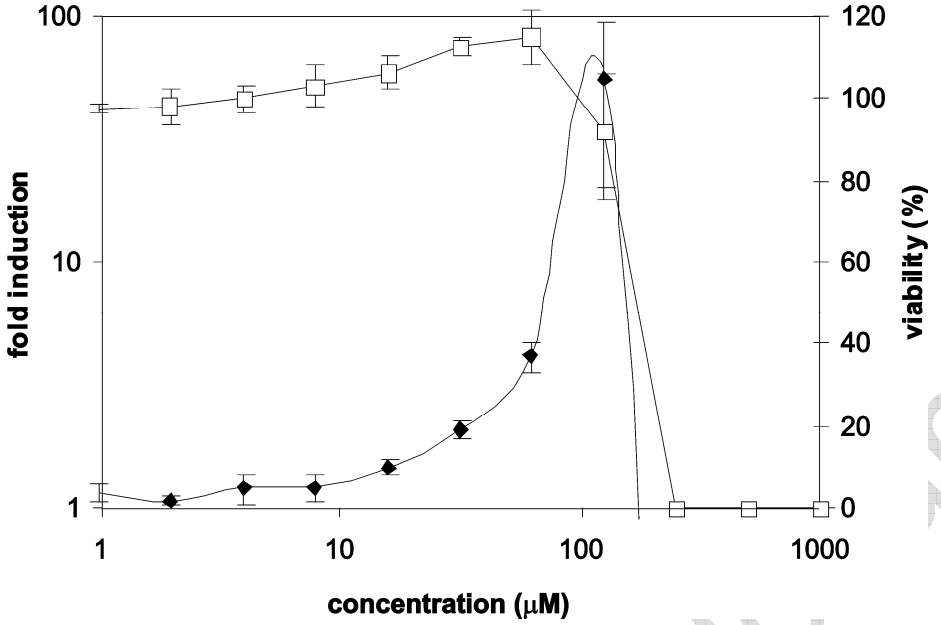

Figure 2. Induction of luciferase activity (closed diamonds) and cellular viability (open squares) for PGE.

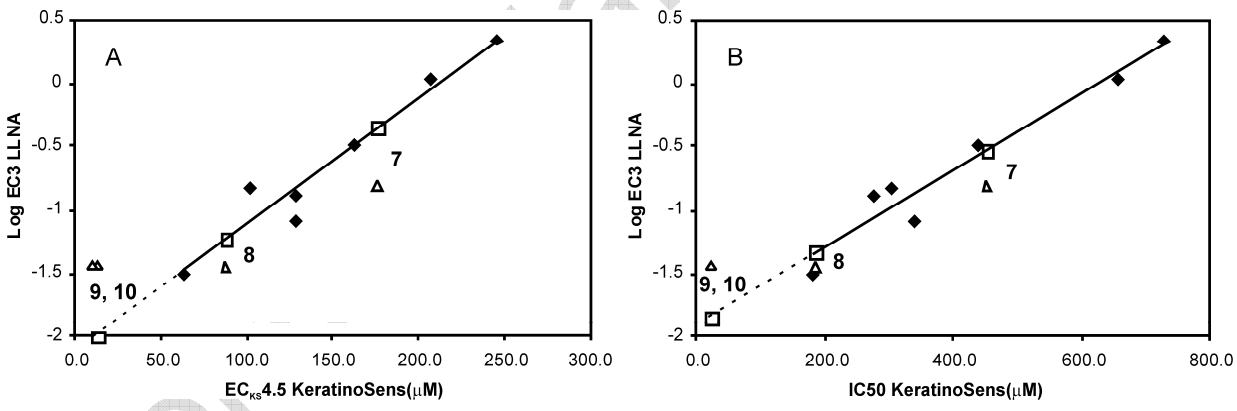

Figure 3. The relationship between the EC3 in the LLNA and the *in vitro* results. (A) The relationship to the luciferase induction expressed as the $EC_{KS}4.5$ value (B) correlation to cytotoxicity expressed as IC_{50} value. Filled diamonds: training set of the seven compounds (PGE, **1** – **6**) with known LLNA data at the time for the correlation analysis. Open squares: Compounds **7, 8, 9** and **10** predicted based on read across; Open triangles: The *in vitro* data for the analogue compounds **7, 8, 9** and **10** plotted vs. the measured *in vivo* data. Note the logarithmic plot for the LLNA result but linear scale for the *in vitro* data.