

Quantitative structure-activity relationships: sulfonate esters in the local lymph node assay

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The biological activity of skin-sensitizing chemicals is related to their ability to react, either directly or after metabolic activation, with appropriate skin proteins. For direct acting electrophilic compounds, this ability can be modelled, using the RAI (relative alkylation index) approach, by a combination of electrophilicity and hydrophobicity parameters. The development of predictive quantitative structure-activity relationships (QSAR) models of skin sensitization, using mechanism-based physicochemical parameters, has been greatly facilitated by the introduction of the murine local lymph node assay (LLNA), which is able to describe the extent of the biological response in objective and quantitative terms. In the present work, sensitization response data in the LLNA is generated for a series of 6 sulfonate esters. An RAI-based hybrid QSAR/dose-response relationship is derived using a negative hydrophobicity coefficient in the RAI expression, to model the effect of retention of the hydrophobic test chemicals in the stratum corneum. Dose-response analyses are used to estimate EC₃ and EC₂₀ values as quantitative indices of skin sensitization potential for each compound, and regression analysis is applied to develop QSARs correlating these EC₃ and EC₂₀ values with an RAI-based parameter. The high statistical quality of these QSARs demonstrates both the consistency of the LLNA method for generating high quality skin sensitization data, and the value of the RAI approach in development of mathematical models for skin sensitization.

Key words: contact sensitization potential; quantitative structure-activity relationships; murine local lymph node assay; mouse sensitization; sulfonate esters; relative alkylation index; dose-response relationships. © Munksgaard, 2000.

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The development of mathematical model-based quantitative structure-activity relationships (QSARs) for contact allergens is the subject of a long-standing research programme in our laboratories. In previous publications, summarized in recent reviews (1, 2), we have presented QSARs correlating quantitative estimates of contact allergenic activity with composite physicochemical parameters derived from the RAI (relative alkylation index) model (3). Recently, the introduction of the murine local lymph node assay (LLNA), as well as offering substantial advantages in terms of animal welfare, has simplified the task of developing QSARs (4). Unlike the long-established guinea pig tests, where there are both induction and challenge treatments (involving different routes and/or vehicles) to consider as well as a subjective endpoint, the LLNA involves a single epicutaneous dose of the test material and provides an objective and quantitative endpoint. The LLNA is described in

detail in numerous publications (5–7); for the present purpose, it suffices to state that it involves repeated topical application of the test chemical to mouse ear skin followed by quantitative assay of the T-cell response in the draining lymph node, measured as a function of the incorporation of tritiated thymidine. The sensitization response is quantified in terms of the stimulation index (SI), this being the ratio of the activity in draining lymph nodes of treated mice compared to that in concurrent vehicle-treated mice. A given chemical is tested over a range of concentrations, such that a dose-response relationship can be determined, from which the sensitization potential, defined in terms of the concentration required to give a specified SI value, can be quantified. Currently, the preference is to estimate the concentration of a chemical required to generate an SI of 3, the EC₃ value (8–10).

In 2 earlier publications, we have described a

QSAR study of the sensitization potential of alkylating sulfonate esters in a modified Magnusson and Kligman guinea pig assay (11, 12). Because both induction and challenge concentrations had to be considered, the correlation took the form of a hybrid dose-response/structure-activity relationship, based on RAI values for both induction and challenge. We now describe the development of a QSAR for the same set of chemicals, tested in the LLNA.

Materials and Methods

Materials

The chemicals were obtained as described previously (11, 12). Their structures and code names are shown in Fig. 1.

Local lymph node assay

Female CBA/Ca strain mice (Harlan UK Ltd., Bicester, Oxford), aged 7–12 weeks old, were used in these studies. Mice were housed in groups of 4 per cage under standard conditions; SDS PCD pelleted diet (Special Diets Services Ltd., UK) and tap water were available ad libitum. In brief, groups of 4 mice were exposed topically on the dorsum of both ears to 25 ml of each concentration of test chemical, or to an equal volume of the appropriate vehicle alone, daily for 3 consecutive days. 5 days after the 1st treatment, all mice were injected intravenously via the tail vein with 250 μ l of phosphate buffered saline (PBS) containing 20 μ Ci of [^{3} H] methyl thymidine (^{3} HTdR; specific activity 2 Ci/mmol; Amersham International,

Amersham, UK). 5 h later the mice were killed and the draining auricular lymph nodes were excised and pooled for each experimental group. Single-cell suspensions of lymph node cells (LNC) were prepared by mechanical disaggregation through 200-mesh stainless steel gauze. Cells were washed 2 $\times$ with PBS and precipitated in 5% trichloroacetic acid (TCA) at 4°C overnight. Pellets were then resuspended in 1 ml 5% TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Hisafe 3', Wallac, Turku, Finland). Incorporation of ^{3} HTdR was measured by β -scintillation counting as disintegrations per minute (dpm) per node for each experimental group. For each group a stimulation index (SI) was derived by determining the increase in ^{3} HTdR incorporation relative to the appropriate (concurrent) vehicle-treated control group.

Mathematical analysis

In general, linear interpolation of the dose-response data is suitable for calculation of the estimated concentration of test chemical required to induce a stimulation index of 3 relative to concurrent vehicle-treated controls, the EC3 value. Ideally the EC3 value is determined by interpolating between 2 points on the SI axis, one immediately above and the other immediately below the SI value of 3 (vehicle-treated control values [SI=1] not being used for the latter). Where the data points lying immediately above and below the SI value of 3 have the co-ordinates (a, b) and (c, d) respectively, then the EC3 value may be calculated using the following equation (10):

$$EC3 = c + [(3-d)/(b-d)] \times (a-c).$$

There are however other methods for evaluation of dose-response data, and these are discussed and compared in more detail in the dose-response analysis section.

Results

Table 1 shows mouse local lymph node assay (LLNA) test results for the sulfonate esters investigated. Several features are apparent from the data. There is a clear dose-response pattern for each of the sensitizers. In most cases response increases monotonically with dose, but there are 2 exceptions. In the case of C12/C1, which has a log p value of 5.49, and to a lesser extent in the case of Δ 2C16/C1, which has a log p value of 7.1, with increasing induction dose beyond a certain limit the sensitization response decreases. This could indicate an overload effect, of the kind sometimes noted in plots of guinea pig sensitization response

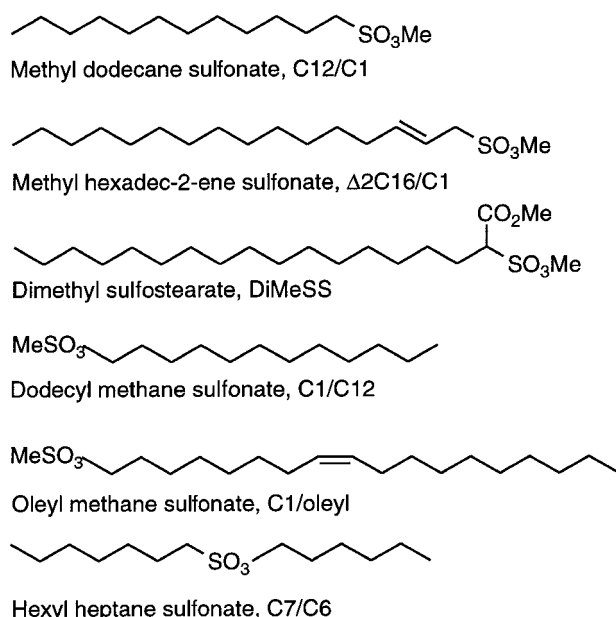


Fig. 1. Sulfonate esters.

Table 1. LLNA sensitization data

Compound		C	RAI	SI
C12/C1	log $p=5.49$	1	1.79	21.6
	log $k=1.48$	2.5	2.19	39.6
	RSP=4.22	5	2.49	48.6
		5	2.49	46.3
		10	2.79	43.6
A2C16/C1		25	3.19	34.1
	log $p=7.10$	5	1.61	26.7
	log $k=1.48$	10	1.91	35.4
DiMeSS	RSP=3.41	25	2.31	32.9
	log $p=8.63$	5	0.75	5.1
	log $k=1.48$	10	1.05	11.6
C1/C12	RSP=2.64	25	1.45	25.6
	log $p=5.49$	5	-0.47	2.1
	log $k=0$	10	-0.17	3.3
C1/oleyl	RSP=1.25	25	0.23	9
	log $p=8.18$	5	-1.93	1
	log $k=0$	10	-1.63	1.3
C7/C6	RSP=-0.09	25	-1.23	3
	log $p=5.49$	5	-0.47	2
	log $k=0$	10	-0.17	2.5
	RSP=1.25	25	0.23	7.5
		50	0.53	10.8

against RAI for induction (2) and which can be represented by a negative RAI^2 term in guinea pig sensitization QSARs (4, 11–13), or some other type of saturation effect. The mechanistic basis of the overload effect is not unequivocally established – the involvement of T-cells with suppressor or regulatory function is a possibility (14, 15). However, since only 3 of the 22 response data points fall into this category, we have not attempted to model the effect, and have excluded these data points from the dose-response plots used to derive the toxicity values.

The higher alkyl transfer agents are in all cases less potent than their methyl transfer analogues having the same log p value. This in itself is not surprising given the greater chemical reactivity of the latter (11, 12, 16).

Increasing log p appears to be correlated with decreasing sensitization potential, contrary to what would be predicted by the basic RAI model. Thus DiMeSS, which has the highest log p value, is the least potent of the 3 methyl transfer agents. This cannot be attributed to an overload effect, since the sensitization response is an increasing function of dose for this compound. Likewise, for the 3 higher alkyl group transfer agents, C1/oleyl, with the highest log p value, has much the lowest sensitization potential.

The absence of an overload effect for DiMeSS, given that there is an overload effect with C12/C1, is another apparent anomaly when viewed from the perspective of the basic RAI model.

Unlike the guinea pig test used in earlier QSAR studies on alkyl alkanesulfonates (11, 12) the LLNA is a single-dose topical application method. Since the alkyl alkanesulfonates studied here are in all cases very hydrophobic, it is reasonable to assume that not all of the dose applied topically will reach the site of action in the timescale of the test, and that the extent to which the chemical is retained in the stratum corneum will be correlated with log p . On the basis of a mathematical modelling study (to be published elsewhere) analysing the influence of stratum corneum penetration ability on sensitization potential, we can model this situation by using a negative log P coefficient in the RAI expression. In the data analysis which follows, the RAI is calculated as:

$$RAI = \log C + 2 \log k - 0.5 \log p,$$

where C is the concentration, in ppm moles, applied topically.

RAI plots

In Fig. 2, all of the SI values are plotted against the corresponding RAI values. The data give the typical response-RAI curve, as described previously for other types of sensitizing chemicals, with some evidence of an overload effect at high RAI values. Fig. 3 shows a linear regression plot of log (SI) against RAI, the overload region points being excluded from the regression analysis. The regression equation:

$$\log (SI) = 0.39 (\pm 0.05) RAI + 0.69 (\pm 0.08) \\ n=20, R^2=0.930, s=0.15, F=240$$

is a hybrid QSAR/dose-response relationship, analogous to that reported for the same chemicals

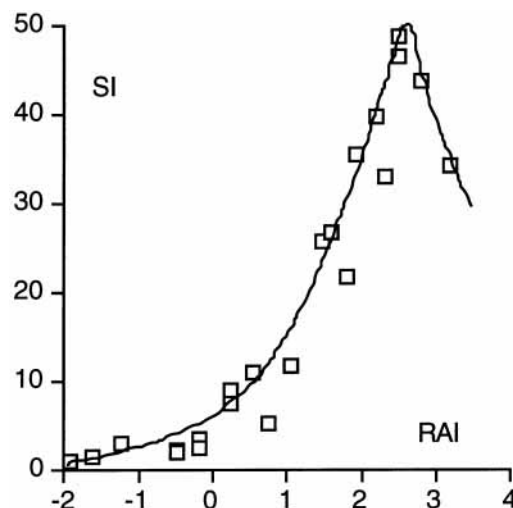
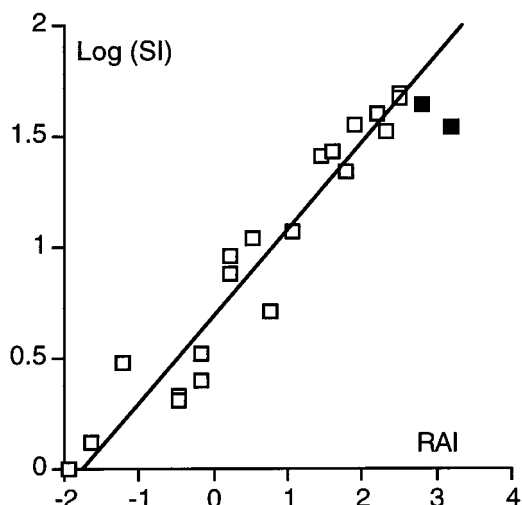


Fig. 2. SI versus RAI.



$$\text{Log (SI)} = 0.39 \text{ RAI} + 0.69 \quad R^2 = 0.930$$

■ C12/C1, overload region, omitted from regression

Fig. 3. Log (SI) versus RAI.

in the guinea pig MSIAT assay. In this case, the equation is simpler because there is no term to model the overload effect and no challenge term.

To develop a "pure" QSAR we need to derive, for each compound, the concentration required to produce a defined SI value. The sensitization potential can then be defined in terms of these concentrations and plotted against physico-chemical parameters. To proceed further we need to analyse the dose-response data for each compound.

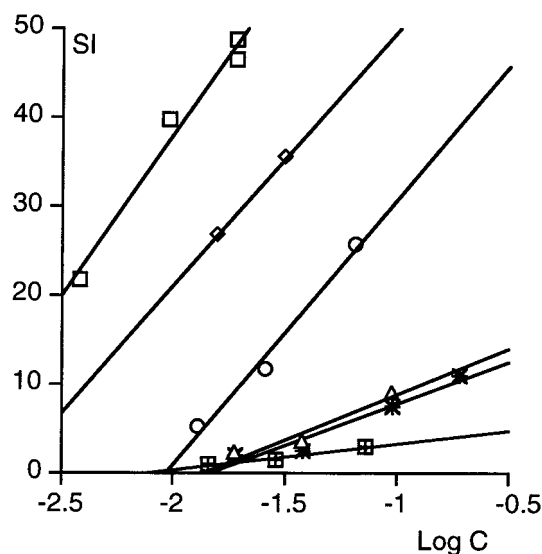
Dose-response analysis

The standard way to determine a quantitative measure of toxicity of any kind is to measure the toxic response over a range of concentrations, and to fit a line to a plot of some function of the toxic response against a function of the concentration. From the equation of this line, the concentration to produce a specified magnitude of response can be estimated. In aquatic toxicity EC50, the concentration required to produce the defined effect (e.g., immobilization in a *Daphnia* toxicity test) in 50% of the test population, is normally used as the basis for quantification of toxicity. EC50 values themselves, expressed in weight concentration units, are often quoted, but for QSAR purposes the toxicity is usually quantified as $\log (1/\text{EC}50)$, with the EC50 being in molar concentration units. In LLNA sensitization studies EC3, the estimated concentration which gives an SI ratio of 3, is particularly useful in the same way as EC50, since an SI value of 3 is defined as the threshold for a positive sensitization response.

For the sensitization data set presented here,

there are some problems in deriving EC3 values. For the 3 methyl sulfonates studied, the observed SI values at all concentrations tested are substantially above 3, so their EC3 values would have to be estimated by extrapolation from the dose-response plots. This leads to a large element of uncertainty from 2 sources. The 1st is statistical: in a linear regression plot, the greater the distance from the mean the wider the error limits on an estimated value. The 2nd is mathematical: although different functions may be approximately co-linear over a narrow range, they will diverge over a wider range. In other words, for interpolation any function which gives a reasonable straight line fit to the data will suffice, but for extrapolation it is important to choose the correct function. These points are illustrated in Table 2, which shows $\log (1/\text{EC}3)$ values and EC3 values derived from plots of SI, $\log (SI)$ and probit (SI) against $\log C$. The dose-response plots for these 3 functions are shown in Figs. 4, 5 and 6.

The probit plots require some explanation. In cases where the toxic response data can be expressed as a % of the maximum possible (e.g., the % of a *Daphnia* test population immobilized by a test chemical at a particular concentration), the probit value corresponding to the % response is the appropriate function to use in a dose-response plot. The theory behind probit values, and their use in analysis of guinea pig sensitization test data,



- C12/C1 $\text{SI} = 36 \log C + 110$; $R^2 = 0.976$
- ◇ $\Delta 2\text{C}16/1$ $\text{SI} = 29 \log C + 79$
- DiMeSS $\text{SI} = 30 \log C + 60$; $R^2 = 0.984$
- △ C1/C12 $\text{SI} = 10 \log C + 19$; $R^2 = 0.924$
- ⊠ C1/oleyl $\text{SI} = 2.9 \log C + 6.2$; $R^2 = 0.910$
- * C7/C6 $\text{SI} = 9.3 \log C + 17$; $R^2 = 0.945$

Fig. 4. Dose-response plots: SI versus $\log C$.

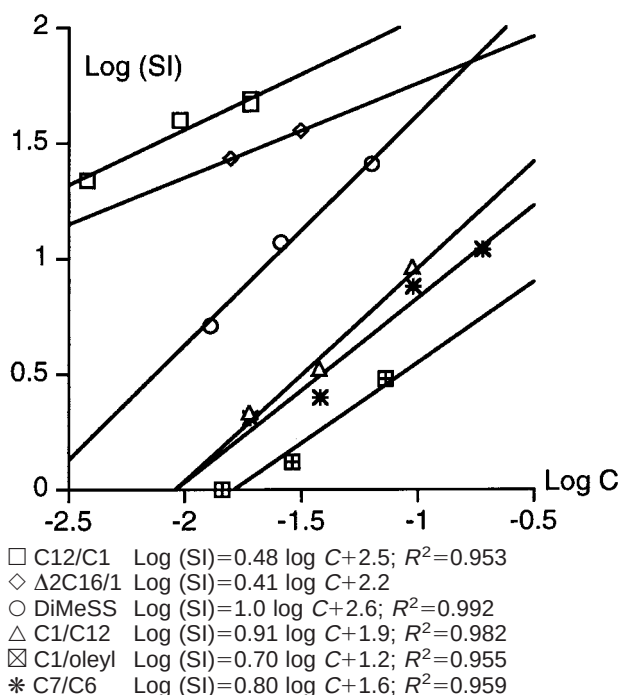


Fig. 5. Dose-response plots: log (SI) versus log C.

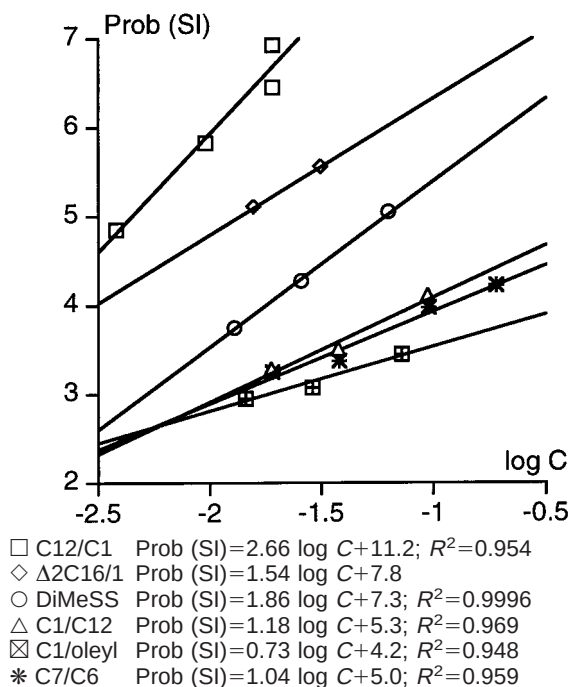


Fig. 6. Dose-response plots: probit (SI) versus log C.

has been discussed previously (2, 11–13). In brief, probit values, which are in standard deviation units, are based on integrals under the standard deviation curve. Direct plots of toxic response against dose are usually S-shaped, and this shape can be linearized by the probit function. The SI

value determined in the LLNA is a measure of the lymphocyte activity provoked by the test material, and in principle there should be an upper SI limit corresponding to the maximum possible lymphocyte activity for the epitope produced by the test compound. If the observed SI value is expressed as a % of the maximum possible value, then these percentages can be converted to the corresponding probit values, which can be used in the dose-response plots. The difficulty is that although there must be a maximum possible SI value, it cannot be precisely quantified. To our knowledge no SI values above 100 have ever been observed in LLNA studies. On the basis of the RAI plot shown in Fig. 2, it seems reasonable to take a rounded figure of 50 as the upper SI limit for the purpose of calculating probits. There are 2 major sources of error in this procedure. Firstly, if 50 is not a good estimate of the SI limit, the probit values will be in error. This error will be greatest when the SI values are highest (i.e., closest to the assumed upper limit). Secondly, and also affecting the highest SI values most, the onset of the overload effect may affect the linearity of the probit based dose-response relationship.

For the 3 higher alkyl transfer agents C1/C12, C1/oleyl and C7/C6, the function used for the dose-response plot does not significantly affect the estimate, by interpolation, of the toxicity. However there are substantial differences in the estimates, by extrapolation, for the methyl transfer agents, particularly the 2 most strongly sensitizing compounds; the dose-response plots based on SI and on prob (SI) give similar estimates, but the log (SI)-based plots give EC3 values more than an order of magnitude lower than the other plots.

Table 2. Log (1/EC3) and EC3 values

Compound		Function plotted against log C		
		SI	log (SI)	prob (SI)
C12/C1	log (1/EC3)	2.96	4.26	2.93
	EC3	0.3	0.01	0.3
Δ2C16/C1	log (1/EC3)	2.62	4.13	2.87
	EC3	0.8	0.02	0.4
DiMeSS	log (1/EC3)	1.94	2.15	2.04
	EC3	4.5	3	4
C1/C12	log (1/EC3)	1.57	1.52	1.54
	EC3	7	8	8
C1/oleyl	log (1/EC3)	1.09	1.10	1.1
	EC3	28	27	27
C7/C6	log (1/EC3)	1.51	1.44	1.46
	EC3	8	10	9

Note. For log (1/EC3), concentration is in mole %. For EC3, concentration is in weight %, and is calculated from log (1/EC3) as $EC3 = M \times 10^{-\log(1/EC3)}$.

Table 3. Log (1/EC20) and EC20 values

Compound		Function plotted against log C		
		SI	log (SI)	prob (SI)
C12/C1	log (1/EC20)	2.49	2.54	2.44
	EC20	1	1	1
Δ 2C16/C1	log (1/EC20)	2.04	2.11	2.02
	EC20	3	2	3
DiMeSS	log (1/EC20)	1.36	1.32	1.34
	EC20	17	19	18
C1/C12	log (1/EC20)	-0.12	0.62	0.43
	EC20	340	63	98
C1/oleyl	Log (1/EC20)	-4.71	-0.08	-0.69
	EC20	17 million	420	1690
C7/C6	log (1/EC20)	-0.31	0.41	0.21
	EC20	540	100	161

Note 1. For log (1/EC20), C is in mole %. For EC20, C is in weight %, and is calculated from log (1/EC3) as $EC20 = M \times 10^{-\log(1/EC20)}$.

Note 2. EC20 values estimated for some of the compounds by extrapolation are above 100%, which implies that it would not in practice be possible to provoke SI values of 20 at any test concentration. Nevertheless it is still valid to use the corresponding log (1/EC20) values as quantitative indices of sensitization potential.

Table 4. Sensitivity analysis on probit values

Compound	Probit values based on SI as:		
	% of 50	% of 60	% of 100
EC3 values (%)			
C12/C1	0.3	0.2	0.1
Δ 2C16/C1	0.4	0.3	0.1
DiMeSS	4	3	3
C1/C12	8	8	8
C1/oleyl	27	27	27
C7/C6	9	9	9
EC20 values (%)			
C12/C1	1	0.9	0.8
Δ 2C16/C1	3	2.9	2.7
DiMeSS	18	18	18
C1/C12	98	93	84
C1/oleyl	1690	1427	1094
C7/C6	161	153	138

The mean SI value from all of the assays is, to the nearest round figure, 20. For the purposes of QSAR development it is therefore convenient to use EC20 as the basis for quantification of sensitization potential; although this involves extrapolation of dose-response plots for all except DiMeSS, the extent of extrapolation (i.e. difference between extrapolated value and nearest observed value) is less than for the EC3 values. Table 3 shows log (1/EC20) values and EC20 values derived from plots of SI, log (SI) and probit (SI) against log C.

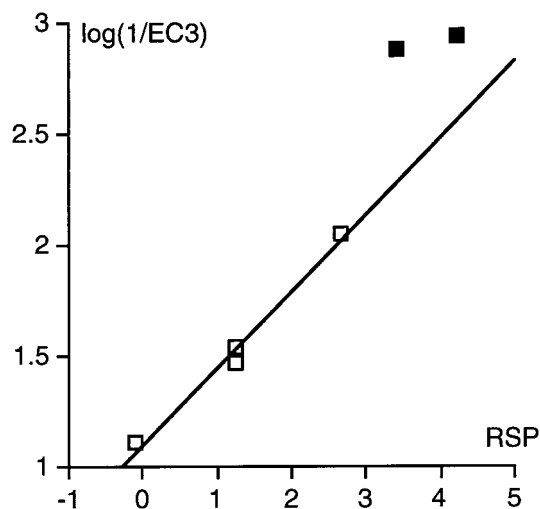
Overall, the agreement between estimates from

the 3 dose-response plots is better for the EC20 values than for the EC3 values, apart from C1/oleyl. For this compound, the SI vs log C plot gives an estimated EC20 value more than 4 orders of magnitude greater than that estimated from the other 2 plots. This major difference is easily rationalized: the SI responses for this compound, the weakest sensitiser in the set, fall on the base part of the S-shaped curve.

Table 4 shows a sensitivity analysis to determine the extent to which the choice of the SI upper limit for the probit plots affects the estimates of EC3 and EC20 from the probit-based dose-response plots. As regards EC3, the estimates for the 2 strongest sensitizers show variation by a factor of up to 4 according to the choice of SI limit, but the estimates for the other 4 compounds are not significantly affected. For EC20, the maximum variation is much less. Since there is a good mechanistic and theoretical basis for the probit function, and since the above sensitivity analysis shows the estimates to be reasonably robust, the toxicity values derived from the probit plots (based on 50 as the SI upper limit) were chosen for use in the development of QSARs.

QSAR development

For pure QSAR development based on the RAI model it is convenient to define a dose-independent relative sensitization parameter RSP. This is calculated exactly as for the RAI, except that the log D



□ Excluding C12/C1 and Δ 2C16/C1

Regression line:

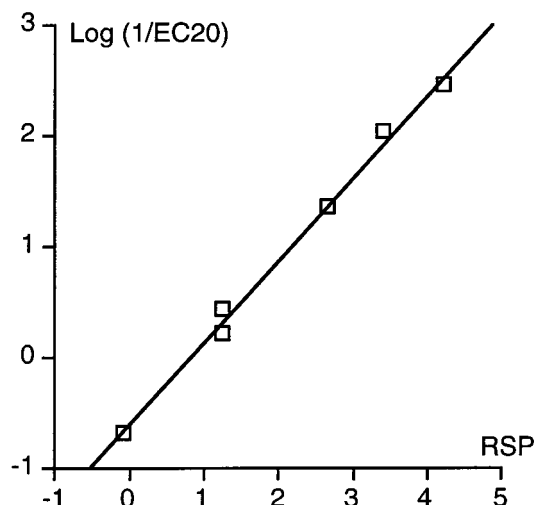
Log (1/EC3)=0.34 RSP+1.10; R^2 (n=4)=0.984

■ C12/C1 and Δ 2C16/C1

Regression including these points gives:

Log (1/EC3)=0.47 RSP+1.00; R^2 (n=6)=0.947

Fig. 7. Log (1/EC3) versus RSP.



Regression line:
 $\text{Log (1/EC20)} = 0.74 \text{ RSP} - 0.61$; $R^2 (n=6) = 0.994$.

Fig. 8. Log (1/EC20) versus RSP.

term is omitted. For the present study, RSP is given by:

$$\text{RSP} = 2 \log k - 0.5 \log p.$$

Fig. 7 shows a plot of $\log (1/\text{EC3})$ against RSP, the EC3 values being derived from the probit-based dose-response plots. Although a quite good straight line fit is obtained by regression analysis for all 6 data points, we decided to treat the 2 points corresponding to C12/C1 and $\Delta 2\text{C16/C1}$ as outliers. As demonstrated above, the estimate of EC3 for these compounds is quite sensitive to the choice of the SI upper limit. Furthermore, the measured SI values for these compounds were closest to the assumed upper limit, where the slope of the probit plots is likely to be affected by the negative contribution from the overload effect. Therefore the EC3 values estimated by extrapolation may be unrealistically low.

These considerations do not apply to the EC20 values, and it is therefore appropriate to include all 6 compounds in the regression analysis for $\log 1/\text{EC20}$ against RSP. This plot is shown in Fig. 8.

The QSAR equations may now be summarised as follows:

For EC3: eq. (1)
 $\text{Log (1/EC3)} = 0.34 (\pm 0.06) \text{ RSP} + 1.10 (\pm 0.10)$
 $n=4$, $R^2=0.985$, $s=0.06$, $F=130$

Outliers:

C12/C1, $\log (1/\text{EC3})$: calc., 2.54; from dose-response plot, 2.93

$\Delta 2\text{C16/C1}$, $\log (1/\text{EC3})$: calc., 2.27; from dose-response plot, 2.87

For EC20: eq. (2)
 $\text{Log (1/EC20)} = 0.74 (\pm 0.06) \text{ RSP} - 0.61 (\pm 0.14)$
 $n=6$, $R^2=0.994$, $s=0.10$, $F=702$

Conclusions

In the context of the search for non-animal predictive models of skin sensitization, *in vitro* test methods are still far from being accepted as valid alternatives, even for the most basic binary hazard identification purposes (17, 18). A recent review suggests that whilst there may be opportunities for the future, these are clearly still some way off, requiring substantial further development (19). In contrast, by utilizing our understanding of the chemistry of skin sensitization, it has proven possible to elaborate a straightforward hazard identification system based on chemical structural alerts, this being the expert system known as DEREK (20–22). Furthermore, for selected series of chemicals, it has been convincingly demonstrated, using relatively simple mathematical models based on a synthesis of our mechanistic understanding of skin sensitization and the appreciation of the physicochemical properties pertinent to this phenomenon, that quantitative predictions of sensitizing potency can be made (1–4, 11–13, 23, 24). However, in order to build such mathematical model-based QSARs, it is preferable to have objective, quantitative data not only to describe the physicochemical properties of the chemicals under investigation, but also to describe the extent of the biological response being assessed. For skin sensitization, such an opportunity has been provided by the development of the LLNA (4, 10, 23).

In the present paper, we have demonstrated using LLNA data that it is possible to construct an excellent QSAR for skin-sensitizing sulfonate esters (as evidenced by the statistical quality of eq. (1) and (2), particularly the very high r^2 value of 0.994 for eq. (2). Furthermore, by implication, the quality of the LLNA data employed may be considered very good and the suitability of the RAI model as a mathematical model has been further vindicated. These tools are now being applied to the analysis of an extensive series of diketones, testing the initial prediction which was recently published (24).

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