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Physical-Chemical and Solvent Considerations in Evaluating the Influence of Carbon Chain Length on the Skin Sensitization Activity of 1-Bromoalkanes

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The murine local lymph node assay (LLNA) is an internationally accepted assay for identification of contact allergens. The LLNA has also been used in research studies to evaluate contact allergen potency, as well as chemical structural—allergenic activity relationships. The 1-bromoalkanes have been used in such a manner as they represent a chemical series with generally the same chemical reactivity but differing in alkane carbon chain length–dependent lipid solubilities. Previous reports noted a biphasic LLNA response with increasing carbon chain length that peaked at the 16-carbon chain (C16) of 1-bromohexadecane (delivered in an acetone-olive oil [AOO] vehicle; 4:1). In the present study, this biphasic LLNA response was confirmed, and 1-bromoalkane chemical-physical factors were explored using both modeling tools and further laboratory studies to help understand this finding. Volatility and effect of vehicle on 1-bromoalkanes' sensitizations were assessed. Selected 1-bromoalkanes were tested in the LLNA using the polar, protic vehicle, tetrahydrofuran-butanol (THF-BuOH; 1:1), to compare to the nonpolar (aprotic) vehicle AOO 1-bromoalkanes-LLNA responses. Enhanced 1-bromoalkane LLNA responses were observed using the THF-BuOH vehicle but with the greatest activity still observed for 1-bromohexadecane (C16). The shorter 1-bromoalkanes were subject to volatile losses upon application with approximately 75% volatile loss from a surface of 1-bromohexane (C6) within 5 min at room temperature. It is concluded that multiple factors, in addition to lipid solubility, including vehicle, solvation, and retention on the skin surface contribute to the apparent potency of 1-bromoalkanes in the LLNA.

Key Words: **bromoalkane; structure-activity relationship; murine local lymph node assay; contact allergen.**

The current hypotheses of skin sensitization (Dupuis and Benezra, 1982; Roberts and Lepoittevin, 1998; Smith and Hotchkiss, 2001) assume that most skin sensitizers are electrophiles capable of reacting with nucleophilic groups located on a protein surface (Smith and Hotchkiss, 2001). A comprehensive analysis of a variety of skin sensitizers conducted by Roberts and Lepoittevin (1998) concluded that the electrophilic properties of a chemical or its potential metabolites are good predictors of skin sensitization activity. Bromoalkanes are a well-known group of electrophilic compounds that are predicted to react with nucleophilic centers located on skin proteins, such as amines and thiols, to form potential immunogens. In addition to the ability to react directly with biological macromolecules, bromoalkanes may potentially undergo microsomal metabolism to release a bromide and possibly reactive metabolites (James *et al.* 1968). Glutathione transferases catalyze the conjugation of monohalides to glutathione which is considered to be a detoxification reaction (Guengerich, 2005).

A systematic evaluation of skin sensitization potential of bromoalkanes was conducted by Basketter *et al.* (1992). They found a biphasic relationship between the sensitization potential and the bromoalkane carbon chain length. Although the EC3 parameter was not defined at the time of publication, the description of the T/C_3 parameter corresponds to the contemporary definition of the EC3 parameter as an estimated dose corresponding to a stimulation index (SI) value of three times control lymph node cell proliferation (Basketter *et al.*, 1999). Basketter *et al.* suggested that the T/C_3 parameter might establish a connection between skin sensitization activity of bromoalkanes, and their relative skin permeation rate as the relative reactivity (i.e., ability to haptenate a protein) of the bromoalkanes should be independent of alkane carbon chain length.

It is very important for the understanding of allergic contact dermatitis to identify the underlying causes of the lack of predictability of local lymph node assay (LLNA) potency within the bromoalkane series and why the physical-chemical parameters presently used for such potency estimations may be inadequate. To that end, the main purpose of this study is to confirm the carbon chain length–dependent biphasic *n*-bromoalkanes LLNA response as observed by Basketter *et al.*

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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(1992) and to examine the physical/chemical characteristics of the *n*-bromoalkanes that may contribute to this observation and play an important role in interpretation of LLNA data for assessment of allergen potency.

MATERIALS AND METHODS

Animals. Female BALB/c mice, 8–12 weeks old, were purchased from Taconic (Hudson, NY). Mice were quarantined for 1 week upon arrival, fed a modified NIH-31 6% irradiated rodent diet (Harlan Teklad #7913) and provided tap water *ad libitum*. Animal facilities were maintained between 18 and 26°C and 25–70% relative humidity with light-dark cycles at 12-h intervals (6:00 A.M.–6:00 P.M.). Cages were cleaned and sanitized weekly. The National Institute for Occupational Safety and Health Animal Facility is an environmentally controlled barrier facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Mice were weighed, tail marked for identification, and assigned to homogeneous weight groups ($n = 5$) before each experiment. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Chemicals. All bromoalkanes were purchased from Sigma-Aldrich Chemical Company (St Louis, MO). For clarity, the 1-bromoalkane carbon chain length will be indicated in parenthesis (e.g., 1-bromohexane [C6] = Br-CH_2-CH_2-CH_2-CH2-CH_2-CH_3). Alpha-hexylcinnamaldehyde (HCA) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). All test articles were prepared in 4:1 acetone-olive oil (AOO), except 1-bromohexane and 1-bromoheptane which were in acetone only and also in 1:1 tetrahydrofuran-butanol (THF-BuOH).

The LLNA. The LLNA was performed following the method described in the Interagency Coordinating Committee on Validation of Alternative Methods Peer Review Panel report [National Institute of Environmental Health Sciences (NIEHS), 1999] with minor modifications. The BALB/c mouse strain is considered a more Th_2 phenotype but has been demonstrated to provide comparable LLNA responses to the more commonly employed DBA/2 and B6C3F1 strains (Woolhiser *et al*., 2000). Briefly, mice were exposed topically to vehicles (acetone, AOO, or THF-BuOH), to bromoalkane in acetone (bromohexane and bromooctane), AOO, or THF-BuOH, or to positive control (30% HCA) on the dorsal surface of each ear (25 μl per ear) for three consecutive days. Animals were allowed to rest for 2 days following the last exposure. On day 6, mice were injected, iv, via the lateral tail vein with 20 μCi ^{3}H-thymidine (Dupont NEN, Boston, MA; specific activity 2 Ci/mmol). Five hours after ^{3}H-thymidine injection, animals were euthanized via CO_2 inhalation, and the left and right cervical draining lymph nodes, located at the bifurcation of the jugular vein, were excised and pooled for each animal. Single-cell suspensions were made, and following overnight incubation in 5% trichloroacetic acid, samples were counted using a Packard Tri-Carb 2500TR liquid scintillation analyzer. SIs were calculated by dividing the mean disintegrations per minute (DPMs) per test group by the mean DPM for the vehicle control group. Three or four concentrations of bromoalkane were evaluated to obtain an EC3 value (concentration of chemical required to induce a threefold increase over the vehicle control). Poor solubility in AOO of some bromoalkanes limited the concentration ranges tested in the LLNA.

EC3 values were calculated using the standard model (Basketter *et al*., 1999), which requires two SI values: one below 3 and another above it. In cases where all SI values were higher than the threefold threshold, the model proposed by Gerberick *et al*. (2004) was applied. For chemicals that did not have an SI value above the three thresholds, an EC3 value was estimated from best-fit curves of the data.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 4.0 (San Diego, CA). All data were analyzed by a one-way ANOVA. In the ANOVA, when significant differences were detected ($p = 0.05$), Dunnett's test was used to compare treatment groups with the appropriate control group. *t*-Test was used to compare LLNA responses between different test compound vehicles.

Bromoalkane volatility studies. Bromohexane, bromononane, bromodecane, bromoundecane, bromotetradecane, bromopentadecane, bromohexadecane, bromoheptadecane, and bromooctadecane were analyzed for volatility by measuring recovery after an open room air incubation (room temperature [RT]). Bromoalkane samples and standards were dissolved in AOO (4:1) at 25% wt/vol. In all, 10 μl of each mixture was placed on glass microscope slides, in duplicate. Ten microliters of standard were diluted with 5 ml acetone, and a 1-ml aliquot was added to a gas chromatographic (GC) sampling vial. The glass microscope slide was left at RT (approximately 20°C) for 1 h. The slide was washed with 5.0 ml of acetone, and a 1.0-ml aliquot was added to a GC sampling vial. Samples and standards (1-μl injection volume) were analyzed using GC-mass spectrometry (MS) (Agilent 6890/6975). The GC was equipped with a 30-m long, 0.25 mm id, and 0.25-μm film thickness HP-5MS capillary column. The inlet temperature was 250°C, and the helium flow rate was 1.0 ml/min. The oven was programmed to operate at an initial temperature hold of 70°C for 2 min, followed by a 20°C/min increase to 310°C, and held there for an additional 2 min. The MS was operated in scanning mode with a low m/z of 40.0 and a high m/z of 550.0. The MS temperature was set at 280°C.

Bromoalkane solubility test. Fifty milligrams of each solid bromoalkane was weighed and placed in screw top glass test tubes. 42.5 μl of BrC6 ($d = 1.125$) and 50 μl of BrC16 ($d = 1.0$) were aliquoted to screw top glass test tubes. Five hundred microliters of the test solvent was then aliquoted to each tube to make a 10% wt/vol solution of each test compound. The tubes were then vortexed, and if the compound was not fully dissolved, another 500 μl of test solvent was added. This was continued until the solute was dissolved or a 1.25% solution was obtained. Additional bromoalkane was added (up to 25% wt/vol) if it was soluble in the test solvent at 10%.

Toxicological modeling. To gain insight into possible mechanisms behind the different skin sensitization activity of bromoalkanes, several molecular properties were calculated using Cerius^2 (Accelrys, Inc., San Diego, CA) and Dragon v5.0 (Taleto, Milan, Italy). Predictions of skin sensitization activity were made using Derek for Windows (LHASA Ltd, University of Leeds, Leeds, UK) and TOPKAT 6.2 (Accelrys, Inc.). In addition, the Gaussian 98 quantum chemical software package (Frisch *et al*., 1998) was used for the *ab initio* calculations of the HOMO-LUMO gap, solvation energies, and dipole moments. The HOMO-LUMO gap stands for energy difference between the highest occupied molecular orbital and the lowest unoccupied molecular orbital. The Meteor metabolic fate prediction program (LHASA Ltd, University of Leeds) was used to predict possible metabolic pathways. To calculate the skin permeation coefficient, the modified Robinson model (Wilschut *et al*., 1995) was applied as implemented in the permeation calculator (http://www.cdc.gov/niosh/topics/skin/skinPermCalc.html). The calculator is an online tool that includes three well-known models of skin permeation: Potts and Guy, modified Robinson, and Frasch (Frasch, 2002; Potts and Guy, 1992; Wilschut *et al*., 1995). The statistical analysis of molecular descriptors was performed using the SAS 9.1 suite of programs.

RESULTS

LLNA Employing AOO or Acetone as the Vehicle

The experimental SI and EC3 values obtained from LLNA studies employing acetone or AOO as the vehicle are shown in Table 1. 1-bromohexane (C6), 1-bromononane (C9), 1-bromodecane (C10), and 1-bromononadecane (C19) produced SI values below 3, for all concentrations tested, classifying these chemicals as nonsensitizers. However, compounds in which EC3 values were estimated produced detectable dose-response trends with an SI of the highest dose

TABLE 1
SI and EC3 Values for Various *n*-Bromoalkanes

		SI values: mean (SE)						
Name	C*n*	0.1%	1%	5%	10%	25%	50%	EC3
1-Bromohexane[a]	C6			1.14 (0.18)	1.58 (0.28)	1.27 (0.14)	2.75 (0.29)	{55}
1-Bromooctane[a]	C8			1.63 (0.12)	1.61 (0.12)	1.37 (0.07)	3.11 (0.24)	48.5
1-Bromononane	C9			1.10 (0.14)	1.19 (0.20)	1.27 (0.21)		
1-Bromodecane	C10			1.08 (0.16)	1.40 (0.18)	1.56 (0.17)		
1-Bromoundecane	C11			1.30 (0.20)	1.41 (0.24)	2.81 (0.33)	4.6 (0.78)	27.5
1-Bromododecane	C12			1.05 (0.14)	2.80 (0.27)	5.71 (0.61)		11.0
1-Bromotridecane	C13			1.44 (0.19)	3.46 (0.34)	11.85 (1.97)		8.6
1-Bromotetradecane	C14			1.82 (0.37)	7.30 (1.08)	11.66 (1.36)		6.1
1-Bromopentadecane	C15			3.56 (0.26)	13.64 (1.65)	14.7 (1.64)		4.8
1-Bromohexadecane	C16	0.79 (0.08)	1.9 (0.66)	7.8 (0.91)				1.75
1-Bromoheptadecane	C17			3.53 (0.26)	8.46 (1.24)	14.70 (1.25)		4.6[b]
1-Bromooctadecane	C18			0.96 (0.10)	1.33 (0.24)	5.58 (0.61)	9.85 (1.20)	16.4
1-Bromononadecane	C19		1.14 (0.11)	1.49 (0.31)	2.13 (0.23)			{17}
1-Bromoeicosane	C20		0.96 (0.10)	1.46 (0.21)	3.89 (0.34)			8.2

Note. C*n* = 1-bromoalkane carbon chain length; EC 3 values in ''{}'' are extrapolated values. These compounds cannot be classified as sensitizers from the present data but have an SI at the highest dose tested > 2 and significantly greater than control.

[a]Test vehicle was acetone; AOO was used for all other compounds.

[b]Estimated EC3 value lower than the lowest concentration tested.

exceeding 2. This ''upward'' trend-based evaluation of the EC3 was conducted exclusively for statistical reasons (Roberts *et al*., 2007), and without exception, these numbers should not be utilized for any other purpose, especially in risk assessment.

Using data from Table 1, a plot was constructed that shows dependency of the SI values upon number of carbons in the aliphatic chain of the bromoalkanes, at three concentrations: 5, 10, and 25% (Fig. 1). Bromoalkanes with sensitization activity were found to have from 12 to 17 carbons in their alkyl groups. The most potent sensitizer was identified to be 1-bromohexadecane (C16) with an EC3 value of 1.75.

Toxicological Modeling

Derek for Windows classified all studied *n*-bromoalkanes as skin sensitizers due to the presence of the halogen. Similarly, TOPKAT identified all studied *n*-bromoalkanes as weak skin sensitizers. Statistical analysis of molecular descriptors failed to find any significant correlation between calculated molecular descriptors and EC3 values. The HOMO-LUMO gap, which is a commonly used measure of chemical reactivity, did not correlate with experimental data (data not shown). Calculated skin permeation coefficients are based on the calculated logP (lipophilicity) parameter. The calculated differences in skin penetration are in a narrow permeability window and do not explain the observed activity pattern for the 1-bromoalkanes in the LLNA using acetone or AOO as the vehicle.

1-Bromoalkane Physical Chemistry

Next we examined the physical properties that might help explain the LLNA data. The bromoalkanes with chain lengths up to C16 are liquids at RT while bromoalkanes > C16 are solids at RT, suggesting that volatility or solubility of these compounds may present a technical problem in the LLNA. The boiling points (Bps) for the liquid bromoalkanes range from 155.3°C for 1-bromohexane (C6) to 335°C for 1-bromohexadecane (C16). These compounds are classified as semivolatile organics. Figure 2 is a plot of the evaporative loss of these bromoalkanes from AOO after being applied to a glass surface for 1 h. Complete loss of bromohexane was noted by 1 h. Approximately 75% loss of bromohexane was found after 5 min RT.

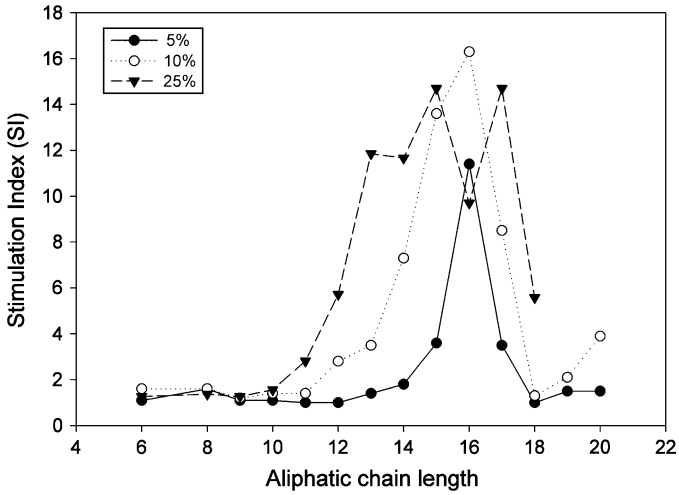

FIG. 1. Local lymph node cell SI versus carbon chain length plotted at LLNA doses of 5, 10, and 25% for studied *n*-bromoalkanes. A clear biphasic response at the 5 and 10% doses is apparent with 1-bromohexadecane (C16) inducing the greatest LLNA proliferative responses.

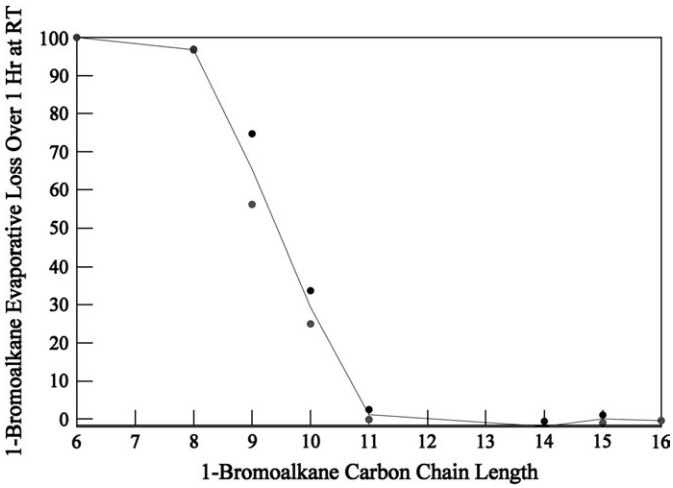

FIG. 2. Assessment of *n*-bromoalkane volatility from AOO. Loss of *n*-bromoalkanes (C6–C16) was assessed by applying 10 μl of a 25% (vol/vol) solution of each bromoalkane on a glass slide and incubating at RT for 1 h. Each slide was rinsed with acetone and assayed by GC-MS. All values are corrected for individual bromoalkane recoveries (the same procedure, but with immediate acetone wash and analyses after application to the glass slide).

Solubility of the individual *n*-bromoalkanes using both AOO and a proposed alternative vehicle, THF-BuOH, and their components were assessed (Table 2). Solubilities in the mixtures (AOO and THF-BuOH) were consistently greater than either of their individual solvent components. In all solvents, solubility decreased with increasing *n*-bromoalkane chain length; however, the *n*-bromoalkanes solubilities were usually greater in THF, butanol, and THF-BuOH. Of the solvents tested, acetone is the most volatile (vapor pressure [Vp] = 181 mm Hg at 20°C), followed by THF (Vp = 131.48 mm Hg at 20°C). The highly volatile nature of acetone resulted in precipitation of the higher, less soluble *n*-bromoalkanes at the tip of the pipette during ejection.

LLNAs Employing THF-BuOH as the Vehicle

Since the physical properties of the bromoalkanes in AOO may have influenced the LLNA results, the studies were repeated using THF-BuOH as the vehicle. Figure 3 shows a comparison of 1-bromoalkane-induced local lymph node cell–stimulated proliferation between AOO and THF-BuOH. Significant enhancement of proliferation was observed when employing THF-BuOH for 1-bromohexadecane (C16), 1-bromooctadecane (C18), and 1-bromononadecane (C19). There was not a significant difference between the SI at the doses tested for 1-bromohexane (C6) and 1-bromoeicosane (C20). 1-Bromodocosane (C22) was tested in the LLNA using THF-BuOH as a solvent resulting in SI of 4.9 ± 0.3 and 7.6 ± 2.0 at 5 and 10% concentrations, respectively. The inset table of Figure 3 compares EC3 values obtained from 1-bromoalkanes dosed with the two carrier solvents. THF-BuOH consistently lowered the calculated EC3 values and produced smaller chain length–related differences and comparative potencies of the bromoalkanes (Fig. 4). THF-BuOH itself produced no LLNA proliferation (as compared to AOO).

DISCUSSION

The 1-bromoalkane data set of Basketter *et al*. (1992) along with several additional 1-bromoalkane LLNA SIs reported by Gerberick *et al*. (2005) have been used extensively in development of *in silico* models and to understand structural activity relationships of chemicals in allergic contact dermatitis (Ashby *et al*., 1995; Estrada *et al*., 2003; Roberts *et al*., 2007). The LLNA SIs of the bromoalkane series using the AOO carrier found in the present study employing BALB/Balb/c mice were quantitatively similar to that reported in the literature using CBA mice. This suggests that the observed trend was not strain dependent. While the 1-bromoalkane biological data sets suggested a biphasic allergic sensitization potency response relative to carbon chain length, most authors have suggested the major determinant of potency of 1-bromoalkanes is logP (lipophilicity). LogP increases with carbon chain length versus the biphasic LLNA proliferative response observed with 1-bromoalkanes. The present study confirmed the biphasic relationship between LLNA potency and 1-bromoalkane carbon chain length (using AOO as the dosing vehicle) and includes additional studies that provide some insight into physical-chemical factors that contribute to this observation.

All the 1-bromoalkanes were predicted to be sensitizers by Derek for Windows and TOPKAT 6.2. We examined commonly used descriptors to predict a contact allergen's reactivity and bioavailability. Analyses of the HOMO-LUMO

TABLE 2
Solubility of 1-Bromoalkanes in LLNA Test Solvents

	Acetone (%)	Olive oil (%)	AOO (4:1) (%)	Butanol (%)	THF (%)	THF-BuOH (1:1) (%)
BrC6	> 25	> 25	> 25	> 25	> 25	> 25
BrC16	5.00	10.00	> 25	> 25	> 25	> 25
BrC18	2.50	2.50	10.00	2.50	> 25	> 25
BrC19	1.25	1.25	5.00	1.25	> 25	> 25
BrC20	< 1.25	1.25	5.00	1.25	> 25	> 25

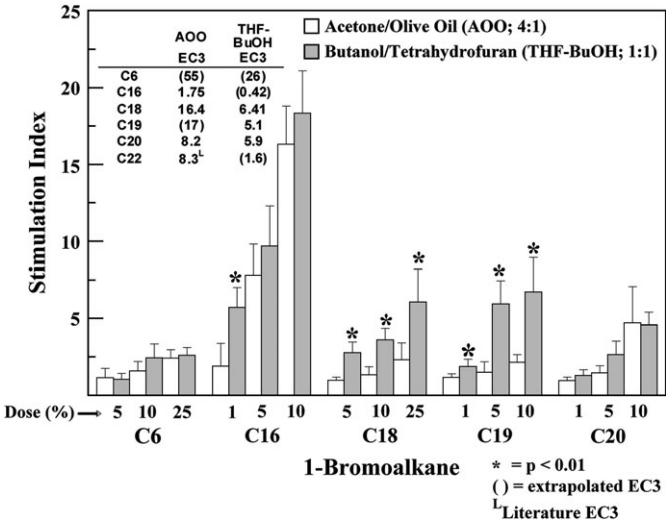

FIG. 3. Effect of application vehicle on local lymph node cell proliferative response. Selected 1-bromoalkanes were compared in the vehicles of AOO (4:1 vol/vol) and THF-BuOH (1:1 vol/vol). 1-Bromohexane (C6) through 1-bromohexadecane (C16) are liquids at RT. 1-Bromooctadecane (C18) through 1-bromodocosane (C22) are solids at RT. The inset table compares bromoalkane EC3 values for the two vehicles. Bromoalkanes applied to the ear in THF-BuOH produced greater proliferative responses in the draining lymph nodes and consistently lower EC3 values. Values are the mean ± SD of $n = 5$ per group. *Denotes statistically significant differences between SI values between 1-bromoalkanes tested in the two vehicles.

gap, solvation energies, and dipole moments using the Gaussian 98 quantum chemical software package (Frisch *et al*., 1998) did not help to explain the biphasic response of 1-bromoalkane with increasing carbon chain length in the LLNA. A direct relationship between lipophilicity and LLNA

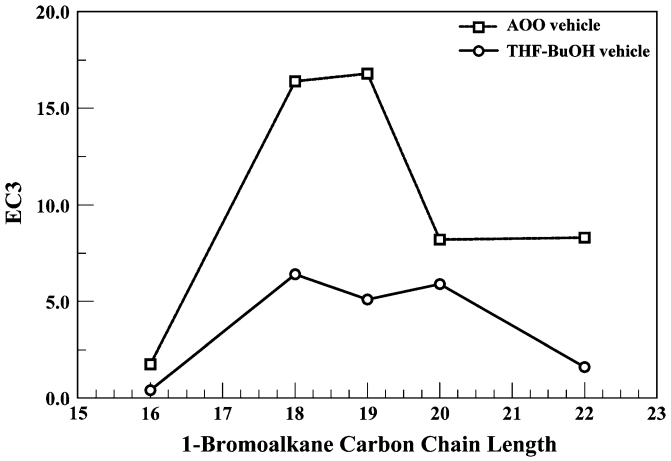

FIG. 4. Effect of dosing vehicle on nonvolatile *n*-bromoalkane EC3 values. 1-Bromohexadecane (C16) was the most potent of the 1-bromoalkanes in both AOO and THF-BuOH; however, greater proliferative responses were obtained when dosing 1-bromoalkanes using the THF-BuOH solvent system, and relative EC3 differences between different 1-bromoalkanes were attenuated.

EC3 was expected from the modeling software and modeling of the chemical characteristics of this series of 1-bromoalkanes.

For purposes of discussion, we are dividing the 1-bromoalkane data set into two separate groups: the semivolatiles (C6–C16; liquids at RT) and the nonvolatile, solids (at RT; 1-bromoalkanes [C18] and larger). The logP of bromohexane (C6) was calculated as 3.721 (Basketter *et al*., 1992). Although bromohexane is the least lipophilic of the bromoalkanes tested in the present study, it is still a liphophilic compound. Cross *et al*. (2003) examined the effect of lipophilicity on dermal permeability and tissue reservoir characteristics using a series of *n*-alcohols. The permeability coefficient (k_p) increased with increasing lipophilicity up to octanol (logP = 3.0) with no further increase at decanol (logP = 4.06). While overall tissue alcohol concentrations increased with lipophilicity, partitioning between the stratum corneum, epidermis, and dermis was different for octanol versus decanol. The lipophilicity of bromohexane is in between that of octanol and decanol, and thus, the lack of observable or extremely weak LLNA responses for the 1-bromoalkanes through bromodecane (C10) cannot be fully attributed to lipophilicity.

Loss of test agent from the skin surface has been largely ignored when comparing potencies of contact allergens. The LLNA test agent is applied without occlusion, and the potential for test vehicle losses from the skin surface due to volatility exist. We hypothesized that bioavailability of the lower chain length (liquid at RT) bromoalkanes may be decreased by volatilization from the skin surface. Bromohexane's (C6) and bromodecane's (C10) Vps are 520 and 7.6 Pa at 25°C, respectively. To put this in perspective, 2,4 toluene diisocyanate, a contact and a respiratory allergen whose vapor is associated with many reports of asthma in the work place has a Vp of 1.07 Pa at 25°C. From known smaller chain bromoalkane Bps and Vps, an exponential relationship between Bp and Vp was found (data not shown) and bromohexadecane's (C16) Vp was estimated to be 0.033 Pa at 25°C ($Vp = 2394866.95 \times 10^{-0.0539\ Bp}$). Figure 3 illustrates the volatile nature of the smaller bromoalkanes (C6–C10) that would be expected to severely limit bioavailability for the LLNA. Lack of potency or any observable stimulation in the LLNA by several of the semivolatile bromoalkanes is most likely due to a loss from volatility (from the skin surface and back out of the stratum cornea) competing with absorption/distribution into the epidermis and bromoalkane haptenation.

While volatility may help explain the effective lack of potency in the LLNA of the shorter chain length 1-bromoalkanes, the dilemma of poor LLNA response to the longer chain length, highly lipophilic, 1-bromoalkanes remained. The 1-bromoalkanes evaluated in this study that were solids at RT were 1-bromooctadecane (C18) and larger. Volatility of the individual bromoalkanes of carbon chain lengths $\geq$ C18 should not be a factor in bioavailability. Vehicle selection, especially for the nonvolatile bromoalkanes, can greatly influence apparent potency in the LLNA. Several reports have indicated

that solvent selection may alter skin absorption and the relative LLNA SI/EC3 (Kanikkannan and Singh, 2002; Lea *et al*., 1999; Ryan *et al*., 2002; Wright *et al*., 2001). Only one vehicle for all test compounds was employed in previous bromoalkane (or other LLNA structural activity studies) studies, regardless of uniform solvent compatibility (test chemical solubility in the solvent) across the chemical spectrum. AOO is a very commonly employed vehicle for chemical structure–LLNA activity studies. AOO cannot be considered a binary solvent as olive oil, itself, is a complex mixture that varies greatly between lots. Solubility of individual 1-bromoalkanes in AOO, acetone, or olive oil varied significantly (Table 2). Solubility decreased with increasing 1-bromoalkane carbon chain length. Due to limited solubility and the very high volatility of acetone, the actual form of the higher carbon chain 1-bromoalkanes were oil slurries or oil-coated large particles (at the higher doses) within a few seconds of application. Skin absorption of a chemical in solution has been demonstrated to be significantly greater than if applied as a dry powder (Romonchuk and Bunge, 2006). Both particulate retention on the skin surface and solubilization limit the ability to evaluate the relationship between LLNA potency and carbon chain length for the nonvolatile 1-bromoalkanes dosed in the AOO vehicle.

Multiple solvents were evaluated for solubility characteristics across the entire spectra of 1-bromoalkanes tested in the present study. The binary mixture, THF-BuOH (1:1), was chosen to evaluate vehicle contribution to the observed LLNA potencies as, in general, it was a better vehicle with respect to 1-bromoalkane solubility. The enhanced solubility can be mainly attributed to THF as solubility in *n*-butanol alone was only slightly better then in olive oil. THF is approximately 28% less volatile than acetone and would be expected to quickly volatilize off the skin. The nonvolatile 1-bromoalkanes tested in the LLNA using the THF-BuOH vehicle significantly enhanced proliferative responses in comparison to dosing in the AOO vehicle. The chemical nature of the vehicle can also alter the reactivity of the test article. Butanol is a polar, protic solvent that would solvate through H-bonding. The AOO vehicle would be an aprotic/nonpolar solvent (although olive oil contains many protic trace components such as the polyols). The protic solvent tends to stabilize the bromoalkane, and the order of reactivity to individual nucleophiles is different than in an aprotic solvent. It is not possible to determine the relative contributions of solubility, solvent effects on dermal membranes, and on 1-bromoalkane reactivity from the present data.

As noted earlier, skin penetration of a chemical increases with increasing lipophilicity, but relative partitioning from the stratum corneum to the epidermis and dermis may decrease with very highly lipophilic compounds due to sequestration in the very lipidic stratum corneum (Babu *et al*., 2004; Cross *et al*. 2003). Langerhans cells reside mainly within the epidermis, and different types of dendritic cells, including plasmacytoid dendritic cells, are found in the more aqueous dermal layer of the skin (Valladeau and Saeland, 2005; Loser and Beissert, 2007). Sequestration of 1-bromoalkanes with lipophilicity > 1-bromohexadecane within the stratum corneum is consistent with the lower observed SI's of these compounds; however, the effect of differential distribution of an allergen on the development of sensitization is not known.

It also must be noted that 1-bromoalkanes are irritants. Irritation and inflammation can alter the barrier properties of the skin, and even slight skin damage can enhance penetration and alter distribution (Nielsen *et al*., 2007). The role of contact allergen chemically induced irritation and damage in LLNA potency determinations presents a strong confounder. This is especially true since the test chemical is repeatedly applied to the same murine skin area for 3 days. This not only alters the barrier properties of the skin but also changes the cellular and biochemical profile of the skin application area.

The murine LLNA is an internationally accepted screening assay for the identification of contact allergens. Contact allergens can be semivolatile, as well as, nonvolatile compounds. Chemicals are dosed on unoccluded mouse skin, and thus, volatilization from the skin may potentially cause a false-negative or much attenuated SI. Although potent chemical allergens may produce sensitization when applied as a dry powder to the skin (Zhang *et al*., 2002), the bioavailability should be much less than when applied to the skin in a solution. To be allergenic, theoretically, a chemical only need to penetrate the stratum corneum as the antigen-presenting cells are immediately below. The biphasic relationship may be related to a chemical's ability to both be absorbed into the lipophilic stratum corneum and then partition into the subsequent more hydrophilic epidermis. The shorter chain bromoalkanes may be limited by both volatility and possibly rate of absorption into the SC, while the longer may partition poorly from the stratum corneum to the epidermis. The present research strongly suggests that these factors, along with effect of application vehicle on chemical reactivity, may all contribute to the apparent potency of a contact allergen in the LLNA.

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