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Toxicity of *N*,*N*,*N*-trialkylammoniododecaborates as new anions of ionic liquids in cellular, liposomal and enzymatic test systems

Tanja Schaffran,* Eugen Justus, Maike Elfert, Tina Chen and Detlef Gabel

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Representatives of *N*,*N*,*N*-trialkylammoniododecaborates, which are anions in a new class of ionic liquids, were tested for their hazard potential. As biological test systems, toxicity against V79 mammalian cells, reproduction inhibition of *Scenedesmus vacuolatus* algae, and inhibition of acetylcholinesterase were studied. EC_{50} values for the toxicity against V79 cells range between 9.1 mM for the trimethylammonio derivative and 0.19 mM for the trihexyl derivative. Reproduction inhibition of *S. vacuolatus* range between over 3 mM for the trimethylammonio derivative down to 0.016 mM for the trihexyl derivative. Fifty percent inhibition of acetylcholinesterase was caused by 21.9 mM of the trimethylammonio derivative, as compared to 0.03 mM for the hexyl derivative The data demonstrate that increasing hydrophobicity leads to higher toxicity and inhibition for straight alkyl chains. All tested ABs are able to induce leakage in liposomes and the capability for triggering it strongly increases with the length of the alkyl chain and consequently with lipophilicity. The leakage experiments could be an indicator for toxic tendencies *in vitro* but they allow no quantitative prediction of EC_{50} values. For branched chains and for derivatives with mixed substitution, a prediction of the toxic potential is not simple. The new class of ionic liquids is in general no more toxic than the ionic liquids presently in industrial applications.

Introduction

Research of ionic liquids (ILs) is one of the most rapidly growing fields in the past years. ILs are salts with melting points below 100 °C. Their properties differ from those of molecular solvents. They have very low vapor pressure, are not flammable, have high electric conductivity, show tolerance to strong acids and have high thermal and chemical stability. They are also able to solubilize different materials.[1,2] Various applications for ILs are noted as *e.g.* solvents, chemical catalysts, biocatalysts or in electrochemistry.[3–6]

The most established ILs consist of a simple anion (chloride, tetrafluoroborate, hexafluorophosphate) and a cation of one of the following classes: tetraalkylammonium, tetraalkylphosphonium, *N*-alkylpyridinium, *N*-methyl-*N′*-alkylimidazolium. Uncommon ILs are *e.g.* meltable stannaborate salts[7] or alkylpyridinium combined with carborane anions.[8]

Recently, we published a new kind of ionic liquids.[1] In Fig. 1, the *N*,*N*,*N*-trialkylammonioundecahydrododecaborates (–) are shown: they consist of the dodecaborate cluster substituted with one *N*-trialkylated ammonium group. We investigated derivatives with three identical alkyl chains from methyl to hexyl and one derivative with an asymmetric substitution (two ethyl chains and one benzyl group). Depending on the cation, these compounds have melting points below 100 °C, and some are liquids at room temperature. We prepared the

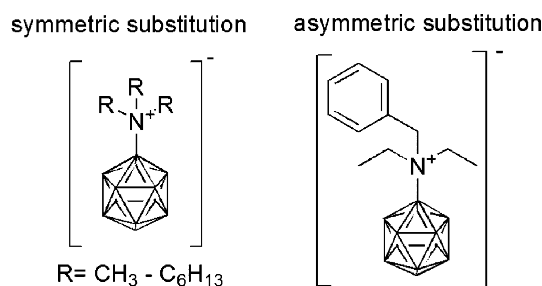

Fig. 1 *N*,*N*,*N*-trialkylammonioundecahydrododecaborates (–).

N,*N*,*N*-trialkylammonioundecahydrododecaborates (–) with a wide range of cations. Interestingly, the potassium and lithium salts also give ionic liquids.

These ILs exhibit high chemical and physical stability and thus are attractive for different applications. They are the first example of a non-corrosive IL with lithium as cation and might, therefore, be used as an electrolyte in lithium batteries. With the new European legislation on chemicals REACH (registration, evaluation, authorization and restriction of chemicals), the knowledge about the toxicological risk potential of chemicals for organisms, plants and the environment increases in importance. Before technical applications can be started, the hazards should be identified.

In this study, we tested the potassium salts of the trialkylammonio derivatives of the recently discovered ILs in various biological test systems. We determined the cytotoxicity of these compounds for one animal cell line, to estimate the effect on humans and animals. In order to assess possible risks for the

Department of Chemistry, University of Bremen, D-28357, Bremen, Germany. E-mail: T.Schaffran@web.de; Fax: +49 421 2182871; Tel: +49 421 2182119

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Table 1 Octanol/water partition coefficients, EC_{50} values in V79 cells and in *S. vacuolatus* algae, and IC_{50} values for AChE for the compounds tested

Compound	Kow	log EC_{50} ± SD	EC_{50}/mM	log EC_{50} ± SD	EC_{50}/mM	log IC_{50} ± SD	IC_{50}/mM
Test system		V79	V79	Algae	Algae	AChE	AChE
MeAB	0.04 ± 0.00	0.96 ± 0.01	9.1	No inhibition at 3 mM	—	1.34 ± 0.02	21.9
EtAB	0.33 ± 0.03	0.22 ± 0.06	1.66	No inhibition at 3 mM	—	0.99 ± 0.03	9.77
PrAB	1.84 ± 0.09	−0.32 ± 0.01	0.48	0.27 ± 0.04	1.86	0.31 ± 0.01	2.04
BuAB	3.04 ± 0.17	−0.51 ± 0.08	0.31	−0.23 ± 0.07	0.59	−0.29 ± 0.02	0.51
HxAB	4.73 ± 0.31	−0.72 ± 0.01	0.19	−1.79 ± 0.03	0.016	−1.49 ± 0.023	0.0325
iPnAB	4.03 ± 0.03	−0.61 ± 0.02	0.25	0.14 ± 0.15	1.4	−1.05 ± 0.01	0.089
Et2BnAB	0.58 ± 0.02	−0.14 ± 0.01	0.72	0.1 ± 0.1	1.33	0.102 ± 0.010	1.26

aquatic environment, we also studied the influence of the ILs on the limnic green algae *Scenedesmus vacuolatus*. The inhibition of acetylcholinesterase (AChE), as an enzyme found in all higher organisms, was also investigated. An inhibition of this enzyme leads to disorders in the neuronal system such as heart diseases (influence of the cardiac response to vagal innervation) and myasthenia.[9,10]

In order to study possible mechanisms of the toxic actions on cells, we investigated the action of the ILs on several simpler models. Liposomes were used as a model for cell membranes.[11]

We measured the octanol/water partition coefficient (Kow), which is the most commonly used parameter to determine the hydrophilic or lipophilic behaviors of a substance,[12] in order to investigate how well a simple physicochemical parameter could be used as predictor for toxic action.

The following ILs were used (with their abbreviations in parentheses): *N*,*N*,*N*-trimethylammonio-undecahydro-*closo*-dodecaborate (–) (MeAB), *N*,*N*,*N*-triethylammonio-undecahydro-*closo*-dodecaborate (–) (EtAB), *N*,*N*,*N*-tripropylammonio-undecahydro-*closo*-dodecaborate (–) (PrAB), *N*,*N*,*N*-tributylammonio-undecahydro-*closo*-dodecaborate (–) (BuAB), *N*,*N*,*N*-triisopentylammonio-undecahydro-*closo*-dodecaborate (–) (iPnAB), *N*,*N*,*N*-trihexylammonio-undecahydro-*closo*-dodecaborate (–) (HxAB), *N*,*N*-diethyl-*N*-benzylammonio-undecahydro-*closo*-dodecaborate (–) (Et2BnAB).

Results

The results of all measurements performed are shown in Table 1. In the following, we present and comment these results in detail for each type of assay.

Octanol/water partition coefficient

We determined the octanol/water partition coefficient, Kow, as a parameter for lipophilicity. The results are shown in Table 1, column 2.

The lipophilicity of the compounds increases from MeAB to HxAB, as expected. The MeAB derivative has a small partition coefficient of 0.04 and distributes almost exclusively into the water phase, compared to that of the HxAB derivative with a value of 4.73, which is found mostly in the octanol phase. The substances from PrAB to HxAB form white interphases between water and octanol, therefore, neither water nor octanol appear to be the optimal solvents for these compounds. iPnAB with its branched alkyl chains follows the common trend and its Kow value lays between BuAB and HxAB. The asymmetric substituted Et2BnAB exhibits a lipophilicity between that of EtAB and PrAB.

Ammonioundecahydrododecaborate $B_{12}H_{11}NH_3^-$ (B12NH3), which is identical in structure to the ILs tested but is not alkylated, has a partition coefficient of 0.103 ± 0.032 and distributes also predominantly into the water phase. It has a slightly higher solubility in octanol than the trimethylated derivative MeAB.

Cytotoxicity in V79 cells

As can be seen from Fig. 2, the cytotoxicity for V79 cells increases with longer alkyl chain lengths. EC_{50} values range from 9.1 mM for MeAB to 0.19 mM for HxAB. The asymmetrically substituted derivative Et2BnAB has a toxic potential between that of the EtAB and PrAB derivatives. All EC_{50} values are shown in Table 1.

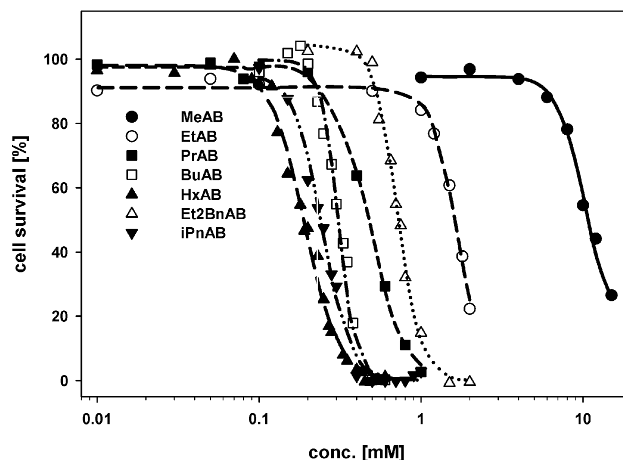

Fig. 2 Toxicity in V79 cells for *N*,*N*,*N*-trialkylammonioundecahydrododecaborates (–) with alkyl chains. The points are the measured values and the lines are the curves fitted with eqn (2).

B12NH3 was also tested and found to have an EC_{50} value of 45 mM. Thus, the structure of the ammoniododecaborate alone is not very toxic in itself, and it seems that the alkyl chains are responsible for the toxicity increase.

In Fig. 3, we compare the toxicity against V79 cells with the Kow partition coefficient. We observe the general trend that increasing lipophilicity (as measured by the Kow value) leads to increasing toxicity. The correlation is not, however, linear; only when plotted in a log–log plot, a regression line with a slope of −0.77 is obtained.

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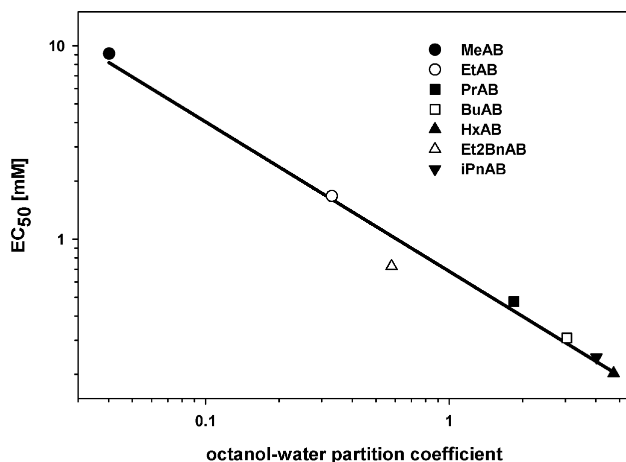

Fig. 3 Correlation between lipophilicity (log Kow) and cytotoxicity in V79 cells (log EC_{50}). The regression line shown has a slope of –0.77 ($r^2 = 0.98$).

Leakage

All trialkylammonio derivatives are able to induce a release of liposomes' contents, in contrast to the unsubstituted B12NH3. In Fig. 4, the results for the leakage induced by the BuAB derivative at different concentrations are shown as representative for the different compounds.

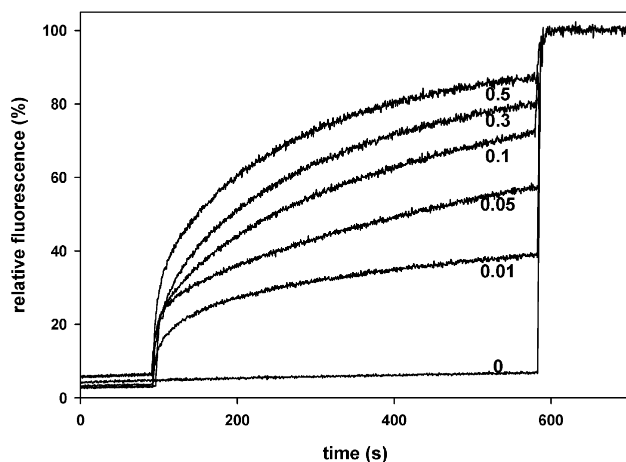

Fig. 4 Leakage of carboxyfluorescein (CF) triggered by the BuAB derivative at 37 °C. Lines from the bottom are for 0, 0.01, 0.05, 0.1, 0.3 and 0.5 mM, respectively.

The velocity of the leakage depends on the dose of the IL. After addition of 0.5 mM BuAB, the release is nearly complete after 580 seconds, while with 0.01 mM, only 38% is set free in this time period.

After addition of the compound, the initial release of the liposomal content is very fast and then slows down. Leakage does not follow a simple first order kinetics. At small concentrations of BuAB of around 0.01 mM, the kinetics appears to be biphasic, with the emergence of transient holes which lead to the observed rapid leakage in the first 20–30 seconds. Leakage has been observed for high concentrations of *N*-methyl-*N′*-alkylimidazolium salts, but only at very much higher concentrations of around 100 mM.[13] Also there, more complex kinetic behavior was found.

The concentrations required to induce around 80% leakage after 8 minutes decrease with increasing hydrophobicity, from 10 mM for MeAB and 0.5 mM for BuAB, to 0.05 mM for HxAB. The data demonstrate that the capability to induce leakage strongly increases with the length of the alkyl chain. In contrast, $B_{12}NH_3$, without any alkyl chains, is not able to induce leakage even at 100 mM. iPnAB requires more than ten times higher concentrations for an 80% leakage than HxAB, and also more than BuAB, indicating that additional factors, such as steric effects, might be required to explain the action of compounds with iso-alkyl chains. A leakage of 80% is obtained with 5 mM of Et2BnAB, which is expected on the basis of its Kow value.

AChE inhibition

The compounds inhibit the enzyme AChE. The measured enzyme activities and curves fitted to the measurements are shown in Fig. 5 and the IC_{50} values are presented in Table 1. The enzyme inhibition increases with increasing alkyl chain lengths. The MeAB derivative inhibits to a 50% at a concentration of 21.9 mM; in comparison, only 32.5 µM of HxAB is required for a 50% inhibition. Interestingly, Et2BnAB inhibits more than PrAB, which is unexpected from its position in the Kow values, and from its effect on V79 cells.

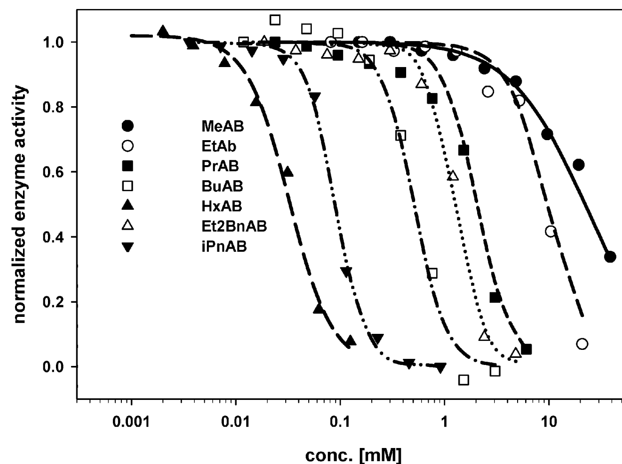

Fig. 5 Response curves for AChE inhibition for the tested compounds. Curves were fitted with eqn (2).

In Fig. 6, we compare the Kow values with the IC_{50} values for enzyme inhibition. Although there is a trend for increasing enzyme inhibition with increasing Kow values, this relation is not simple. With longer alkyl chains, the inhibitory power increases much more than the lipophilicity. Et2BnAB does not fit into this trend; its enzyme inhibitory power is larger than expected from its Kow value.

Reproduction inhibition of *S. vacuolatus*

MeAB and EtAB have no inhibitory effect on the algae reproduction up to a concentration of 3 mM. In Fig. 7, the individual data from EtAB are shown as one representative; those of MeAB are similar but are omitted for clarity. When replacing one ethyl group by a benzyl group, Et2BnAB, a 50% inhibition is observed at 1.33 mM. As can be seen in Fig. 7, PrAB inhibits the reproduction by 50% at a concentration of 1.86 mM.

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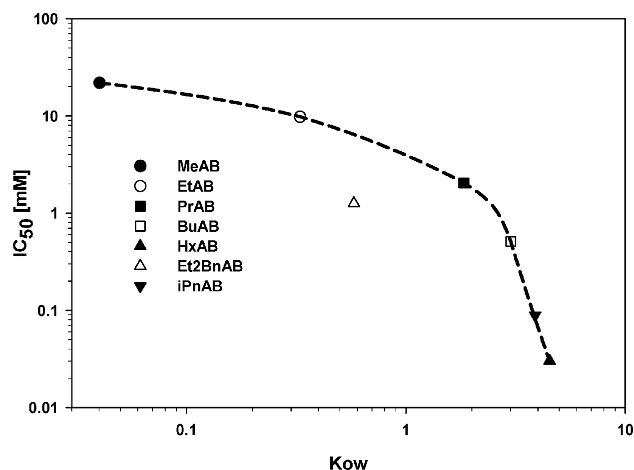

Fig. 6 Correlation between (log Kow) and (log IC_{50}) for enzyme inhibition. The dashed line is only intended as a guide to the eye.

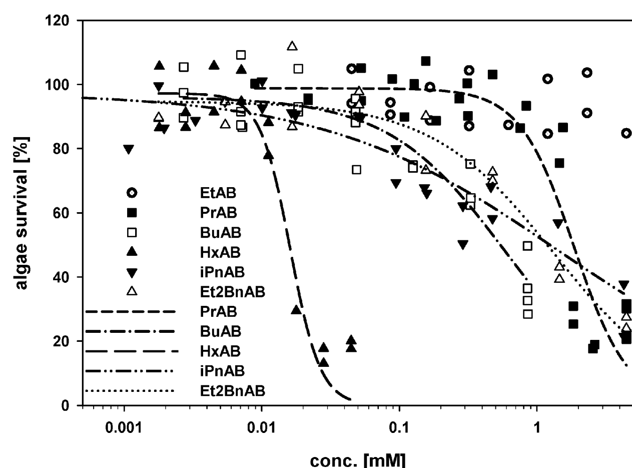

Fig. 7 Reproduction inhibition of *S. vacuolatus* in the presence of EtAB, PrAB, BuAB, HxAB, iPnAB and Et2BnAB. The lines are the curves fitted with eqn (2). The squared correlation coefficients, r^2, are 0.88 for PrAB and BuAB, 0.93 for HxAB, 0.81 for iPnAB and 0.93 for Et2BnAB. The hollow circles are the data points for EtAB, for which no line is shown.

BuAB and HxAB are more effective, and 16 μM of HxAB leads to a 50% reproduction inhibition. iPnAB, with branched chains, shows again a lower inhibition effect than BuAB, with *n*-alkane chains.

The data demonstrate that the inhibition increases with the increasing lipophilicity of the compounds containing straight alkyl chains. This common trend is observed for toxicity and enzyme inhibition as well, although the quantitative dependence differs between the test systems.

Discussion

Several recent studies deal with cytotoxic effects of ILs. Kumar *et al.*[14] have looked at a few examples of pyrrolidinium, piperidinium, and pyridinium ILs. In mammalian cells, they found that the toxicity increased with increasing alkyl chain lengths, but toxicity was also influenced by the anion present. Cho *et al.*[15] found the same tendency on phytoplancton for the four alkylmethylimidazolium ILs investigated. Ranke *et al.*[16] have assessed the hydrophobicity of a great number of representatives from different classes of ILs by chromatography. They found a general correlation between the degree of hydrophobicity, as measured by chromatography, with the toxicity against a mammalian cell line.

In general, we found that the concentrations of the ABs required to affect mammalian cells, algae, AChE, and liposomes decreased with an increasing octanol/water partition coefficient Kow. Thus, our study falls qualitatively in line with data from the literature, despite the fact that we have investigated anions of different hydrophobicity, whereas the ILs referenced above all have cations of different hydrophobicity.

We found that the Kow value had the best predictive power for toxicity on V79 cells. In general, increasing hydrophobicity leads to an increased toxicity in V79 cells, in about the same way as observed by Ranke *et al.*[16] for other mammalian cells. Plotting log EC_{50} values *versus* log Kow, a linear correlation is obtained similar to that by Ranke *et al.*[16]

For the other test systems used, an equally good quantitative prediction as that for the effect on V79 cells is not possible. For acetylcholinesterase, the lipophilicity/toxicity correlation is not linear, which is in contrast with the relationship found by Arning *et al.*[17] The correlation in our case is more complex, and extrapolation to longer alkyl chains is difficult.

For algae, a definite predictive relationship cannot be established; for this, more data points for longer alkyl chains are necessary. Stolte *et al.*[18] found a good correlation between hydrophobicity and effect, but they investigated only straight chain ILs of a different structure.

In our investigation, the effects of two compounds, iPnAB and Et2BnAB, were more difficult to predict from their Kow values. iPnAB has three identical, but branched alkyl chains and Et2BnAB have two different side chains, one of which is aromatic. A number of additional compounds, in which the substituents should be varied in a more systematic and comprehensive way, would have to be investigated before any general conclusions about asymmetrically or branched side chains can be drawn.

The influence of some ILs on aquatic organisms has been reviewed by Kulacki *et al.*[19] Data for various algae (*Oocystis submarina*, *Cyclotella meneghiniana, Pseudokirchneriella subcapitata*) have been presented by Latala *et al.* [20] and Wells and Coombe,[21] and for the algae *S. vacuolatus* by Matzke *et al.*[22] and Stolte *et al.*[18] The response of the algae, when exposed to ionic liquids, is slightly different depending on the species; this has been attributed to differences in the cell wall composition. For imidazolium salts (side chain: ethyl to octadecyl), the observed EC_{50} values range from micromolar to nanomolar depending on the side chain; longer alkyl side chains lead to higher lipophilicity and concomitantly higher toxicity. When compared to the imidazolium salts, the ABs with short chains are less toxic, the first substance for which toxicity in the micromolar concentration range is found is BuAB.

The correlation between toxicity in V79 cells and in *S. vacuolatus* is poor. In the algae, the most hydrophobic HxAB is by far the most toxic of the compounds tested, being about 100 times more toxic than PrAB. In V79 cells, HxAB is only about 2.5 times more toxic than PrAB. For the same algae, a similar trend was observed for methylimidazolium ILs.[22] These

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differences might be a result of the different structures of mammalian and algal cells.

For moderately lipophilic ABs, we did not find a marked enzyme inhibition. In contrast, the more lipophilic ABs iPnAB and HxAB were potent inhibitors. At a first glance, it is surprising that an anion should inhibit an enzyme whose regular substrate carries a positive charge. The enzyme has three distinct sites where molecular interactions can take place: the peripheral anionic site (PAS), a lipophilic channel located in the entrance of the gorge and the hydrolytic site as the active center.[23,24,17]

Arning *et al.*[17] interpreted the enzyme inhibition by ILs with the (positively charged) imidazolium and pyridinium structures as electrostatic interactions between the positively charged cation with negatively charged amino acids in the PAS. The ABs investigated by us contain also a positively charged nitrogen which might interact with the PAS. In view of the negative net charge of the compounds, binding to the PAS might, however, be considered less probable. Also, cation–π interactions formed by the ILs and a tryptophan residue in the hydrolytic site might lead to inhibition.[17] This might, in principle, also be possible for the ABs. The third site of inhibition includes the channel of the narrow gorge, where hydrophobic interactions between the ILs and the amino acid residues lining the channel are important.[17] As the inhibitory power of the ABs increases with increasing *n*-alkyl chain lengths and, accordingly, increasing lipophilic properties, this kind of inhibition appears to be the most probable of the three possibilities mentioned here.

Liposomes have frequently been used as a model for cell membranes.[11] Disturbance of the liposome membrane or disordering of the bilayer lead to leakage of the liposomal content. This model can help to identify the cell membrane as one target of toxic interactions. We found that the addition of hydrophobic ABs leads to leakage of the liposomes' contents. The capability to induce leakage increases with the length of the alkyl chains and, hence, with lipophilicity. This qualitative tendency is in line with the results we obtained for the other biological systems. A more thorough investigation of membrane defects through which leakage occurs, or a detailed analysis of the kinetics of leakage, such as described by Schubert *et al.*,[25] possibly together with molecular dynamics simulations, would be needed to fully understand the events on the liposomal membrane. Leakage of liposomal contents triggered by ILs has first been described by Evans *et al.*,[13] who investigated *N*-methyl-*N′*-alkylimidazolium cations. For *N*-methyl-*N′*-octylimidazolium chloride, around 100 mM was needed to induce a 50% leakage in liposomes after 250 s. In contrast, *N*-methyl-*N′*-octylimidazolium chloride had an EC_{50} value of about 0.1 mM.[16] As pointed out by Evans *et al.*,[13] the possibility of micelle formation of the alkylimidazolium cations cannot be excluded at the high concentrations required for leakage and, thus, the effective compound concentration for leakage induction is not known. Smaller concentration than 100 mM had not been tested in the experiment. On the basis of the existing data, no correlation between toxicity and leakage exists for ILs containing imidazolium cations because relative higher concentrations are necessary to trigger leakage than for a toxic effect. Thus, it seems that leakage effects play no major role in view of the toxicity in cells in the case of imidazolium ILs.

For the similarly toxic HxAB (EC_{50} = 0.19 mM), concentrations of 0.05 mM, well below its EC_{50} value, are required for nearly complete leakage of the liposomal content within minutes. Therefore, the leakage experiment does not allow a quantitative prediction of EC_{50} values for the V79 cells. In contrast to Evans *et al.*,[13] we found, however, that both leakage and toxicity increased with increasing lipophilicity. This might indicate that effects on the membrane might partially be responsible for toxic actions. Ranke *et al.*[16] and Stolte *et al.*[18] also explained the observed toxic effects in mammalian cells and algae by the assumption that the ILs adsorb or intercalate into the membrane resulting in membrane perturbation.

Conclusion

The ILs investigated here have toxicity values similar to those of other, more widespread ILs based on organic cations with simple inorganic anions. Trends can be seen so far as more hydrophobic ILs tend to be more toxic. For safe handling of ILs of this class, the data presented here give hints about possible risks associated with manufacturing, use, and disposal.

Materials and methods

Materials

Dipalmitoylphosphatidylcholine (DPPC) was a gift from Lipoid, Ludwigshafen (Germany). Carboxyfluorescein (CF) was from Kodak. WST-1 dye was from Roche Diagnostics (Mannheim, Germany). Bovine serum albumin was from Sigma-Aldrich (Steinheim, Germany) and acetylthiocholine iodide from Fluka (Buchs, Switzerland). Acetylcholinesterase (AChE, EC 3.1.1.7) from the electric organ of the electric eel (*Electrophorus electricus*) type VI-S was purchased from Sigma-Aldrich (Steinheim, Germany). The activity was determined to be 463 U per mg protein.

5,5′-Dithio-bis-(2-nitrobenzoic acid) (DTNB) was from Sigma (Steinheim, Deutschland). Alga *S. vacuolatus* (strain 211–15, SAG culture collection of algae) was from the University of Göttingen, Germany.[18,22]

Compounds

The dodecaborate cluster was synthesized according to Komura *et al.*[26] or purchased from BASF (Ludwigshafen, Germany). $B_{12}H_{11}NH_3^-$ (B12NH3) was prepared from $B_{12}H_{12}^{2-}$ with hydroxylamine-*O*-sulfonic acid.[27] Trialkylated products were prepared as described by Justus *et al.*[1]

Liposome preparation

DPPC was dissolved in chloroform/methanol (2 : 1) and a lipid film was obtained after evaporation and drying in vacuum. Then, the lipid film was hydrated and dispersed by vortexing in a 100 mM carboxyfluorescein solution (100 mM CF dissolved in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (10 mM), pH 7.4). The resulting suspension was extruded 21 times through a polycarbonate membrane with a pore diameter of 100 nm (Avestin, Mannheim, Germany) at a temperature of 54 °C.[28,29] Free CF was removed by size

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exclusion chromatography on a pre-packed Sephadex G-25 M column (GE Healthcare Bio-Sciences, Uppsala, Sweden). The equilibration and elution buffer was HEPES buffered saline, pH 7.4 (150 mM NaCl, 10 mM HEPES). The lipid concentration was determined with the Stewart assay.[30]

Leakage experiments

CF was encapsulated passively in a self-quenched concentration (100 mM) into liposomes. Leakage was determined through an increase of the fluorescence after compound addition, where CF leaked out and was diluted to a fluorescent concentration (excitation 490 nm, emission 515 nm). To obtain a 100% fluorescence value, TRITON X-100 (final concentration: 0.1%) was added at the end of measurement.

The fluorescence of CF was quenched by the MeAB and Et2BnAB derivatives, therefore, the curves were corrected with the following equation:

$$F_t = F_0 + (F - F_0)f_{\text{compound}} \quad (1)$$

where F_t is the corrected fluorescence without any quenching effects, F_0 is the start fluorescence and F is the fluorescence at a defined time point in the experiment. f_{compound} is a correction factor and comprises the following ratio: $\frac{100\%}{\text{end fluorescence } [\%]}$. Here, the end fluorescence is defined by the fluorescence reached after addition of the test compound (MeAB or Et2BnAB). The end fluorescence is calculated as a percent by the following formula: $\frac{\text{end fluorescence} \times 100\%}{\text{fluorescence after TRITON addition}}$, in which the fluorescence after the TRITION addition describes the maximum reachable fluorescence and is consequently the 100% value.

Partition coefficient octanol/water (Kow)

Partition coefficients were determined using the shaking-flask method (SFM).[31] Fourier transform infrared (FTIR) spectroscopy was used for the determination of the boron content in the water and the octanol phase. This simple and rapid method has been described for BSH by Kageji *et al.*[32]

A stock solution of each boron compound was prepared in a volume mixture of water/octanol (equal volume). The compound concentration was 80 mM in this mixture. The solutions were vortexed for some minutes. After the phase separation, the boron concentration was determined in each phase.

For the FTIR detection we used a Bio Rad FTS 155 spectrometer with a Mid-IR DTGS detector at a resolution of 16 cm^{-1}. For each measurement, 256 scans were collected. To determine the boron concentrations in solution, a variable path length demountable SL-2 cell kit from International Crystal Laboratories (Garfield, New Jersey, United States) with calcium fluoride windows was used, with a path length of 0.5 mm for octanol and 0.1 mm for water. The background was recorded as single-beam spectrum of the solvents and then subtracted from the test spectrum. Each spectrum was baseline corrected before the boron level was quantified.

A calibration curve was established to determine the boron concentration in the water and octanol phases. For this, samples with defined boron concentrations were prepared. For the water phase, $Na_2B_{12}H_{11}SH$ and $Na_2B_{12}H_{12}$, respectively, were used and for the octanol phase, the tetrabutylammonium salt of $B_{12}H_{11}SH^{2-}$ was used. The position of the maximal B–H absorption was picked. In water, the absorption was in the wave number range of 2485–2500 cm^{-1} and in octanol, at 2500 cm^{-1}. Finally, the boron concentrations were plotted against the absorption values. The values obtained from the linear regression were used to determine compound concentrations in each phase; their ratio was calculated to give the partition coefficient.

Toxicity in animal cell culture

Cell survival was detected by the enzymatic reduction of the WST-1 dye from living cells to a yellow formazan salt whose absorbance could be measured.[33]

The cell line V79 (lung fibroblasts of Chinese hamster) was used and cultivated with Ham's F10 medium and 10% newborn calf serum at 37 °C and 5% CO_2. Cells (11 000) per well were seeded into 96-well plates and grown for 24 h. Then, the cells were incubated with different concentrations of compounds for 24 h. The cell survival was determined with the WST-1 test system. The supernatant was removed and the wells were filled with 100 µl each of a WST-1 stock solution (1 : 4 diluted with phosphate buffered saline (PBS) and additionally 1 : 10 with medium). After 4–6 h at 37 °C, the absorbance at 450 nm was measured.[29]

The cell survival values were obtained by fitting a sigmoidal curve with the following equation:in which f is the percentage

$$f = \frac{a}{(1 + e^{-(x - xo)/b})} \quad (2)$$

survival of cells, a the highest point of response, x the concentration of the tested substance, b the slope of the response curve, and xo the concentration of the tested substance that provokes 50% cell death. The fitting was performed using the non-linear fitting module of Sigmaplot 2001 (SPSS, Erkrath, Germany).

Enzyme inhibition

The test system to determine the acetylcholine inhibition is based on a reduction of the dye 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) by the enzymatically formed thiocholine moiety from the AChE substrate acetylthiocholine iodide. The reduction can be followed colorimetrically.

The modified assay, as described in detail in Stock *et al.*,[34] was used. Different dilutions of the tested compound were prepared in a 96-well plate with phosphate buffer (0.02 M, pH 8.0) and 0.75% DMSO. DTNB (2 mM, 0.185 mg mL^{-1} $NaHCO_3$ in phosphate buffer pH 8.0) and the enzyme (0.26 U mL^{-1}, plus 0.25 mg mL^{-1} bovine serum albumin in phosphate buffer pH 8.0) were added to each well. The reaction was started with the addition of 25 µl acetylthiocholine iodide (2 mM in phosphate buffer).

The final concentration of DNTB was 0.5 mM, of acetylthiocholine iodide 0.5 mM, and of AChE 0.0512 mM.

The changes of optical density (ΔOD) at 405 nm were measured in a microplate-reader (MRX Dynatech). The first measurement was taken after a time delay of 150 seconds and

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then every 30 seconds for a total time period of 5 minutes. A linear regression line through all data points gave ΔOD min^{-1}.

The inhibition value was obtained by fitting, with Sigmaplot 2001, a sigmoidal curve with the same equation (eqn (2)) for toxicity determinations.

Reproduction inhibition assay with the limnic green alga *S. vacuolatus*

The synchronized unicellular limnic green algae *S. vacuolatus* was used as test system. The algae were cultivated under photoautotrophical conditions at 28 °C in an inorganic, sterilized medium (pH 6.4) according to Faust *et al.*[35] with white light (intensity of 22 to 33 kilolux, Lumilux Daylight L 36 W-11 and Lumilux Interna L 36 W-41, Osram, Berlin, Germany) and 1.5 vol% CO_2. For the synchronization, a day-and-night cycle of 14 h and 10 h was used. The stock culture was diluted every day to a density of 5×10^5 cells mL^{-1}. The methods for stock culturing and testing are described in detail by Faust *et al.*[35]

The protocol for the assay described by Altenburger *et al.*[36] was used, but in a modified procedure. For the start of the test, autospores were collected and were incubated with the test compounds for 24 h. The tested substances were dissolved in medium with 0.1% DMSO. For the whole time period, the algae were stirred and cultivated under the same conditions as for synchronization except for the CO_2 source where, instead, 150 µl of $NaHCO_3$ solution (1.5 M) was added to the test system. The assay was performed in sterilized glass tubes (20 mL Pyrex tubes sealed with caps containing a gas tight Teflon membrane).

The cell number of treated samples after 24 h was counted with a Coulter Counter Z2 (Beckmann, Nürnberg, Germany) and compared to the cell number of controls (untreated samples), the ratio gave the reproduction inhibition.

The inhibition value was obtained by fitting a sigmoidal curve with eqn (2).

Fluctuations of the algae growth were found between the measurement days and they are responsible for the scatter of the data points. Data sets of three different measurements were chosen for the graphs and for the fitting of the curves.

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