Structure-Activity Relationships for an Ecotoxicological Hazard Assessment of Selected Isothiazol-3-one Biocides and Ionic Liquids

A Mode-of-Action-Based Strategy

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'Lord Curryfin:...We ought to have more wisdom, as we have clearly more science.

The Rev. Dr. Opimian: Science is one thing and wisdom is another..."

Thomas Love Peacock, *Gryll Grange*, chapter 19

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AchE	Acetylcholinesterase
ATP	Adenosine triphosphate
EC_{50}	Effective Concentration for wich half of the maximal effect is observable
EINECS	European Inventory of Existing Commercial Chemical Substances
EPA	Environmental Protection Agency
FAD	Flavin adenine dinucleotide
GR	Glutathione Reductase
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione-S-transferase
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory Concentration for which half of the maximal acticity is observable
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PEC	Predicted Environmental Concentration
PNEC	Predicted No Effect Concentration
QSAR	Quantitative Structure Activity Relationship
ROS	Reactive Oxygen Species
REACH	EU legislation for the Registration, Evaluation, Authorisation and Restriction of Chemicals
SAR	Structure Activity Relationship
TR	Toxic Ratio
T-SAR	Thinking in Terms of Structure-Activity Relationships

SUMMARY

Especially since the new EU legislation for the registration, evaluation, authorisation and restriction of chemical substances (REACH) was put into force in June 2007, efficient testing strategies are needed to handle the enormous number of chemicals, for which toxicological and ecotoxicological data must be generated in the near future. For example, actually around 30,000 substances of high priority are identified by the EU that need to be tested within the next 10 years. Therefore, ecotoxicologists are asked to develop "intelligent testing strategies" that allow for a rapid screening of the hazard potential of chemical entities. Furthermore, the goals formulated in the Agenda 21 challenge chemists and the chemical industry to develop sustainable chemical products and processes. This task requires the "design" of new chemicals that are inherently safer regarding their toxicological and ecotoxicological impacts, but at the same time they have to meet their desired technological features.

For both fields, the ecotoxicological hazard assessment and the development of sustainable chemicals, a detailed knowledge on structure-activity relationships is needed since every toxic effect finds its origin in certain molecular interactions a chemical entity exerts on the molecules of life (*e.g.* proteins, DNA or membrane structures). Thus, it was the objective of this thesis to contribute to a deeper insight in such molecular structure-activity relationships underlying certain modes of toxic action. Furthermore, it was the aim to validate and develop cellular and subcellular test systems for a rapid screening of these toxic modes of action. These test systems should extend the existing flexible ecotoxicological test battery at the Centre for Environmental Research and Sustainable Technology (UFT) at the University of Bremen, especially aiming at screening tools for the assessment of reactive electrophiles.

To reach these goals two case studies - represented by four published or submitted manuscripts - were performed analysing the hazard potential of four environmentally relevant isothiazol-3-one biocides and of selected ionic liquid structures. The isothiazol-3-ones have been chosen since they represent highly reactive electrophilic organic chemicals. Additionally, their varying substitution pattern allows for detailed structure-activity studies. The ionic liquids were selected since they resemble a non-reactive but structurally highly diverse group of substances and hence they function as an ideal example to analyse the impacts of substructural variations on the hazard potential of chemicals. Furthermore, for these technologically promising ionic liquids only little is known about their ecotoxicological behaviour.

For the presented case studies a tiered testing strategy was applied. Firstly, the hazard potential of the compounds was predicted using the "thinking in terms of structure-activity relationships" (T-SAR) approach. Subsequently, according to this analysis, cellular and subcellular test systems were specifically selected and developed to verify the predicted modes of toxic action.

The isothiazol-3-one biocides were tested in three cellular test systems comprising a human Hep G2 liver cell line, the marine luminescence bacterium *Vibrio fischeri* and the unicellular green alga *Scenedesmus vacuolatus*. The measured cytotoxicities were analysed using the toxic ratio approach. It could be shown that the integral cellular endpoints provided hints for two different modes of toxic action for the biocides tested. The N-methylisothiazol-3-one (MIT) and especially its chlorinated analogue 5-chloro-N-methylisothiazol-3-one (CIT) exhibited consistently drastic excess toxicities in each of the three test systems. In contrast, the hydrophobic N-octylisothiazol-3-one (OIT) and its double-chlorinated derivative 4,5-dichloro-N-octylisothiazol-3-one (DCOIT) were found to act predominantly as baseline toxicants. To further analyse these possible modes of action, the tripeptide glutathione (GSH) was selected as molecular target since it represents the most abundant cellular nucleophile. In three molecular and subcellular assays the reactivity of the isothiazol-3-ones towards glutathione, the inhibition of glutathione reductase (GR) and finally the impact of the biocides on the total cellular glutathione content (in Hep G2 cells) were analysed. As expected from the T-SAR analysis, it could be shown that different chlorine substitution at the aromatic ring system modulated the intrinsic electrophilicity of the isothiazol-3-ones and hence their reactivity. Both chlorinated species (CIT and DCOIT) reacted readily with GSH and the reaction of DCOIT with GSH was even that fast that no reaction rate constant could be measured. Additionally, CIT and DCOIT completely inhibited cellular and isolated glutathione reductase activity and depleted cellular GSH. In contrast, the non-chlorinated biocides MIT and OIT showed no or only weak effects in the molecular test systems. Combining the results from the cellular and molecular assays it was concluded that the excess toxicities of CIT and MIT can most likely be explained by the generally high thiol reactivity of these biocides. Even more important, the observed high intrinsic reactivity of DCOIT in the molecular test systems, which could not be confirmed at the cellular level, revealed that for highly hydrophobic organic chemicals baseline toxicity can mask further and more specific modes of action in acute cellular toxicity screening assays. This led to the conclusion that cellular screening assays need to be accompanied by a T-SAR analysis to identify structural alerts - like the chlorine substitution at the isothiazol-3-ones - in the compounds tested that point to more specific toxic mechanisms. Otherwise there is a high risk to miss or overlook - and hence to underestimate the hazard potential - these mechanisms that might exert severe chronic or sublethal effects.

Comparable to the biocides, the ionic liquids were screened for their basal cytotoxic effects using a little differentiated and highly sensitive IPC-81 rat leukaemia cell line. By correlating the measured cytotoxicities with a HPLC-derived hydrophobicity parameter it could be demonstrated that the ionic liquids tested act most likely as baseline toxicants as it was expected owing to their high structural similarity with cationic surfactants. The only exception was found for the N,N-dimethylaminopyridinium head group where a certain excess toxicity pointed to further and more specfic modes of action. Furthermore, hydrophobic and/or fluoride containing anions (*e.g.* $[(CF_3SO_2)_2N]^-$, PF_6^-

and BF_4^-) exerted significant cytotoxic effects most likely due to baseline toxicity or the release of HF after hydrolytic cleavage.

In general, the side chain of the ionic liquid cations was identified to predominantly modulate the hydrophobicity and hence the toxicity of the corresponding ionic liquids. The introduction of polar functional groups (hydroxy, ether and nitrile functions) into hydrophobic alkyl side chains led to a drastic reduction of the observed cytotoxicity.

According to a previous study and the performed T-SAR analysis, the enzyme acetylcholinesterase (AchE) was selected as molecular test system to analyse in more detail the impacts of the three substructural elements head group, side chain and anion on the enzyme´s activity. In contrast to the cellular tests, the enzyme inhibition assay identified the cationic head group to be the key substructural element dominating the inhibitory potential of the corresponding ionic liquid. Especially, the quinolinium and N,N-dimethylaminopyridinium head groups exhibited drastic inhibitory effects with IC_{50} values < 5 μM. Additionally, it could be demonstrated that for one particular head group the hydrophobicity of the side chain modulated significantly the inhibitory potential of the corresponding ionic liquid. From the measured IC_{50} values of a series of imidazolium-based ionic liquid structures a quantitative structure-activity relationship could be derived that revealed that the inhibitory potential increased with increasing hydrophobicity of the side chain. Again the introduction of functionalised polar side chains reduced the inhibitory effect. For the anion species tested, it could be shown that only fluoride and fluoride-containing anions that readily undergo hydrolytic cleavage exerted a certain inhibitory effect on the enzyme´s activity.

The found inhibition of AchE activity by ionic liquids demonstrated that even non-reactive chemicals can - beside baseline toxicity - exert toxic effects by molecular interactions with certain target proteins. This case study clearly demonstrates that these interactions and their modulation by substructural elements can be predicted and understood at the T-SAR level.

Summarising the results obtained from both case studies, it could be shown that the applied tiered and mode of action based testing strategy in combination with a flexible test battery is a valuable tool to identify and screen modes of toxic action as well as structure-activity relationships of reactive and non-reactive chemicals. The identified hazard potentials of isothiazol-3-one biocides and ionic liquids reduced the uncertainty of their toxicological and ecotoxicological impacts and thus the obtained results can lead to a refined multidimensional risk assessment of these substances. Additionally, the uncovered structure-activity relationships can be used to design more inherently safer and hence more sustainable biocides and ionic liquids. The used T-SAR-based approach could be identified as a promising candidate for the above cited "intelligent testing strategies" in the hazard assessment of environmentally relevant chemicals.

Furthermore, the Hep G2 cell viability assay, the glutathione reactivity test, the cellular glutathione reductase inhibition assay and the subcellular GSH assay were successfully validated and implemented into the existing pool of test systems. Hence,

the flexible test battery at the UFT could be extended to test systems that are able to screen and evaluate the hazard potential of electrophilic chemicals.

However, to further develop and refine such mode of action based approaches for the hazard assessment of chemical substances, more basal cellular, organ- and tissue-specific molecular targets need to be identified and characterised to reduce the risk of missing important structure-activity relationships and their corresponding modes of action.

Provided this and a paradigm shift in the chemical industry, structure-activity-based strategies in ecotoxicology will represent a useful tool that can be integrated from the beginning in the development process of sustainable chemical products. Furthermore, this strategies can be used - *e.g.* under REACH - as a highly efficient approach to preselect substances with high priority out of large substance libraries. These preselected substances cansubsequently be tested in more complex test systems like organisms or simulated ecosystems.

ZUSAMMENFASSUNG

Insbesondere die neue EU Verordnung zur Registrierung, Evaluierung, Autorisierung und Beschränkung chemischer Stoffe (REACH), die im Juni 2007 in Kraft gesetzt wurde, verlangt nach effizienten Teststrategien, die es ermöglichen, toxikologische und ökotoxikologische Datensätze einer riesigen Anzahl von Industriechemikalien zu generieren. Allein auf der Prioritätsliste der EU sind 30.000 Substanzen gelistet, für die innerhalb der nächsten zehn Jahre komplette Datensätze von den Herstellern bei der EU Registrierungsbehörde vorzulegen sind. Besonders die Ökotoxikologie ist daher herausgefordert, neue sogenannte „intelligente" Teststrategien zu entwickeln, die ein schnelles „Screening" der Gefahrenpotenziale von Chemikalien für Mensch und Umwelt ermöglichen.

Des Weiteren verlangen die in der Agenda 21 festgeschriebenen Ziele von der chemischen Industrie die Entwicklung nachhaltiger Produkte und Prozesse. Chemiker und Verfahrenstechniker stehen also vor der Herausforderung, eigensichere Chemikalien und Produkte mit einem reduzierten Gefahrenpotenzial für Mensch und Umwelt zu „designen", die aber gleichzeitig optimale technische Eigenschaften aufweisen müssen.

Für beide Bereiche, der Identifizierung ökotoxikologischer Gefahrenpotenziale und der Entwicklung von nachhaltigen Produkten, ist ein breites Wissen zu Struktur-Wirkungsbeziehungen von Chemikalien unerlässlich, da jede toxische Wirkung einer Substanz auf bestimmte molekulare Wechselwirkungen mit den zellulären Strukturen und Molekülen des Lebens (z.B. DNA, Proteine oder Membranstrukturen) zurückgeführt werden kann.

Es war daher das Ziel dieser Arbeit, einen Beitrag zu einem besseren Verständnis dieser molekularen Wechselwirkungen und den daraus resultierenden Wirkweisen toxischer Substanzen zu leisten. Dazu sollten zelluläre und subzelluläre Testsysteme entwickelt und validiert werden, die es ermöglichen, schnell und zuverlässig die molekularen Wirkweisen von Chemikalien zu identifizieren und zu analysieren. Diese Testsysteme sollen die bereits am Zentrum für Umweltforschung und Nachhaltige Technologien (UFT) der Universität Bremen bestehende flexible toxikologische und ökotoxikologische Testbatterie erweitern. Besonderes Augenmerk sollte dabei auf Testsysteme und Endpunkte gelegt werden, welche die Bewertung reaktiver, elektrophiler Chemikalien erlauben, da diese eine zentrale Substanzklasse umweltrelevanter Toxine bilden.

Um diese Ziele zu erreichen, wurden in zwei Fallstudien die Gefahrenpotenziale von vier Isothiazol-3-on-Bioziden und einer Reihe ausgewählter Ionischer Flüssigkeiten analysiert und in vier zum Teil bereits veröffentlichten oder zur Veröffentlichung eingereichten Manuskripten im Detail diskutiert. Die Isothiazol-3-one wurden ausgewählt, da sie hochreaktive, elektrophile Substanzen darstellen, die aufgrund ihrer Verwendung als Biozide von hoher ökotoxikologischer Bedeutung sind. Außerdem

erlauben die systematischen Substitutionsmuster dieser Verbindungen eine detaillierte Struktur-Wirkungsanalyse. Als zweite Substanzklasse wurden die Ionischen Flüssigkeiten ausgewählt, da sie im Gegensatz zu den Bioziden als nichtreaktiv gelten, und durch ihre große strukturelle Vielfalt eine besondere Herausforderung an ökotoxikologische Teststrategien stellen. Hinzu kommt, dass für diese Verbindungen bisher kaum ökotoxikologische Daten vorlagen, sie aber als neue Generation vielversprechender Industriechemikalien gehandelt werden.

In dieser Arbeit wurde eine abgestufte Teststrategie verfolgt, bei der die Substanzen zunächst auf theoretischer Ebene mittels des Struktur-Wirkungs-Denkens (T-SAR, Thinking in Terms of Structure-Activity Relationships) auf mögliche Gefahrenpotenziale und daraus resultierenden Wirkweisen hin analysiert wurden. In einem zweiten Schritt wurden diese dann in gezielt ausgewählten zellulären und subzellulären Testsystemen aus der flexiblen Testbatterie verifiziert.

Die Isothiazol-3-on-Biozide wurden zunächst in drei zellulären Systemen, einer humanen hepatoblastom Zelllinie (Hep G2), dem marinen Bakterium *Vibrio fischeri* und der limnischen Grünalge *Scenedesmus vacuolatus*, getestet. Die Analyse der gemessenen basalen Zytotoxizitäten mittels des „Toxic Ratio"-Konzepts führte zu der Identifizierung von zwei möglichen Wirkweisen der Isothiazol-3-one. Für das N-methylisothiazol-3-on (MIT) und sein chloriertes Analogon, dem 5-chlor-N-methylisothiazol-3-on (CIT) wurden in allen drei Testsystemen vor allem für das CIT drastische „Excess Toxicities" gefunden, die auf einen spezifischen Wirkmechanismus hindeuten, der über reine Basislinien-Toxizität hinausgeht. Im Gegensatz dazu zeigten die hydrophoben N-octylisothiazol-3-on (OIT) und 4,5-dichlor-N-octylisothiazol-3-one (DCOIT) Derivate in den akuten Zytotoxizitätstests nur basislinien toxische Effekte. Um die vermuteten spezifischen Toxizitäten der Isothiazol-3-one zu identifizieren, wurden die Substanzen in einem nächsten Schritt in drei molekularen und subzellulären Testsystemen auf ihre Reaktivität gegenüber Glutathion, ihr Potenzial zelluläre und isolierte Glutathionreduktase zu hemmen und auf ihren Einfluss auf den zellulären Gesamtglutathiongehalt (in Hep G2 Zellen) hin untersucht. Glutathion wurde als molekulares Target ausgewählt, da es das bedeutenste und am weitesten verbreitete zelluläre Nukleophil darstellt, das maßgeblich an der Detoxifizierung von elektrophilen Xenobiotika beteiligt ist. In diesen Testsystemen konnte die T-SAR Vorhersage, dass das Chlorierungsmuster entscheidend die elektrophile Reaktivität der Biozide beeinflusst, bestätigt werden. Beide chlorierten Spezies (CIT und DCOIT) reagierten extrem schnell mit Glutathion, wobei für das doppelt chlorierte DCOIT die Reaktionsgeschwindigkeit so hoch war, dass die verwendete Methode keine zeitliche Auflösung gestattete. Zusätzlich wurde gezeigt, dass beide chlorierten Verbindungen die Aktivität von zellulärer und isolierter Glutathionreduktase komplett hemmen konnten, und beide Biozide führten zu einer extremen Verarmung an zellulärem Glutathion und damit zu einem Zusammenbruch des zellulären Redoxpotenzials. Die nicht chlorierten Verbindungen MIT und OIT zeigten in allen molekularen Testsystemen lediglich schwache Effekte.

Aus diesen Ergebnissen für die zellulären und subzellulären Testsystemen wurde gefolgert, dass insbesondere die hohe „Excess Toxicity“ von CIT wahrscheinlich auf dessen hohe Reaktivität gegenüber biologisch essentiellen Thiolgruppen, wie z.B. im Glutathion oder der Glutathionreduktase, zurückgeführt werden kann. Interessanterweise konnte die hohe intrinsische Reaktivität des DCOIT, die in den molekularen Testsystemen klar identifiziert werden konnte, nicht in den zellulären Systemen beobachtet werden. Dies führte zu der ökotoxikologisch wichtigen Schlussfolgerung, dass besonders für sehr hydrophobe, reaktive organische Verbindungen die Wirkweise „Basislinien-Toxizität“ weitere spezifische Wirkweisen in integralen, akuten Zytotoxizitätstests maskieren kann. Es ist daher absolut notwendig, diese zellulären „Screening“-Tests mit einer T-SAR Analyse der zu untersuchenden Substanzen zu begleiten, um sogenannte „Structural Alerts“ zu identifizieren, die Hinweise auf mögliche spezifische toxische Wirkmechanismen liefern. Nur so kann gewährleistet werden, dass das Gefahrenpotenzial von Chemikalien nicht unterschätzt wird und mögliche spezifische Effekte, die sich eventuell erst nach chronischer oder bei subletaler Exposition zeigen können, nicht übersehen werden.

Die Ionischen Flüssigkeiten wurden ebenfalls in einem zellulären und einem subzellulären, molekularen Testsystem untersucht. Mit Hilfe der nur wenig differenzierten und sehr empfindlichen IPC-81 Rattenleukämie-Zelllinie und einem chromatographisch abgeleiteten Hydrophobieparameter konnte gezeigt werden, dass die untersuchten Ionischen Flüssigkeiten als basislinien toxische Stoffe zu charakterisieren sind. Diese Wirkweise wurde vorab in der T-SAR Analyse aufgrund der großen Strukturähnlichkeit der Ionischen Flüssigkeiten zu kationischen Tensiden vorhergesagt. Lediglich für die N,N-dimethylaminopyridinium-Kopfgruppe wurde eine „Excess Toxicity” identifiziert, die auf weitere spezifischere Wirkweisen dieses Kations hinweist. Des Weiteren wurde auch für hydrophobe und/oder fluorierte Anionen (z.B. $[(CF_3SO_2)_2N]^-$, PF_6^- und BF_4^-) eine deutliche Zytotoxizität gefunden, die ebenfalls über Basislinien-Toxizität (Hydrophobie) oder die hydrolytische Freisetzung von HF aus diesen Anionen erklärt werden kann. Die detaillierte Struktur-Wirkungsanalyse der Ionischen Flüssigkeiten zeigte deutlich, dass die Hydrophobie und damit die Toxizität dieser Verbindungen maßgeblich durch die Seitenkette des Kations bestimmt wird. So konnte gezeigt werden, dass das Einbringen von polaren funktionellen Gruppen (z.B. Hydroxy-, Ether- oder Nitrilgruppen) in die hydrophoben Alkylseitenketten die Zytotoxizitäten der resultierenden Ionischen Flüssigkeiten drastisch reduzieren konnte. Der gleiche Effekt zeigt sich bei einer Verkürzung der Seitenkettenlänge.

Als molekulares toxikologisch und ökotoxikologisch relevantes Testsystem wurde ein Acetylcholinesterase-Hemmtest ausgewählt, da bereits in einer früheren Studie und in der T-SAR Analyse gezeigt werden konnte, dass Ionische Flüssigkeiten mehrere „Structural Alerts“ für eine Interaktion mit dem aktiven Zentrum dieses Enzyms aufweisen. Der hier untersuchte Testkit sollte dazu dienen, die einzelnen Substrukturen der Ionischen Flüssigkeiten genauer hinsichtlich ihres Einflusses auf dieses Inhibitionspotenzial zu charakterisieren. So konnte gezeigt werden, dass im Gegensatz zu der Zytotoxizität für die Enzymhemmung die Kopfgruppe die entscheidende

Substruktur darstellt. Besonders die N,N-dimethylaminopyridinium- und die Quinolinium-Kopfgruppen zeigten mit IC_{50} Werten < 5 µM ein sehr hohes Inhibitionspotenzial, das mit Hilfe der T-SAR Analyse auf den quaternären Stickstoff und die breit delokalisierten aromatischen Systeme in diesen Kopfgruppen zurückgeführt werden konnte. Die Analyse einer einzelnen Kopfgruppe in Kombination mit mehreren Seitenketten zeigte deutlich, dass die Hydrophobie dieser Seitenkette das Inhibitionspotenzial weiter modulieren kann. Aus diesen Daten konnte für die Imidazolium-Kopfgruppe eine QSAR-Korrelation abgeleitet werden, die deutlich zeigt dass mit steigender Hydrophobie der Seitenkette die IC_{50} des korrespondierenden Kations sinkt. Zusätzlich konnte gezeigt werden, dass von den getesteten Anionen lediglich Fluorid und hydrolyseempfindliche fluorierte Anionen (BF_4^- und PF_6^-) einen inhibitorischen Effekt auf die Enzymaktivität hatten.

Das Beispiel der Acetylcholinesterase-Hemmung durch eine Vielzahl von Ionischen Flüssigkeiten zeigt, dass auch nichtreaktive Verbindungen spezifische toxische Wirkmechanismen durch molekulare Wechselwirkungen mit Biomolekülen zeigen können, die über die reine Basislinien-Toxizität hinausgehen. Die hier durchgeführte Fallstudie konnte beispielhaft zeigen, dass solche Effekte und deren Modulation durch molekulare Substrukturen mit Hilfe des T-SAR Ansatzes vorhergesagt und verstanden werden können.

Zusammenfassend lässt sich also feststellen, dass die in dieser Arbeit verwendete abgestufte und wirkmechanismenbasierte Teststrategie ein leistungsfähiges Werkzeug darstellt, um das ökotoxikologische Gefahrenpotenzial von reaktiven und nichtreaktiven Chemikalien zu erfassen. Die aufgezeigten Struktur-Wirkungsbeziehungen der Isothiazol-3-on-Biozide und der Ionischen Flüssigkeiten konnten die Unsicherheit dieser Substanzklassen hinsichtlich ihrer toxikologischen und ökotoxikologischen Effekte reduzieren, und so zu einer verbesserten Risikobewertung dieser Stoffe beitragen. Auch können die identifizierten Struktur-Wirkungsbeziehungen zum „Design“ neuer, eigensicherer und damit nachhaltiger Ionischer Flüssigkeiten und Biozide genutzt werden. Der T-SAR basierte Ansatz konnte so in der hier präsentierten Fallstudie als vielversprechender Kandidat für die oben genannten „intelligenten“ Teststrategien identifiziert werden, mit dem schnell und effizient die Gefahrenpotenziale selbst von strukturell hochvariablen Substanzklassen erfasst und bewertet werden können.

Weiterhin ist es in dieser Arbeit gelungen, die Hep G2 Zelllinie, den Glutathionreduktase-Aktivitätstest und den zellulären Glutathiongehaltstest in die bestehende flexible Testbatterie zu integrieren. Wie am Beispiel der Isothiazol-3-on-Biozide gezeigt werden konnte, sind diese Testsysteme geeignet, um sensitiv elektrophile Umweltchemikalien zu analysieren.

Um allerdings in Zukunft die Möglichkeiten solcher wirkmechanismenbasierter Teststrategien voll nutzbar zu machen, ist es vor allem nötig, dass Toxikologen und Ökotoxikologen weitere basale zelluläre und organ- und gewebespezifische Targets identifizieren und charakterisieren, da nur so weitere Testsysteme entwickelt werden

können, mit denen möglichst umfassend die Gefahrenpotenziale von Chemikalien erfasst werden können. So kann in Zukunft die Gefahr, wichtige spezifische Struktur-Wirkungsbeziehungen zu übersehen, minimiert werden.

Dies und einen Paradigmenwechsel in der chemischen Industrie vorausgesetzt, können Struktur-Wirkungsbeziehungen in ökotoxikologischen Teststrategien von Anfang an in Entwicklungs- und „Design"-Prozesse neuer, nachhaltiger Chemikalien mit einbezogen werden und so einen entscheidenden Beitrag zu einem prospektiven Umgang mit Gefahrenpotenzialen leisten. Auch können solche Teststrategien genutzt werden, um z.B. nach REACH aus der riesigen Menge noch zu testender Industriechemikalien diejenigen zu selektieren, für die eine weiterführende Testung in Tierversuchen besonders nötig ist. So könnte es durch gezielte Vorauswahl zu einer drastischen Reduktion aufwändiger Organismen- und Populationstests kommen.

PART I:

RATIONALE AND SCIENTIFIC BACKGROUND OF THE THESIS

1 Introduction

1.1 Sustainable chemistry and the need for ecotoxicology

From an environmental point of view, chemistry and the related industries are often linked solely with severe pollution and disastrous accidents. In history, some sad but famous examples are the outbreak of the Minamata disease in the 1950s owing to organic mercury pollution of Japanese coastal waters or the Itai-Itai disease caused by the contamination of soil with cadmium from metal mining wastewater from 1940 to 1960 in the Japanese Toyama Prefecture. As a consequence of these deliberate releases of contaminants into the environment because of lack of knowledge or even indifference, thousands of people were killed. Also, devastating accidents have demonstrated that the production and use of chemicals cannot be fully controlled but can pose considerable hazards to man and the environment. Examples for the fatal effects that accidents with chemicals can cause are the dioxin pollution in Seveso (Italy) in 1976 or the poisoning of workers in Bhophal (India) with the highly toxic chemical methylisocyanate in 1984. Still today, chemical substances interfere with ecological structures and functions with serious consequences for ecosystems including humans. For instance, in the early 1990s it was discovered that the widely applied marine antifouling agent tributyltin (TBT) exerts harmful effects on estuarine molluscs (Gibbs *et al.*, 1991). The severe toxicity of TBT resulted in its worldwide ban in 2003. Even substances that are known to be hazardous for a long time remain problems of high concern for human health such as the mercury burden in fish - especially relevant in South America where mercury is emitted by gold mining operations (Reuther, 1994). This "classical" pool of environmentally harmful chemical substances is nowadays supplemented with new compounds and substance classes like persistent brominated fire retardants, synthetic estrogens and pharmaceuticals in surface waters or antimicrobial products (*e.g.* isothiazol-3-ones) that are widely released from personal care products into the biosphere (Hale *et al.*, 2001).

One major reason for all the negative impacts of chemical substances on man and the environment can be found in the prevalent paradigm that any substance or product predominantly has to fulfil its expected technological features. Thus, substances and products are and always have been designed exclusively to fit as well as possible a certain application. The fate or possible harmful effects of chemicals or their metabolites for man and the environment still are of secondary concern. In the past, the most prominent example of this problem is the story of DDT (dichlorodiphenyltrichloroethane). This insecticide - discovered in 1939 by Paul Hermann Müller - combines excellent technological properties: it was found to be highly toxic to target organisms, it exhibited a high chemical stability leading to a desired long-term biocidal effect and it was easy and cheap to produce. Hence, DDT was applied in vast amounts all over the world and in 1948 Müller was even awarded the Nobel Prize in medicine for his discovery. It was until the late 1950s that it was discovered that DDT and its metabolites DDD (1,1-dichloro-2,2-bis[p-

chlorophenyl]ethane) and DDE (dichlorodiphenyldichloroethylene) bioaccumulate to acute toxic concentrations in various species (Dolphin, 1959). Also, it turned out that DDT and DDE are strong inhibitors of certain Ca-ATPases in the shell gland of birds (Cooke, 1973) resulting in shell thinning of their eggs entailing a severe reduction of bird populations in Alaska and other regions of the United States (Hickey and Anderson, 1968). In 1972, DDT finally was banned in the United States and in the following years also in nearly all other countries. The case of DDT became the first publicly discussed ecological disaster, also thanks to the novel "Silent Spring" written in 1962 by the American biologist Rachel Carson drawing peoples' attention to the consequences of ignoring the hazards of xenobiotic substances in ecological systems. The deep fall of DDT from the Nobel Prize crowned "miraculous" insecticide to a worldwide ban underlines three important findings forming the base of sustainable chemistry today:

i. Besides the technological features of a chemical entity its possible toxicological and ecotoxicological impacts have to be of equal concern in the design and development of chemical products.
ii. Special attention should be drawn to the persistence of pollutants and their metabolites since bioaccumulation can lead to unexpected acute or chronic toxic effects.
iii. Molecular structure driven interferences of chemical entities with the cellular structures and functions of life are the origin of all adverse effects, even at the population or ecosystem level as it was shown by the reduction of bird populations owing to egg shell thinning caused by the inhibition of certain Ca-dependent ATPases by DDT and DDE.

Especially this latter issue is of high relevance for the development of sustainable chemical products, since it illustrates that toxicological and ecotoxicological impacts of a chemical entity can be understood by investigating structure-activity relationships on the molecular level. Explicitly avoiding molecular structures that are known to be responsible for a certain mode of toxic action, offers the chance to develop new chemical substances and products with a reduced hazard potential. The introduction of inherently safer chemical products represents a milestone on the way towards sustainablility in chemistry as it demonstrates that chemistry can actually be used in a safe and responsible manner.

Sustainability or sustainable development – as it was defined in 1987 by the Brundtland Commission – meets the needs of the present without compromising the ability of future generations to meet their own needs. This implies that economic and social development cannot proceed at the expense of the environment. All three dimensions of sustainability - economic, social and environmental aspects - equally have to be taken into account without privileging one of them. In June 1992, the Rio Declaration and the Agenda 21 - a global action programme for sustainable development - were signed by 179 countries from all over the world at the United Nations Conference on Environment and Development held in Rio de Janeiro. The Rio

Conference initiated the development of extensive international and local agendas for the integration of sustainability aspects in economic, scientific and social processes. Chapter 19 of the Agenda 21 is completely dedicated to toxic chemicals and their impacts on the environment. But what does sustainability actually mean for chemistry and related industries? The three dimensions of sustainability and what they imply for chemical substances are presented in Figure 1.

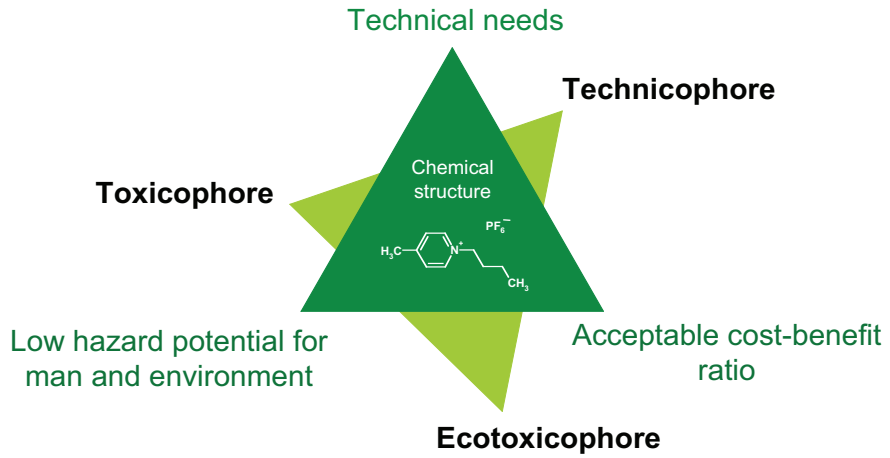

Figure 1 The sustainability triangle of chemistry according to Jastorff *et al.* (2003). It demonstrates how the three dimensions of sustainability are related to chemical structures. Accordingly, the hazard potential can be related to certain substructural elements - the "toxicophores" and "ecotoxicophores" - as well as the technical properties, that can be described as the so called "technicopohres".

Especially with regard to the environmental dimension of sustainable chemistry, the use and design of inherently safer substances is fundamental. In order to identify and avoid chemicals containing a high hazard potential right from the research and development stage, the generation and use of knowledge on their toxicological and ecotoxicological properties is required. This knowledge plays a key role for a paradigm shift towards the precautionary management of chemicals.

The economic dimension of sustainable chemistry is also closely related to the design and development processes of new substances, products and applications. Sustainable chemical processes are as effective and cost efficient as possible. This entails the design of chemicals that are optimised to fit a specific technical application, the development of more efficient production pocesses and the reduction of waste. For instance, production processes should be designed for low energy consumption, for the use of renewable feedstocks and for maximal atom economy with a concomitant reduction of side products. These factors are crucial for economically sustainable chemical processes.

Finally, the impact of chemistry on the social dimension of sustainability is enormous, since social prosperity is more or less related to chemical products or chemical research. Thus, the chemical industry bears a high social responsibility to protect not only workers but also the users of their products as well as ecosystems from

harmful effects of chemical substances. Again, this goal can efficiently be achieved by the introduction of inherently safer compounds. Especially in less-developed countries, people are vulnerable to the exposure of dangerous chemicals, since cost-intensive technologies and - even worse - skills how to handle hazardous substances are often not available (Djerassi, 2004). Therefore, societies in developing countries will benefit significantly from sustainable chemicals.

However, in order to achieve sustainability in chemistry there is a strong need for a paradigm shift in the chemical industry as well as in academic research and education. Scientists and industry have to accept that not only the technological features of a chemical are the important parameters defining "good" chemistry, but also that the fate and toxicity of chemical products in man and the environment are crucial. Hence, environmental and health aspects have to be integrated into industrial and academic research as well as - with special emphasis - into the education of young scientists. Chemists have to take responsibility for their research and their products, even though a substance has left the lab or the plant. The entire life cycle of a substance has to be taken into account in order to achieve the sustainable design of chemicals.

By publishing the 12 principles of "Green Chemistry" in 1998 Paul Anastas and John C. Warner demonstrated that a paradigm shift in chemistry towards sustainable development will be possible, if some fundamental guidelines are taken into account (Table 1) (Anastas and Warner, 1998).

Table 1 The 12 principles of Green Chemistry (Anastas and Warner, 1998)

1. Prevent waste: Design chemical syntheses to prevent waste, leaving no waste to treat or clean up.
2. Design safer chemicals and products: Design chemical products to be fully effective, yet have little or no toxicity.
3. Design less hazardous chemical syntheses: Design syntheses to use and generate substances with little or no toxicity to humans and the environment.
4. Use renewable feedstock: Use raw materials and feedstock that are renewable rather than depleting. Renewable feedstocks are often made from agricultural products or are the wastes of other processes; depleting feedstock is made from fossil fuels (petroleum, natural gas, or coal) or are mined.
5. Use catalysts, not stoichiometric reagents: Minimize waste by using catalytic reactions. Catalysts are used in small amounts and can carry out a single reaction many times. They are preferable to stoichiometric reagents, which are used in excess and work only once.
6. Avoid chemical derivatives: Avoid side products using blocking or protecting groups or any temporary modifications if possible. Derivatives use additional reagents and generate waste.
7. Maximize atom economy: Design syntheses so that the final product contains the maximum proportion of the starting materials. There should be few, if any, wasted atoms.

8. Use safer solvents and reaction conditions: Avoid using solvents, separation agents, or other auxiliary chemicals. If these chemicals are necessary, use innocuous chemicals. If a solvent is necessary, water is a good medium as well as certain eco-friendly solvents that do not contribute to smog formation or destroy the ozone.
9. Increase energy efficiency: Run chemical reactions at ambient temperature and pressure whenever possible.
10. Design chemicals and products to degrade after use: Design chemical products to break down to innocuous substances after use so that they do not accumulate in the environment.
11. Analyse in real time to prevent pollution: Include in-process real-time monitoring and control during syntheses to minimize or eliminate the formation of byproducts.
12. Minimize the potential for accidents: Design chemicals and their forms (solid, liquid, or gas) to minimize the potential for chemical accidents including explosions, fires, and releases to the environment.

These principles show in detail, how chemistry can be made more sustainable. They – especially item two and three – underline the significance of designing chemicals that inherently exhibit little or no toxicity to man and the environment. This difficult task often leads to a conflict of aims between technological and ecotoxicological features that can only be solved by a close cooperation between scientists from various disciplines (chemistry, toxicology, biology, engineering sciences), related producing industries, downstream users and consumers of chemical products (Jastorff *et al.*, 2005).

In this context, the science of ecotoxicology constitutes one of the most important branches of academic research. As a synthetic, problem-orientated science, ecotoxicology combines physical sciences (*e.g.* physical and analytical chemistry, geography, climatology or geology) and the life sciences (*e.g.* biochemistry, physiology, ecology or population biology) in order to address questions on the interference of chemicals with biological structures ranging from the molecular up to the population and even to the ecosystem level. Such highly interdisciplinary research is implicitly necessary, since for an effective environmental stewardship, it is important to understand fates and effects at all levels (Newman, 2001). From a historic point of view, ecotoxicology is a young science. It arose in the late 1970s, when the scientific community began to realise that there was a need to better understand the fate of chemical substances in the biosphere. The original definition of the term "ecotoxicology" dates back to 1977 and was given by Truhaut, who introduced ecotoxicology as *"the branch of toxicology concerned with the study of toxic effects, caused by natural and synthetic pollutants, to the constituents of ecosystems - animals (including human), vegetable and microbial - in an integrated context"* (Truhaut, 1977).

Even though Truhaut's definition is the oldest one, it is one of the most comprehensive descriptions that can be found today for ecotoxicology. It explicitly includes adverse effects of pollutants to human beings. Furthermore, it states an integral approach – that means that the molecular up to biosphere levels as well as local up to

global effects are considered. Therefore, the field of ecotoxicology represents an ideal scientific basis to address the questions and challenges of evaluating the effects chemicals can have on man and the environment. The following section will discuss in more detail, how ecotoxicology actually can facilitate the hazard assessment of chemical products and how this work fits into this general concept.

1.2 Risk and hazard assessment of chemical substances

For sustainable chemistry, a risk assessment of chemicals is essential. Risk is commonly defined as the product of probability and the extent of effect. With regard to chemicals the probability is equivalent to the given exposure of species or ecosystems with a substance and the extent and type of effects can be subsumed under the corresponding hazard potential. All factors are linked in the following relationship:

$$Risk = Exposure * Hazardpotential$$

Consequently, the hazard assessment of chemicals is a part of the more integral risk assessment. While the exposure strongly depends on the specific application, on the technological environment the substance is used in and on spatio-temporal release factors, the hazard potential is inherent to every single chemical entity. Hence, the hazard potential can be analysed and deduced from physicochemical parameters (*e.g.* octanol-water partition coefficient and chemical reactivity) in combination with data from toxicity assays.

Based on these considerations, today the PEC/PNEC concept is the predominant concept for assessing the risk of chemical substances. PEC is the ***P****redicted* ***E****nvironmental* ***C****oncentration* and can be estimated using the parameters from the exposure analysis. PNEC stands for the ***P****redicted* ***N****o* ***E****ffect* ***C****oncentration*. It can be calculated on the basis of the physicochemical parameters of a substance and the measured toxicity data. In order to account for uncertainty arising from the transfer of artificial laboratory data to complex ecosystems, a factor is considered in the estimation of the PNEC. Thus, if the derived risk quotient PEC/PNEC for a certain substance will range above one, harmful effects in the environment cannot be excluded indicating the need for regulatory action. However, this pragmatic, widely applied and easily traceable PEC/PNEC concept has some essential drawbacks, because uncertainty - which is of high relevance in decision making processes - cannot be reflected separately from other parameters influencing the estimation of the PEC or PNEC (Böschen *et al.*, 2003). To tackle this problem, an alternative approach for assessing the risk potential of chemicals was proposed by Ranke and Jastorff (2000) based on a multidimensional and comparative risk analysis (Figure 2).

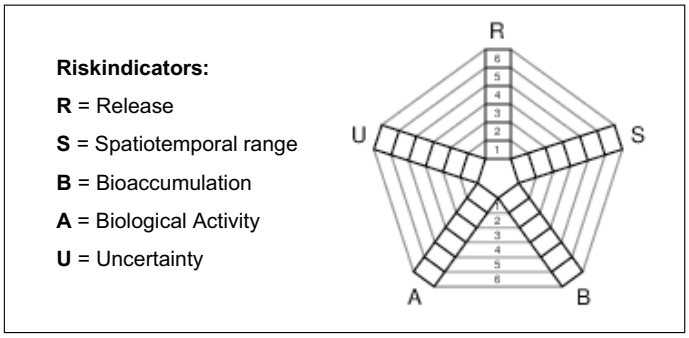

Figure 2 Scheme of a multidimensional risk assessment of chemicals. In contrast to the PEC/PNEC approach, the different risk indicators are weighted independently on a relative scale ranging from 1 to 6 (Ranke, 2002).

Within this concept, the five risk indicators (release, spatio-temporal range, bioaccumulation, biological activity and uncertainty) can be evaluated separately. The risk indicators "biological activity" and "bioaccumulation" summarise the hazard potential of a chemical substance. Ecotoxicological research can help to characterise the risk indicators "biological activity" and "bioaccumulation" for a certain substance in more detail. Additionally, a growing general knowledge in the field of ecotoxicology reduces the uncertainty of these two risk indicators. This is of high importance as especially reducing uncertainty of those substance-inherent risk indicators "bioaccumulation" and "biological activity" is the most potent strategy to reduce the risk indicator "uncertainty". A decrease of uncertainty leads to a more accurate and sound prediction of chemical hazards. The remaining risk indicators "release" and "spatio-temporal range" depend on parameters (*e.g.* uncontrolled release, distribution between different compartments) that are much more difficult to predict and hence are implicitly correlated with a high uncertainty.

On the whole, it becomes obvious that the above described academic challenges of a sustainable chemistry and the pragmatic requirements for the hazard assessment of chemical substances, urgently need and will benefit from efficient ecotoxicological testing strategies.

1.3 Mode of action based testing strategies for analysing the hazard potential of chemicals

Especially since the new EU chemical legislation for the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) was put into force in June 2007, the chemical industry is facing the problem to provide toxicological and ecotoxicological data for all products and substances. Under REACH the distinction between substances listed in the EINECS (*European Inventory of Existing Chemical Substances*) index and new compounds was abolished so that from now on toxicological and ecotoxicological data have to be submitted for every single substance produced at a certain scale. Additionally, the responsibility for providing these data now lies with the industry and no longer with

national authorities as it used to be the case for the risk assessment of EINECS substances.

As a result, REACH triggered a strong demand for efficient and effective testing strategies. These have to facilitate the rapid generation of reliable toxicological and ecotoxicologcal data sets that are suitable to be used in the hazard assessment of chemical entities under REACH. In this context, many efforts are made to establish quantitative structure-activity relationships (QSARs) as rapid and effective screening tools of similar substances or substance classes. QSARs require a detailed knowledge of the molecular descriptors that cause an observed toxic action of a certain compound. In order to promote the application of QSARs in hazard assessment, the EU Commission established many research projects (*e.g.* www.osiris.ufz.de) to develop "intelligent testing strategies" on the molecular *in vitro* basis. Furthermore, Annex XI of the REACH directive explicitly states that in the future, data from *in vitro* test systems can be used to reduce the commonly performed animal testings in the hazard assessment of chemical substances.

For ecotoxicologists this development implies that there is an increased need for test systems and testing strategies on the molecular and cellular level that allow for the establishment of mode of action based structure-activity relationships. QSARs applying mode of toxic action based descriptors have proved to be more consistent and reliable compared to the commonly applied "chemical class approach", which groups chemicals exclusively according to physicochemical descriptors (Bradbury, 1994).

To illustrate the power of a mode of action based approach for the rapid screening of large substance libraries, in the following the pros and cons of such a molecular approach will shortly be discussed.

For the current regulatory risk management the knowledge of effect concentrations of integral endpoints - such as death or growth inhibition - is normally sufficient. In contrast, for the development of mode of action based structure-activity relationships the question why the test organism is dying or showing abnormal behaviour is of crucial importance. This question can only be answered, if the integral testing endpoint "death of an organism" is broken down to molecular or cellular endpoints that can answer the question: "What molecular interactions of a certain substance with the molecules of life are exactly responsible for the observed toxicity?". Answering this question is a fundamental prerequisite to establish reliable QSARs for a predictive hazard assessment of chemicals. A second striking advantage of molecular and cellular test systems is directly related to the demand to generate ecotoxicological data for large substance libraries. Molecular assays are normally short-term assays (seconds to days) and as they can easily be automated they represent an ideal tool for the high throughput screening of chemicals.

Nevertheless, from an ecotoxicological point of view there are also some drawbacks of molecular test systems. Most importantly, short-term molecular test systems normally are unable to reveal chronic or long-term effects (*e.g.* mutagenic, teratogenic and reproduction toxicity effects) of chemicals. Furthermore, results from molecular test

systems are difficult to be transferred to organisms or populations, because the effects of factors such as bioavailability, toxicokinetics, biomagnification, the general sensitivity of organisms and the effect of further environmental stressors is not sufficiently mapped at the molecular level.

Thus, to get a complete ecotoxicological profile for a chemical entity it is necessary to take all levels of biological complexity into consideration, because *"processes at one level take their mechanisms from the level below and find their consequences at the level above"* (Caswell, 1996). In Figure 3 the different levels of biological complexity and the relation to their ecotoxicological weight are presented.

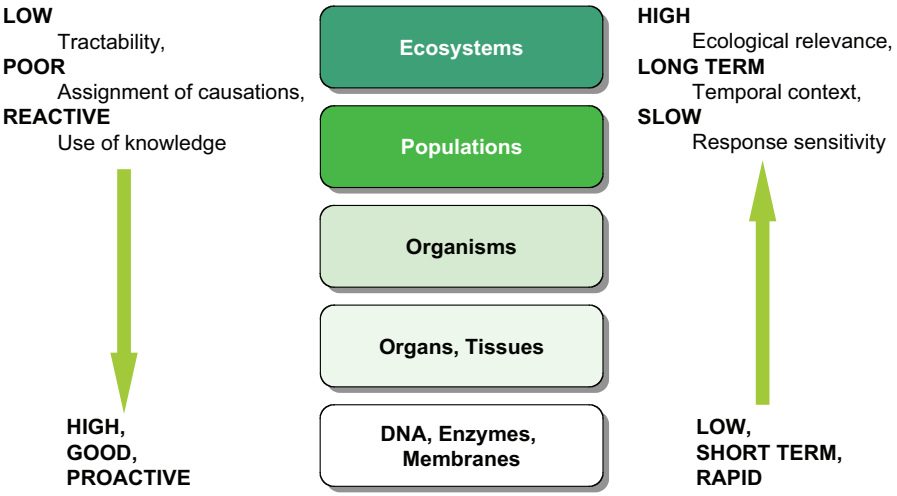

Figure 3 The different levels of biological complexity and their relation to ecological relevance, general tractability, ability to assign causations, general use of knowledge, temporal context of consequence and temporal sensitivity of response according to Newman and Unger (2002).

Since such a comprehensive testing is only possible for a small number of substances, every testing strategy should be accompanied by a mechanism that identifies compounds of high hazard potential for testing at higher levels of biological complexity. How mode-of-action-based test systems can support such strategies as an integral part of a flexible toxicological and ecotoxicological test battery will be discussed in section 2.1.

On the whole, the lowest level of biological complexity - *i.e.* the interaction of chemicals with the molecules of life (*e.g.* proteins, lipids or nucleic acids) - represents a promising starting point for the development of inherently safer chemical products and for a pragmatic structure-activity guided hazard assessment. Therefore, mode-of-action-based molecular testing strategies need to be further developed, refined and integrated into regulatory hazard assessment procedures but always with considering their limits to avoid *"falling into the traps of naive reductionism"* (Caswell, 1996).

1.4 Objective and structure of the thesis

Considering the need for toxicological and ecotoxicological data sets and the challenges of a shift towards sustainable chemistry, explains why mode of action based strategies for assessing the hazard potential of chemicals will be gaining more and more importance. Furthermore, efficient mechanisms to systematically select, analyse and test chemical substances or classes of substances are necessary to deal with the enormous number and variability of existing chemical entities. For example, just the EINECS index is listing around 106,000 substances that need to be evaluated (out of these, actually around 30,000 were selected to be tested with priority owing to their production volume). In 2007 over 16 million different chemical products are commercially available today (Chemical Abstracts Service, 2007).

Motivated by these challenges, the Centre for Environmental Research and Sustainable Technology (UFT) of the University of Bremen developed and implemented a flexible ecotoxicological test battery to assess the hazard potential of chemicals comprising subcellular, cellular and organismic test systems (*e.g.* Doose *et al.*, 2004; Siol, 2002). The tiered concept of this test battery starts with a systematic preselection of substances and the subsequent composition of test-kits according to the T-SAR (Thinking in terms of Structure-Activity Relationships) approach (Jastorff *et al.*, 2007) and the so-called "test kit concept" (Jastorff *et al.*, 2003). Subsequently, these test kits are tested in test systems selected from the available pool of different assays to screen and identify possible hazard potentials of the chemicals.

Within this framework, the rationale of this thesis was to extend the pool of available test systems of the flexible test battery to cellular (human liver cell culture) and subcellular endpoints (glutathione metabolism) that allow for the screening of toxic mechanisms of electrophilic organic substances. In two case studies, these newly established test systems combined with already implemented cytotoxicological assays are applied to get a deeper insight into the toxic modes of action of the highly electrophilic substance class of isothiazol-3-one biocides and of selected ionic liquids, which are termed to be chemically inert. These case studies also are aiming at reducing uncertainty in the hazard assessment of the environmentally relevant and broadly applied isothiazol-3-one biocides and for the new and technologically promising ionic liquids. Finally, based on this case studies it will be discussed how the applied tiered and mode-of-action-based test strategy can support the future needs to efficiently manage and test large substance libraries and how such an approach can be used as an integral part in the development process of sustainable chemical products.

This thesis is divided into three parts. In the following theoretical part, the T-SAR based test strategy will be presented in more detail. Furthermore, the isothiazol-3-one biocides and the substance class of ionic liquids are introduced and scrutinized for structural alerts pointing to possible modes of toxic action[1]. Based on this analysis,

[1] In the context of this thesis the term "mode of toxic action" is used as described by (Escher and Hermens, 2002) and stands for the effect a toxicant can exert on basal cellular structures and functions *e.g.* the disruption of biological membranes.

integral cellular and molecular test systems were selected from or implemented into the existing flexible test battery to verify the assumed modes of toxic action and to reveal the molecular mechanisms underlying them. These test systems will be presented and it will be discussed, to which structural alerts they react especially sensitively in order to identify the presumed modes of toxic action.

In the second part, the case studies investigating the highly reactive isothiazol-3-ones and the non-reactive ionic liquid structures will be presented by two published and two submitted manuscripts. Paper No. 1 and No. 3 analyse and discuss in detail the cytotoxicity of isothiazol-3-one biocides and selected ionic liquids. Paper No. 1 presents the impacts of isothiazol-3-one biocides on the human liver cell line Hep G2, the marine bacterium *Vibrio fischeri* and on the limnic green alga *Scenedesmus vacuolatus*. Paper No. 3 illustrates the cytotoxic modes of action of selected ionic liquids using an IPC-81 rat leukaemia cell culture. The two remaining manuscripts (paper No. 2 and No. 4) deal with molecular endpoints. They report on the impact of isothiazol-3-ones on cellular glutathione (GSH) metabolism and glutathione reductase (GR) activity. Furthermore, structure-activity relationships for the inhibitory potential of ionic liquid head groups, side chains and anions on acetylcholinesterase (AchE) are presented.

Finally, in the third part of the thesis the results of the case studies will be discussed with regard to the potential of molecular mode-of-action-based test strategies to contribute to the hazard assessment of environmentally relevant chemicals. An outlook is given by summarising the merits and limits of the presented approach and by highlighting the future needs and challenges of molecular ecotoxicology.

2 Theory

2.1 The T-SAR approach and the concept of a flexible test battery

The T-SAR (Thinking in terms of Structure-Activity Relationships) analysis of a chemical entity is based on the three-dimensional structural formula representing the "identity card" of every single substance. From this starting point, the most important molecular interaction potentials, the stereochemistry and functional groups causing a certain reactivity can be identified. For example, the potential of a chemical to act as hydrogen bond donor or acceptor can easily be revealed as well as its hydrophobic interaction potential. Furthermore, the flexibility, the three-dimensional space filling and the various bonding angles of a molecule - describing the spatial orientation of the interaction potentials - can be estimated. Especially this spatial pattern of molecular interaction potentials is of high relevance for the interaction of substances with biomolecules like enzymes or receptor proteins. Jastorff *et al.* (2004; 2007) give a detailed description of the algorithm for the theoretical analysis of the structural formula of a chemical entity. In Figure 4, the T-SAR analysis of a chemical is presented schematically including the colour-coding scheme used to highlight the most important interaction potentials substances can exert.

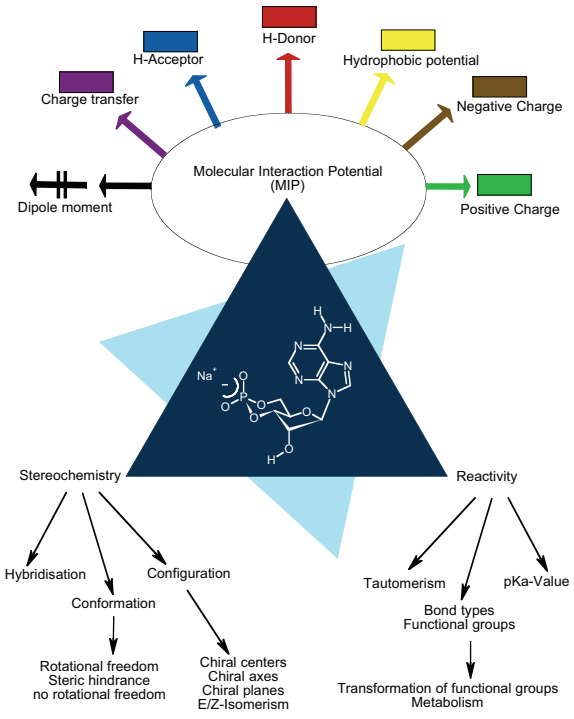

Figure 4 The T-SAR triangle according to Jastorff *et al.* (2007). It provides an overview of the T-SAR analysis of a chemical structure. Additionally, the colour-coding scheme to highlight molecular interaction potentials is presented.

Subsequently, the T-SAR analysis allows for the formulation of working hypotheses regarding physicochemical properties (*e.g.* partition coefficients, vapour pressure, solubilities), possible biotic and abiotic metabolisation pathways as well as of toxicological and ecotoxicological impacts. These working hypotheses can then be verified in toxicity assays selected from a flexible test battery. The crucial advantage of such a tiered and differentiated approach compared to the routinely applied testing in only a few standardised and mostly integral organismic test systems is that the T-SAR analysis yields in test systems that are able to provide valuable data, which can be used to accept or reject certain working hypotheses. Thus, this testing strategy can efficiently increase the quantity and quality of knowledge within the framework of ecotoxicology in the sense of what Kuhn called "normal science" research work (Kuhn, 1970).

Another more practical benefit of this flexible testing approach arises from the fact that it is capable of revealing general mechanisms and trends of harmful impacts of chemicals by simply selecting a few "lead structures" from a large pool of substances. The selection of such lead structures - representing the key structural and substructural elements that characterise a certain substance class - forms the basis of the so-called "test-kit concept" (Jastorff *et al.*, 2003). The knowledge generated from testing these test-kits then can be transferred to compounds that have not been explored. Thereby, it is supporting the implementation of qualitative and quantitative structure-activity relationships (SARs and QSARs). SARs and QSARs represent a powerful and highly efficient tool in the comparative hazard assessment of chemicals, since it helps to reduce the number of necessary tests and costs dramatically.

However, a mode-of-action-based test strategy and the application of a corresponding flexible test battery require a broad biological target spectrum to cover different endpoints that address the three modes of toxic action a chemical can exert in biological systems. These can be classified as follows: i.) unspecific interaction with membrane structures (named as baseline toxicity or narcosis), ii.) specific interaction with proteins (*e.g.* enzyme inhibition or binding to receptor proteins) and iii.) chemical reactivity towards the molecules of life (Escher and Hermens, 2002). Especially with respect to the last issue the two subcellular endpoints "ratio of glutathione (GSH) to glutathione disulphide (GSSG)" and "inhibition of glutathione reductase (GR)" were incorporated into the flexible test battery. As it will be presented in the following, these endpoints are highly sensitive and hence useful to detect harmful effects of electrophilic organic chemicals.

2.2 Selection and T-SAR analysis of the test substances

2.2.1 Isothiazol-3-one biocides

The isothiazol-3-one biocides were selected for this case study since they represent a substance class of highly electrophilic and broadly applied industrial chemicals. Thus, these substances serve as a modell for reactive electrophilic organic chemicals and owing to their broad commercial application; a deeper insight into their hazard potential is of high ecotoxicological relevance. Furthermore, the availability of different substitution

patterns at the aromatic core structure (Figure 5) allows for structure-activity investigations. The four biocides N-methylisothiazol-3-one (MIT), 5-chloro-N-methylisothiazol-3-one (CIT), N-octylisothiazol-3-one (OIT) and 4,5-dichloro-N-octylisothiazol-3-one (DCOIT) were selected to analyse the influence of the chlorine substitution and the hydrophobicity on the cytotoxicity of these substances.

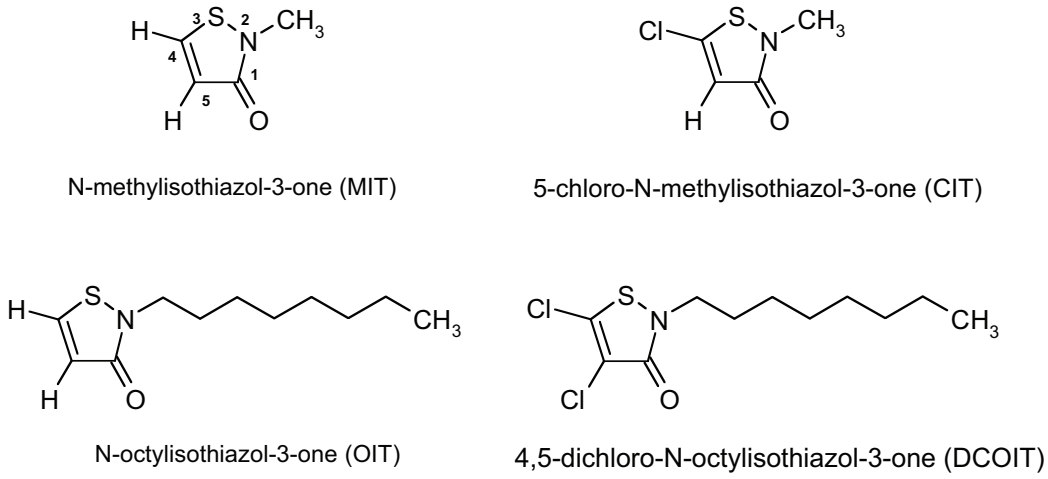

Figure 5 Structures of the selected isothiazol-3-one biocides. The chemical names are presented with the used acronyms in parenthesis. The numbers given in the MIT structure refere to the atoms forming the isothiazol-3-one ring-system.

The substance class of isothiazol-3-ones was introduced in the early 1970s as potent biocides showing a high activity against a broad spectrum of microorganisms and fungi (Lewis *et al.*, 1971). Hence, MIT, CIT and OIT are listed in the EINECS index. The only exception is DCOIT that was developed in the early 1990s to serve - under the trade mark Sea-Nine® - as a surrogate for the banned TBT (tributyl tin) compounds in marine antifouling paints. In this context DCOIT even received the US EPA "Presidental Green Chemistry Award" in 1996.

In general, the highly reactive isothiazol-3-one biocides find manifold commercial applications in wall paintings (OIT), antifouling paintings (DCOIT) and as stabilisers in cosmetics (MIT and CIT) (Du *et al.*, 2002; Jacobson and Williams, 2000; Jacobson and Willingham, 2000). This group of biocides is assumed to exert its high toxicity towards microorganisms and fungi by diffusing through the cell membrane and subsequently reacting with essential intracellular sulphur-containing proteins or smaller biomolecules like glutathione (Chapman and Diehl, 1995; Collier *et al.*, 1990). Quantum chemical calculations and *in vitro* reaction studies using thiol containing compounds (*e.g.* 2-methyl-2-propanethiol) provide strong hints that the first reaction step is based on a nucleophilic attack of a sulfhydryl group at the sulphur atom of the electrophilic isothiazol-3-one ring structure (Morley *et al.*, 2007). Furthermore, the irreversible reaction of isothiazol-3-ones with cysteine residues in proteins was identified to be the reason for the known skin-sensitising potential of these substances in humans (Alvarez-

Sanchez *et al.*, 2003; Alvarez-Sanchez *et al.*, 2004). This skin sensitising potential was uncovered in epidemiological studies and finally led to a reduction of the application of isothiazol-3-ones in cosmetic or household products (Ellis, 1995). Nevertheless, still today these biocides can be found as preservatives in various products such as shampoos, cleaning agents and wall paintings.

Data from different cellular assays testing commercially available mixtures of MIT and CIT (*e.g.* KATHON^{TM}) indicate that the observed cytotoxicity is related to the interaction of the biocides with the cellular glutathione (GSH) metabolism leading to apoptotic or necrotic cell death (Di Stefano *et al.*, 2006; Du *et al.*, 2002; Ettorre *et al.*, 2003). In these literature references it was also found that the chlorinated CIT species exerts stronger effects compared to its non-chlorinated analogue MIT. Thus, there are first hints that the substitution pattern at the aromatic isothiazol-3-one core structure is able to modulate the toxicity of these biocides. To analyse these substitution effects in more detail the presented test-kit (see Figure 5) was combined to assess the effects of an increasing hydrophobicity (MIT to DCOIT) and of the chlorine substitution (MIT *vs.* CIT and OIT *vs.* DCOIT).

From the T-SAR analysis and the known reactivity of the isothiazol-3-ones the following structural alerts can be identified with respect to possible modes of toxic action:

- A hydrophobic interaction potential, especially in the chlorinated and octyl substituted compounds (CIT, OIT and DCOIT).
- A highly electrophilic isothiazol-3-one ring system with the sulphur found to be the atom exhibiting the highest positive polarisation and thus the highest Lewis-acidity.

These structural alerts can be transferred to the following working hypotheses addressing the hazard potential of the isozhiazol-3-ones:

- Can the intrinsic toxicity of the isothiazol-3-ones - based on the highly electrophilic sulphur atom – be confirmed as predominant mode of toxic action in cytotoxicity assays using different endpoints and species? If yes, a significant excess toxicity of the biocides should be observable compared to solely narcotically acting chemicals. Furthermore it is expected that the different substitution patterns modulate the observed cytotoxicity.
- Can unspecific hydrophobic interactions owing to the hydrophobicity of the biocides with membrane structures be identified as a second mode of toxic action (baseline toxicity)? If yes, one should observe significant differences when comparing the excess toxicities of the methyl substituted biocides (MIT and CIT) with their octyl substituted analogues. To answer this question, again cytotoxicity assays can be used.
- Can the high electrophilic reactivity be monitored using molecular endpoints? If yes, the soft Lewis-acidic isothiazol-3-ones should preferably interact with GSH, which represents the most prominent soft Lewis-basic molecule inside living cells.

Again it is assumed that the different substitution pattern should modulate these interactions.

To verify these working hypotheses in part two of the thesis, paper No. 1 deals with different cytotoxicity assays (human liver cell culture, marine bacterium and limnic green alga) and analyses the obtained data using the toxic ratio approach with respect to structure-activity relationships. The molecular aspects are discussed in detail in paper No. 2 where the impacts of the isothiazol-3-ones on cellular GSH metabolism and on GR activity were investigated.

2.2.2 Ionic liquids

Under the term "ionic liquids" a large and highly heterogeneous substance class of salts is subsumised whose unifying physicochemical parameter is a melting point smaller than 100°C. Some structures out of this large pool of ionic compounds were chosen as a second substance class for the presented case study. This selection is based on the fact that ionic liquids are gaining more and more importance in manifold technological applications - that are reviewed in detail in Li *et al.* (2006) and Zhao (2006) - but the knowledge about their hazard potential is still very restricted. The current knowledge about possible hazard potentials of ionic liquids and the resulting ecotoxicological impacts are reviewed in Ranke *et al.* (2007).

In general, ionic liquids are often termed to be non-reactive and of high chemical stability (Dupont and Suarez, 2006) in contrast to the above introduced isothiazol-3-one structures. Additionally, the high structural variability of this heterogeneous substance class allows on the one hand for detailed structure-activity relationship studies, but on the other hand it challenges the applied testing strategy and the test systems. Thus, ionic liquids represent an ideal substance class to validate and refine the concept of a flexible test battery and additionally, ecotoxicological data are needed to fill this gap for an advanced hazard assessment of these chemicals. Furthermore, the knowledge about possible modes of toxic action of ionic liquids is of high relevance for the design of new and inherently safer ionic liquids for industrial applications (Jastorff *et al.*, 2005).

The investigated ionic liquids all consist of an organic cation that can further be subdivided into the head group carrying the positive charge and the side chains connected to this cationic core moiety. For the presented case study different head group structures (imidazolium, pyridinium, dimethylaminopyridinium, pyrrolidinium, piperidinium, morpholinium, quinolinium and quaternary ammonium and phosphonium structures) were combined with either alkyl side chains of varying chain length (methyl to octadecyl) or with functionalised alkyl side chains containing hydroxy, nitrile or ether functions. These cations were combined with various anions ranging from halides (Cl^-, Br^- and I^-) to complex anion species containing an inorganic central atom (*e.g.* BF_4^-, PF_6^-, $[N(SO_2CF_3)_2]^-$ or complex borate anions). Thus, a test-kit of ionic liquids was combined that allowed for a systematic analysis of the influence of the three structural elements head group, side chain and anion on the hazard potential of the corresponding ionic liquids. The detailed structures of the tested ionic liquids are presented and discussed in

manuscript No. 3 and No. 4. In general, the T-SAR analysis of the test-kit compounds revealed the following striking structural alerts pointing to possible modes of toxic action:

- Depending on the side chain length and functional groups present in these side chains nearly all investigated ionic liquid structures exhibit a moderate to high hydrophobic interaction potential combined with a cationic head group.
- The majority of the investigated ionic liquids contains a quaternary ammonium or phosphonium moiety in the cationic head group often combined with an aromatic system providing a p-p interaction potential.

Again these structural alerts can be converted into working hypotheses to assess possible hazard potentials:

- Is the hydrophobic interaction of ionic liquids with biological membranes and proteins the predominant mode of toxic action as it is assumed owing to their high structural similarity to common surfactants? If yes, in different cytotoxicity studies the effects of the selected ionic liquids should be comparable to those of baseline toxicants.
- Is it possible to identify structure-activity relationships for the influence of the different substructural elements head group, side chain and anion on the observed toxic effects? If yes, this should give some insights into the toxic mode of action of ionic liquids.
- Since it is known that some ionic liquid cation structures are potent acetylcholinesterase inhibitors (Stock *et al.*, 2004) the question arises whether it is possible to implement qualitative and quantitative structure-activity relationships to describe the influence of the head group, the side chain and anion species on this inhibitory potential?

To verify these hypotheses the ionic liquids were tested in a toxicity assay using an IPC-81 rat leukaemia cell line and the results are discussed in detail in paper No. 3 focusing on the modes of toxic action and structure-activity relationships. To get a deeper insight into acetylcholinesterase inhibition by ionic liquid substances, paper No. 4 deals with a structure-activity study of a broad set of different head group structures, functionalised side chains and anion species to reveal the impacts of these structural elements on the inhibitory potential of the corresponding compounds.

2.3 Selection and characterisation of the test systems

2.3.1 Cellular test systems

In the following section four different cytotoxicity assays – based on unicellular test species - are presented. In general, cellular test systems represent an integral biological framework where nearly all interactions of chemicals that are taken up by organisms take place. That implies that these test systems can be used to identify general effects a toxicant can exert on the cellular level. Among these effects the disturbance of

membrane structures and function owing to hydrophobic interactions, the interaction with cellular signal transduction pathways, the disruption of the energy metabolism or interference with the protein biosynthesis machinery can be found. The endpoints of the four cellular test systems used all reflect an integral parameter to assess the general cell viability and thus they can be used to screen general impacts of substances on basal cellular structures and functions. The advantages and limitations of cellular test systems in the assessment of the hazard potential of chemicals are illustrated within the basal cytotoxicity concept. Introduced by Ekwall *et al.* (1993), this concept proposes that all chemical toxicity to organisms can be classified according to the following three main categories: i.) organisational or extracellular toxicity ii.) organ-specific cytotoxicity, *i.e.* injury to organotypic cell functions and structures and iii.) basal cellular toxicity, *i.e.* injury to structures and functions that can be ubiquitously found in cells. Furthermore, the main hypothesis of the basal cytotoxicity concept states that the majority of toxicological effects caused by chemicals on the level of organisms find their reasons in basal cytotoxic events. These effects can be revealed by using either undifferentiated cell lines or differentiated cell cultures if basal toxicity endpoints are monitored. But the above stated hypothesis also implies that these cell cultures cannot be used to identify organ-specific or tissue-specific toxicities. To uncover such effects and toxicokinetic aspects further special test systems and assays on higher levels of biological complexity are needed. Additionally, the detailed molecular mechanisms underlying the observed general cytotoxicity can normally not be investigated on the level of cellular assays.

However, as mentioned in section 2.1, according to Escher and Hermens (2002) basal cytotoxic events can be further subdivided into three modes of toxic action: i.) unspecific interaction with membrane structures (*i.e.* baseline toxicity/narcosis), ii.) specific interaction with proteins and iii.) chemical reactivity towards the molecules of life. Since baseline toxicity[2] is the minimal and well predictable toxicity (Veith and Broderius, 1990) every chemical entity exerts, for an accurate hazard assessment it is of high relevance to discriminate baseline toxic effects from other more specific modes of toxic action. It is assumed that over 60 % of common industrial chemicals are baseline toxicants (Veith *et al.*, 1983). Hence, efficient screening tests are necessary to identify excess toxicities of substances and to focus ecotoxicological testing efforts on these substances. Screening assays using cell cultures and known baseline toxicants as reference substances represent a powerful tool to identify substance specific excess toxicities[3]. Once identified, the reasons for these excess toxicities can be analysed in detail using molecular and subcellular endpoints.

To achieve such a tiered approach, in the present work cellular test systems were used to reveal the general toxicity pattern of selected isothiazol-3-one biocides and ionic liquids and to identify excess toxicities that need to be further investigated at the molecular level. To cover a broad set of general cellular mechanisms and functions not

[2] In this work the terms "baseline toxicity" or "narcosis" are used to describe the minimal toxicity a chemical can exert in cellular test systems due to hydrophobicity driven interactions with lipid structures and/or hydrophobic domains in proteins.

[3] According to Lipnick *et al.* (1987) the term "excess toxicity" describes any additional toxic effects of a substance compared to its predicted or measured baseline toxicity.

only mammalian (human and rat) cell cultures were used in the two case studies but also prokaryotic (*Vibrio fischeri*) and photosynthetically active algae (*Scenedesmus vacuolatus*) cells were investigated. The following sections present these test systems in more detail.

The Hep G2 cell culture

The human hepatoblastoma cell line Hep G2 was established and characterised by Aden *et al.* (1979) who first isolated these cells from a liver biopsy of a child. In contrast to other differentiated liver cell lines, Hep G2 cells still share many functions, histological markers and morphological features with normal primary human hepatocytes. Especially the cytochrome P450 monooxygenase system - an essential enzyme family regarding cellular metabolism and detoxification processes - is still active and can be induced in Hep G2 cells (Dawson *et al.*, 1985). Thus, this cell line represents an established model system to investigate the toxification, detoxifiation and the metabolism of chemical substances in human liver tissue. Additionally, even human acute toxicities for certain substances can be predicted at the base of a Hep G2 cytotoxicity assay (Dierickx, 2005).

For this case study the Hep G2 cell line was selected and implemented into the flexible test battery because the liver is the main target organ for toxic effects of chemicals in humans and other mammals. Furthermore, observed toxic effects in this cell culture are of high concern, since the Hep G2 cells are well equipped by nature to deal with toxic chemicals and thus, they normally can tolerate a higher toxicant burden than other cell types. Finally, the metabolic activity of these cells allows for the identification of degradation products and possible toxification effects of substances owing to metabolic reactions. The detailed culture conditions for the Hep G2 cells used and the incubation protocols are described in paper No. 1.

The cell viability of Hep G2 cells was measured photometrically after a 48 h preincubation with toxicants using the WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt) dye (Ishiyama *et al.*, 1995). This assay is based on the extracellular enzymatic reduction of a red tetrazolium dye yielding the corresponding yellow formazan derivate (Berridge *et al.*, 1996). Hence, this assay reflects well the membrane integrity of the cells and the general state of reduction equivalents, which are directly linked to the cellular energy metabolism as basal cellular structures and functions.

The IPC-81 cell culture

The promyelotic rat leukaemia cell line IPC-81 was isolated in 1983 from brown Norway rats in the group of Dr. Michel Lanotte (Lacaze *et al.*, 1983). Since these cells originate directly from pluripotent stem cells they are only little differentiated (Hochhaus and Hehlmann, 2001) and thus, they represent an ideal model system to study basal cytotoxic effects. The IPC-81 cells are used as well established test system in the flexible test battery - they were successfully applied to assess the toxicity of various environmentally relevant chemicals (Doose *et al.*, 2004; Ranke *et al.*, 2004; Siol, 2002;

Stock, 2004). Additionally, it could be shown that this cell line responds highly sensitive to various chemicals compared to other commonly used NB4, C6 or HeLa cells (Ranke *et al.*, 2004; Stock, 2004). Hence, in the presented case study this cell line was chosen as a mammalian model system to analyse the harmful effects of certain ionic liquid structures.

The cell viability was measured as an integral cytotoxicological endpoint using the WST-1 assay described above. The detailed culture conditions and the incubation protocol are summarised in paper No. 3.

The marine bacterium *Vibrio fischeri*

The gram-negative marine bacterium *Vibrio fischeri* was discovered in 1889 by Martinus Willem Beijernick and can be found in all oceans around the world. Normally these flagellated bacteria live in symbiosis with higher marine organisms, *e.g.* they can be found in the light organs of the squid *Euprymna scolopes*.

As a luminescence bacterium, *Vibrio fischeri* is able to convert part of its metabolism energy into light. Responsible for this bioluminescence is the enzyme luciferase - a flavine-dependent monooxygenase - that catalyses the oxidation of luciferine to the corresponding oxyluciferine. The eneregy of this redox reaction is emitted as light showing the maximal peak in the emission spectrum at 490 nm. The total energy consumption of this redox step is high since around 60,000 molecules of ATP are needed per second and cell to generate 104 light quanta per second and cell. Thus, the luminescence intensity of the bacteria is directly related to their general cellular energy metabolism and can be used as a sensitive indicator for cell viability. The photometrical measurement of the bioluminescence of *Vibrio fischeri* is one of the most commonly applied ecotoxicological test systems to monitor aqueous toxicities of various chemicals, and hence a broad data set of reference compounds is available in the literature. Furthermore, the bacteria react in short time frames (minutes) highly sensitive to different toxicants and thus they are frequently used in OECD standardised 5, 15 and 30 minutes acute toxicity screening assays.

Due to this the 30 minutes *Vibrio fischeri* assay was chosen as a prokaryotic and highly sensitive cellular test system to assess the aquatic toxicity of the isothiazol-3-ones. A detailed description of the culture and assay conditions for the commercially available freeze-dried bacteria is given in paper No. 1.

The limnic green alga *Scenedesmus vacuolatus*

The limnic green alga *Scenedesmus vacuolatus* (in earlier literature also termed as *Chlorella fusca*) is a spherical unicellular micro alga with a diameter of around 10 µm that belongs to the group of chlorothphyta. It can be found in limnic plankton communities as well as in moisty soils. The most important charateristics of this alga are their thin but extremely stable cell wall composed of different cellulose layers and their well developed photosynthesis system, which is comparable to that of higher plants. Additionally, the content of cellular chlorophyll in these algae is high entailing extremely high

photosynthesis rates. Because the reproduction rate of *Scenedesmus vacuolatus* is in the range of 16 - 20 h these algae can be used in reproduction screening assays to evaluate the aqueous toxicities of various chemicals. Thus, in the literature, broad data sets for single substances and mixtures can be found. Furthermore, the algae are routinely used in bioassays monitoring wastewater qualities.

To assess the cytotoxicity of the selected isothiazol-3-ones towards these algae a 24 h reproduction inhibition assay was used. This integral endpoint also reflects general cell viability and includes - in addition to the other cellular test systems used - the photosynthesis system as a further basal cellular mechanism toxicants can interfere with. The assay protocol and the conditions for the synchronised algae cultures are described in detail in paper No. 1.

2.3.2 Molecular test systems

As pointed out in section 1.3 molecular test systems are powerful tools to reveal the detailed mechanisms and molecular interactions underlying certain modes of toxic action. Thus, these test systems are of high value in establishing structure-activity relationships that can support the comparative risk assessment and the design of inherently safer chemical substances.

However, to generate useful data that help to verify structural alerts in chemicals it is implicitly necessary to choose molecular targets (*e.g.* enzymes, receptor proteins or smaller biomolecules like GSH) that bear the inherent potential to interact with the substances of interest. Since molecular and subcellular test systems are highly specific and - in contrast to the integral cellular test systems - cover only a tiny piece of biochemical mechanisms and pathways it is important to avoid the trap asking a "molecular" question the test system cannot respond to even if it is well established. Thus, the T-SAR guided selection of molecular test systems is an important part of the test strategy, too. Here the T-SAR approach helps to identify structural alerts present at the target site rendering a test system sensitive to the molecular interactions that are presumed to be responsible for a toxic mode of action. In the following the selected molecular and subcellular test systems used in the case study are presented and their structural alerts forming the base for this selection are briefly discussed.

Cellular glutathione metabolism

The tripetide glutathione (γ-Glu-Cys-Gly, GSH) is one of the most important low molecular weight biomolecules that can be found in high concentrations up to 20 mM in nearly all cell types and species. In general, GSH and its oxidised disulphide GSSG exert two important functions maintaining cell viability and integrity. Firstly, the electrophilic conjugation of functionalised xenobiotics to GSH in the phase II metabolism of toxicants and pharmaceuticals represents the main cellular detoxification and excretion pathway. Secondly, the GSH/GSSG redox pair represents the most important cellular redox buffer system. In healthy cells the high GSH/GSSG ratio

(aprox. 100:1) protects lipids and proteins from oxidation by reactive oxygen species (ROS).

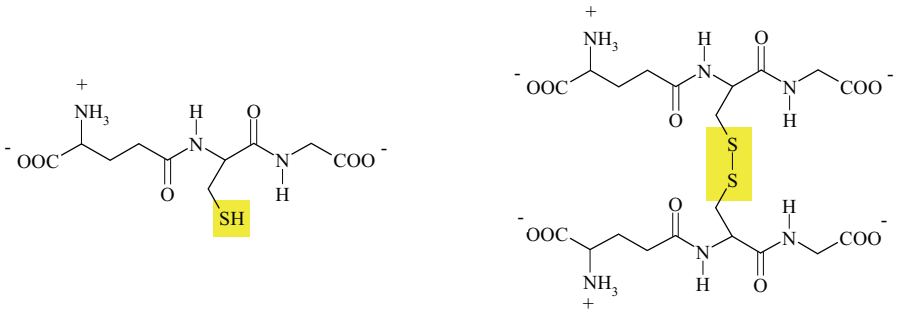

Figure 6 Glutathione (GSH) and its oxidised disulphide (GSSG). The essential thiols are highlighted in yellow.

Furthermore, the GSH dependent reduction potential of the cell is essential for the structure and function of many redox-sensitive enzymes and proteins (*e.g.* proteins carrying essential cystein residues). To ensure this high GSH to GSSG ratio, cells are well equipped with several enzymatic systems that are able to recycle the oxidised GSSG to GSH. Among these enzymes the glutathione reductase (GR) - that is discussed in the following section - plays a pivotal role in maintaining the high cellular GSH excess.

Because the GSH concentration is represented by a quadratic term within the Nernst-equation describing the GSH/GSSG equilibrium, especially GSH depletion by electrophilic toxicants exerts a dramatic influence on the cellular reduction potential (Jacob *et al.*, 2003). As mentioned above the breakdown of this reduction potential influences the redox state of essential proteins and additionally the mitochondrial membrane potential. Thus, GSH depletion by reactive xenobiotics or a shift in the GSH/GSSG ratio due to an enrichment of GSSG can yield in severe cellular changes entailing apoptotic or necrotic cell death.

Because of its sulphydryl group, GSH represents a "soft" Lewis-basic nucleophile, which is - owing to its high cellular concentration - the main target for "soft" Lewis-acidic electrophiles. Since the isothiazol-3-ones are known to be very potent "soft" Lewis-acidic electrophiles that can react readily and irreversibly with sulfhydrylgroups, GSH was chosen as molecular target in this case study to evaluate this highly specific reactivity of the biocides tested. As a molecular endpoint the pseudo first order reaction rate constants for the reaction of the four isothiazol-3-ones with GSH was determined in a cell free buffer system. Additionally, the concentration and time dependent influence of each of the biocides on the cellular GSH metabolism was measured using Hep G2 cells. Here, the total cellular glutathione content (GSH + [2 × GSSG]) and the GSH/GSSG ratio were chosen as molecular endpoints. A detailed description of the applied assay conditions and incubation protocols is given in paper No. 2.

Glutathione reductase

Glutathione reductase (GR) is a cytosolic flavine-dependent enzyme that carries FAD^+ as prosthetic group near its active site. This active site is highly conserved for many species. GR is a key enzyme in maintaining the high GSH/GSSG ratio within cells by binding GSSG as natural substrate and reducing it in a two step reaction yielding two molecules of GSH. The electrons needed for this reaction step are provided by the cofactor NADPH. The mechanistic cycle of this enzyme can be divided into several steps. In its oxidised and inactive state the enzyme contains an essential disulfide bridge between Cys-58 and Cys-63 in its active centre (Boese *et al.*, 1997). In the first step of the catalytic cycle the co-substrate NADPH is bound near the active site of the enzyme and the disulfide bridge is reduced via an electron transfer from the NADPH via the FAD^+ moiety to the active centre (Picaud and Desbois, 2002). This results in a highly nucleophilic thiolate anion at Cys-63, which then attacks the natural substrate GSSG. This disulfide exchange reaction yields in an intermediate where Cys-63 forms a new disulphide bridge with one part of the GSSG molecule and the other part is released as GSH molecule. In the last step Cys-58 undergoes another disulphide exchange reaction with Cys-63 leading to the oxidised inactive state of the active site and the release of the second GSH molecule. In parallel, the now oxidised $NADP^+$ leaves the enzyme and is replaced by a NADPH molecule initiating a new catalytic cycle.

From this catalytic cycle, as a key structural alert the highly nucleophilic thiolate moiety formed at Cys-63 in the first step was identified. This "soft" Lewis-basic anion renders the active site of GR extremely vulnerable for an inactivation by the reaction with electrophilic, "soft" Lewis-acidic organic chemicals. Indeed, it was shown that GR can be inhibited by various electrophilic organic chemicals (Cheung *et al.*, 2002; Savvides *et al.*, 2002).

Thus, GR was chosen as second molecular target to assess the toxicity of the isothiazol-3-one biocides, which is mediated by their intrinsic electrophilic reactivity. Additionally, GR was chosen since it plays a crucial role in the cellular GSH metabolism and an inhibition of this enzyme with a concomitant depletion of GSH by the isothiazol-3-ones would dramatically amplify the toxic effects of the biocides in a feedback cycle.

As endpoint, the concentration dependent inhibition of the enzyme´s activity was measured in a cell free system as well as in cell lysates obtained after the incubation of Hep G2 cells with the biocides. The enzyme inhibition assays used and the impact of the biocides on GR are discussed in detail together with the GSH metabolism in paper No. 2.

The molecular target used for the assessment of ionic liquids - the enzyme acetylcholinesterase - is presented and discussed in the following section.

Acetylcholinesterase

Acetylcholinesterase (AchE) belongs to the group of serine hydrolases and can be found in nearly all higher organisms including humans. It is located - extracellularly

bound to the post-synaptic membrane - in the synaptic cleft between neurons. AchE catalyses the rapid degradation of the neurotransmitter acetylcholine - one of the key mechanisms in neurotransmission - to terminate and regulate the signal processing between neurons and between neurons and muscles. Thus, an inhibition of this enzyme leads to various adverse effects in neuronal processes, such as heart diseases or myasthenia in humans (Chemnitius *et al.*, 1999; Pope *et al.*, 2005). Furthermore, AchE represented the main target in the development of potent insecticides based on phosphoric acid esters (*e.g.* Parathion®) and carbamates (*e.g.* Carbendazim®). The activity pattern of this enzyme in different biological matrices and tissues is used as an established biomarker to monitor the pesticide burden in non-target species (Eder *et al.*, 2004; Fulton and Key, 2001; Rickwood and Galloway, 2004).

The highly conserved active site of AchE is located at the bottom of a narrow gorge. This gorge is lined with hydrophobic aromatic amino acid residues and its entrance is built up with negatively charged residues. The active centre itself can be divided into the catalytic esteratic site where the acetyl group of the substrate is bound and the anionic site where the quaternary ammonium moiety of the acetylcholine is stabilised via a cation-π interaction with an essential tryptophan residue (Trp 84) (Harel *et al.*, 1993). Additionally, a peripheral anionic site (PAS) could be identified at the entrance of the narrow gorge where the substrate acetylcholine is bound to Trp 279 again via cation-π interactions and is thereby orientated towards the active centre (Bourne *et al.*, 2003).

The catalytic cycle of the enzyme can be described in three steps. Firstly, the substrate acetylcholine is attracted by the negative surface potential surrounding the entrance of the gorge and binds to Trp 279. The so orientated substrate molecule is subsequently transferred through the hydrophobic gorge and bound at the active centre with the positively charged nitrogen moiety interacting with Trp 84 and the acetyl group lying at the esteratic site. The ester bond is hydrolysed and the resulting choline moiety leaves the catalytic site via the gorge. In the last step a water molecule regenerates the acetylated enzyme and the acetate anion is expelled via the channel formed by the hydrophobic gorge (Colletier *et al.*, 2006).

Thus, competitive inhibitors of acetylcholinesterase can act via two distinct mechanisms. Firstly, they can bind directly to the active site and thereby inhibit the cleavage of the natural substrate or secondly, inhibitors can bind to the PAS and block substrate traffic into and out of the catalytic centre by steric interference with or allosteric alteration of the enzymes active centre (Bourne *et al.*, 2003).

AchE was chosen as molecular target to screen the hazard potential of ionic liquids, since these substances exhibit the key structural alerts to act as potent AchE inhibitors. Similar to the natural substrate acetylcholine most of the ionic liquids contain a positively charged quaternary ammonium moiety. Furthermore, the aromatic head groups provide a p-p interaction potential pointing to strong interactions with the essential tryptophan residues at the PAS or at the anionic active site, respectively. Finally, some ionic liquids contain long hydrophobic side chains that can interact with the hydrophobic amino acid residues lining the gorge to the active centre of AchE. With respect to this Stock *et al.* (2004) could show that imidazolium and pyridinium ionic

liquids act as strong AchE inhibitors. In the present case study AchE activity was measured as molecular endpoint in a cell free system to further reveal the particular influences of the three ionic liquids substructural elements head group, side chain and anion on this inhibitory potential. Additionally, it was the intention to derive a QSAR equation describing the inhibitory potential of certain ionic liquid structures. The results dealing with AchE inhibition as well as the assay conditions used to screen the enzyme´s activity are presented in paper No. 4.

PART II:

CASE STUDIES – ASSESSMENT OF ISOTHIAZOL-3-ONE BIOCIDES AND IONIC LIQUIDS WITH CELLULAR AND MOLECULAR METHODS

PAPER NO. 1:

ANALYSING CYTOTOXIC EFFECTS OF SELECTED ISOTHIAZOL-3-ONE BIOCIDES USING THE "TOXIC RATIO" CONCEPT AND THE "T-SAR" APPROACH

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Abstract

To investigate the toxic mode of action of isothiazol-3-one biocides as an example for reactive electrophilic xenobiotics, the four compounds N-methylisothiazol-3-one (MIT), 5-chloro-N-methylisothiazol-3-one (CIT), N-octylisothiazol-3-one (OIT) and 4,5-dichloro-N-octylisothiazol-3-one (DCOIT) were tested for their effects on the human hepatoblastoma cell line Hep G2, on the marine bacteria *Vibrio fischeri* and on the limnic green alga *Scenedesmus vacuolatus*. All four biocides exhibited extremely high toxicities, in which the two chlorinated species CIT and DCOIT were found to be significantly more toxic than their non-chlorinated analogues MIT and OIT. In each of the three test systems, toxic effects were observed in a consistent pattern. In order to analyse these observed effects, we use a "Thinking in terms of structure activity relationships (T-SAR)" guided examination of the four biocides as well as the toxic ratio (TR) approach to determine the modes of toxic action of each of the substances tested. For the isothiazol-3-ones, we could identify two mechanisms of toxic action. The small and polar MIT and CIT exhibited pronounced toxic effects - a so-called excess toxicity - in each of the three test systems that presumably are triggered by their intrinsic reactivity towards cellular thiols. In contrast, the hydrophobic OIT and DCOIT showed mainly toxicities as could be expected for pure baseline toxicants - *i.e.* toxic effects are solely caused by hydrophobic interactions with membrane structures or proteines. Hence, for OIT and DCOIT baseline toxicity - also referred to as narcosis - seems to be the predominant mode of toxic action. However, comparing these results with data that indicate a dramatic depletion of cellular glutathione levels after incubation with DCOIT, reveals that for highly hydrophobic substances baseline toxicity - which is only relevant for acute toxic effects - can lead to the neglect of other more specific modes of toxic action. Since specific reactivities may exert dramatic long-term effects even at concentrations that proved to be subtoxic in acute toxicity assays, this fact should be taken into account in the hazard assessment of hydrophobic and also reactive chemicals.

Introduction

The new EU legislation for the registration, evaluation authorisation and restriction of chemical substances (REACH)[1] demands large data sets to assess the toxicological and ecotoxicological impacts of chemical substances and products containing them on man and the environment. Thus, there is a special need for efficient testing strategies in order to develop reliable screening assays for the rapid identification and measurement of possibly toxic chemical structures. Accordingly we pursue a T-SAR (thinking in terms of structure-activity relationships) guided strategy to systematically analyse the hazard potential of a chemical entity[2]. This theoretical approach not only helps to identify structural alerts in chemical entities that are responsible for possible toxic modes of action, it also supports the selection of test systems that are suitable to confirm and

describe the assumed toxic mechanisms. Hence, the T-SAR approach can be used to identify the molecular origins of the observed toxic effects. Such knowledge is essential for establishing mode of action-basisd, quantitative structure activity relationships (QSARs), which represent a promising tool for an effective toxicity screening of large substance libraries[3].

Based on these considerations, we applied a tiered testing strategy to assess the toxicity of four differently substituted isothiazol-3-one biocides as an example for highly reactive and environmentally relevant toxicants (Figure 1).

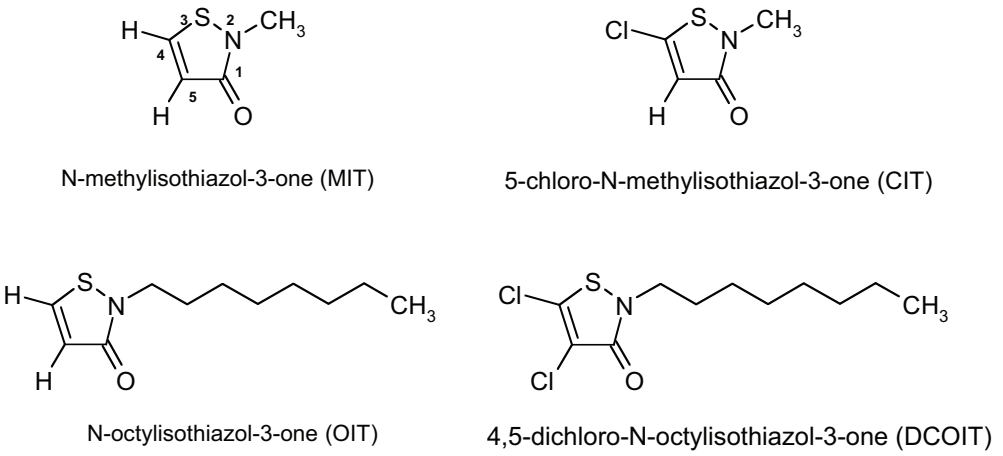

Figure 1 Structures of the selected isothiazol-3-one biocides. The chemical names are presented with the used acronyms in parenthesis. The positions of the isothiazol-3-one ring atoms are indicated with numbers (MIT).

Firstly, the substances were systematically selected - owing to the different chlorine substitution and chain lenght at the nitrogen - according to the test kit concept[4]. Secondly, the assumed toxicities of these compounds were verified in three test systems chosen from a flexible toxicological and ecotoxicological test battery[5]. As test systems the viability of the human hepatoblastoma cell line Hep G2, the luminescence inhibition of the marine bacterium *Vibrio fischeri* and the reproduction rate of the limnic green alga *Scenedesmus vacuolatus* were investigated after incubation with various concentrations of each of the four isothiazol-3-ones.

Isothiazol-3-ones are well known and highly reactive electrophilic biocides, which find manifold commercial applications, *e.g.* in wall paintings (OIT), antifouling agents (DCOIT) and as stabilisers in cosmetics (MIT and CIT)[6-8]. This group of biocides is assumed to exert its high toxicity towards microorganisms and fungi by diffusing through the cell membrane and subsequently reacting with essential intracellular thiol-containing proteins or smaller biomolecules like glutathione[9,10]. Furthermore, isothiazol-3-ones have been identified to act as strong skin-sensitisers in humans via the irreversible reaction with cysteine residues in proteins[11,12]. Data from various cellular

assays for commercially available mixtures of MIT and CIT (*e.g.* KATHON®) indicate that the observed cytotoxicity is related to the interaction of the biocides with the cellular glutathione (GSH) metabolism leading to apoptotic or necrotic cell death[13,14]. Here, it was also reported that the chlorinated CIT exerts higher effects compared to its non-chlorinated analogue MIT. However, so far some toxicity and ecotoxicity data for single substances are only available for DCOIT, whereas for the remaining isothiazol-3-ones most data are published for commercially available mixtures. Therefore, we isolated and purified the four compounds from these mixtures to get a deeper insight into the influence of different substitution patterns on the observed cytotoxic effects in each of the three test systems.

The used TR concept is based on the assumption that every chemical entity exhibits a certain minimal toxicity - the so-called baseline toxicity or narcosis - via the interference with lipid structures in biological systems, which is only determined by the compound´s hydrophobicity. This minimal toxicity - that can be drastic for highly hydrophobic substances - of a compound can easily be predicted by QSAR approaches and is therefore of minor concern for new test systems supporting the hazard assessment of chemical substances[15]. However, apart from this baseline toxicity chemicals can additionally exert specific intrinsic toxic effects owing to *e.g.* electrophilic reactivity or steric fit to specific targets[21]. This results in a higher toxicity - the so-called excess toxicity[15] - over that it would be expected for solely narcotic acting substances. It was proposed[16] to take a TR of 10 as benchmark for separating excess toxicity (TR $\geq$ 10) from baseline toxicity (TR < 10).

The octanol-water partition coefficient of the four isothiazol-3-ones was used to determine their hydrophobicity. Applying the TR concept[17] to quantify the excess toxicity of a substance resulting from its intrinsic chemical reactivity we could identify different modes of toxic action for MIT and CIT compared to OIT and DCOIT. The less hydrophobic substances MIT and CIT showed the highest excess toxicity. Thus, we assumed the specific thiol reactivity of these isothiazol-3-ones to be most likely the reason for the observed cytotoxicities. In contrast, for the hydrophobic OIT and DCOIT we found hardly any excess toxicity. Therefore, their hydrophobicity seems to be the dominating molecular descriptor for the observed toxic effects. Nevertheless, we could also show[18] that especially DCOIT leads to a dramatic decrease of cellular GSH levels. That means that for highly hydrophobic substances the hydrophobicity basisd mechanisms of toxic action (disturbance of lipid membrane structures owing to the partitioning of hydrophobic substances into biological membranes) - *i.e.* the so called baseline toxicity or narcosis[19,20] - may mask other more specific modes of toxic action in acute toxicity assays. In particular, this fact should be considered in the hazard assessment of reactive chemicals, since intrinsic reactivities may cause long-term effects even at concentrations that prove to be subtoxic in acute toxicity screenings.

Furthermore, we could confirm the T-SAR derived assumption that the chlorinated isothiazol-3-one species CIT and DCOIT should exhibit higher cytotoxicities than their non-chlorinated analogues MIT and OIT in each of the three test systems used.

RESULTS

The results for the impact of the isothiazol-3-ones on the human hepatocarcinoma cell line Hep G2, on the marine bacterium *Vibrio fischeri* and on the limnic green alga *Scenedesmus vacuolatus* are presented. Additionally, octanol-water partition coefficients and half-life times for the hydrolytical stability of the substances are shown to evaluate the obtained toxicity data and to derive QSARs for the baseline toxicity of each substance in all three test systems.

Physicochemical data

The decadic logaritms of the octanol-water partition coefficients (log $P_{O/W}$) measured for the four biocides are presented together with the experimentally observed half-life times for the hydrolytical stability ($t_{1/2\ hydro}$) of the isothiazol-3-ones tested (Table 1).

Table 1 CAS registry numbers, decadic logarithm of the measured octanol-water partition coefficients and derived half-life times (h) from the performed hydrolytical stability tests for the four isothiazol-3-one biocides. a = Value derived from the described HPLC method, b = Value obtained using the standardised OECD shake-flask method.

Substance	CASRN	log $P_{O/W}$	$t_{1/2hydrol.}$ (h)
N-methylisothiazol-3-one (MIT)	2682-20-4	-0.49 ± 0.01	>1000
5-chloro-N-methylisothiazol-3-one (CIT)	26172-55-4	0.53 ± 0.01	866 ± 22
N-octylisothiazol-3-one (OIT)	26530-20-1	3.30 ± 0.01	>1000
4,5-dichloro-N-octylisothiazol-3-one (DCOIT)	64359-81-5	4.79 ± 0.01[a] 4.68 ± 0.16[b]	115 ± 8

The octanol-water partition coefficients of the investigated isothiazol-3-ones nearly covered five orders of magnitude, ranging from -0.49 ± 0.01 for the non-chlorinated and methyl-substituted MIT up to 4.79 ± 0.01 for the double-chlorinated and octyl-substituted DCOIT. The high, HPLC-derived log $P_{O/W}$ for the DCOIT could be confirmed by using the shake flask method resulting in a log $P_{O/W}$ of 4.68 ± 0.16.

With regard to hydrolytical stability, the obtained data from the test at 37 °C in PBS buffer (pH 7.4) showed that the half-life times of all four biocides were > 4 days. Among the four isothiazol-3-ones the two chlorinated substances CIT and DCOIT exhibited the lowest half-life times.

Toxicity data

To derive EC_{50} values for the isothiazol-3-ones, concentration-response data were recorded for the particular endpoints in each of the three test systems. Subsequently, the decadic logarithm of the EC_{50} values was calculated from the corresponding regression fit using the best fit of sigmoidal probit- or logit-models. The experimental data and the fitted Concentration-response curves for each test system are presented showing the obtained raw data with their corresponding standard deviation (Figure 2).

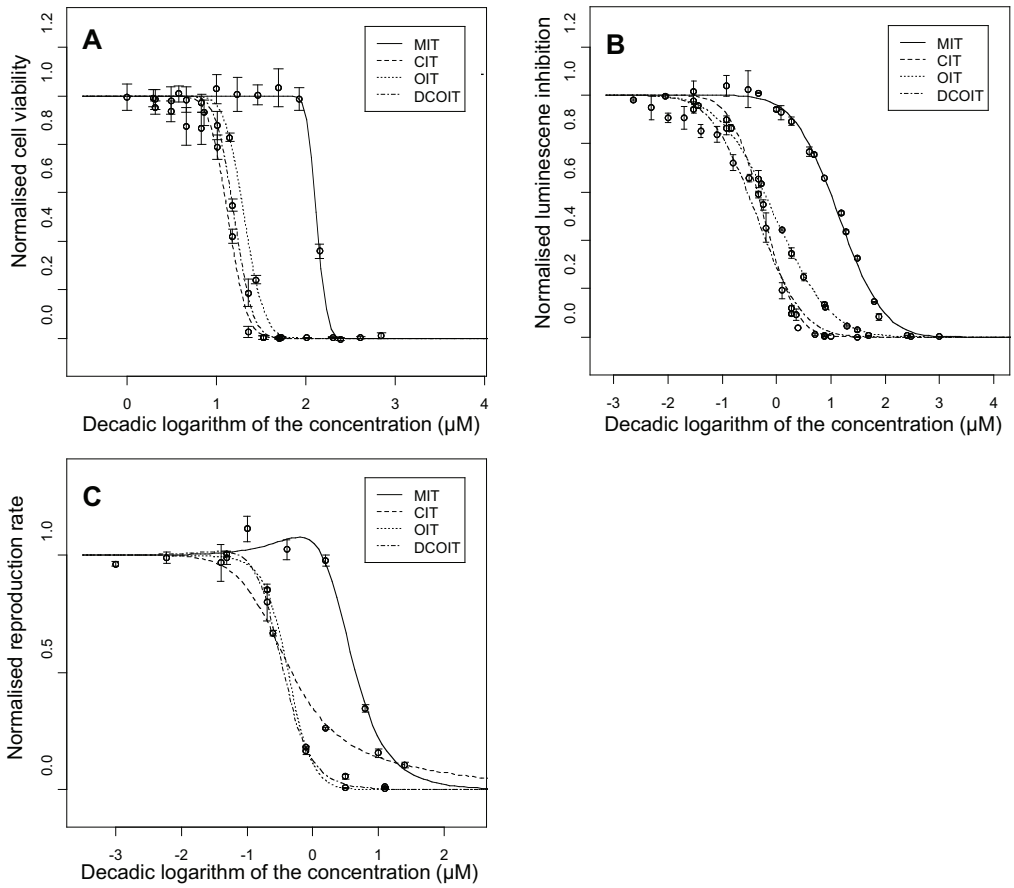

Figure 2 Concentration-response curves for each isothiazol-3-one obtained for the cell viability assay with Hep G2 cells (A), for the luminescence inhibition test with the marine bacterium *Vibrio fischeri* (B) and for the reproduction inhibition of the limnic green alga *Scenedesmus vacuolatus* (C). The data are presented as means of at least six independent experiments with error bars representing their corresponding standard deviation.

The corresponding calculated EC_{50} values in µM for the biocides obtained for each test system are given in Table 2.

Table 2 EC_{50} (µM) values derived from the measured concentration-response curves for each biocide in all three test systems. The 95% confidence intervals are given in parenthesis.

Substance	Hep G2 cells	Vibrio fischeri	Scenedesmus vacuolatus
	EC_{50} (µM)	**EC_{50} (µM)**	**EC_{50} (µM)**
MIT	132 (129 - 135)	14 (13 - 15)	4.5 (3.5 - 5.6)
CIT	13 (12.9 - 14)	0.6 (0.5 - 0.6)	0.5 (0.4 - 0.6)
OIT	20 (19.9 - 21)	0.9 (0.9 - 1.0)	0.4 (0.4 - 0.5)
DCOIT	16 (15.8 - 16.2)	0.43 (0.4 - 0.5)	0.38 (0.3 - 0.4)

Concentration-response curves demonstrate that on the whole, the four investigated isothiazol-3-one biocides showed similar patterns of toxic action in all of the three test systems (Figure 2). All four biocides exhibited high toxicities with EC_{50} values ranging from 132 µM for MIT in the Hep G2 cell viability assay to 0.4 µM for DCOIT in the reproduction assay with the limnic green alga *Scenedesmus vacuolatus* (Table 2). In all test systems, the non-chlorinated and hydrophilic MIT (log $P_{O/W}$ = -0.49 ± 0.01) was found

to be the least active compound exhibiting EC_{50} values, which were consistently one order of magnitude higher than those for the other biocides. The OIT (log $P_{O/W}$ = 3.3 ± 0.01) and the two chlorine substituted substances CIT and DCOIT all showed significantly lower EC_{50} values ranging either from 0.4 µM to 0.9 µM (*Vibrio fischeri* and *Scenedesmus vacuolatus*) or around 15 µM (Hep G2 cell culture), respectively.

Comparing the three test systems the Hep G2 cell viability tests exhibited the lowest sensitivity towards isothiazol-3-one activity demonstrated by EC_{50} values for all four biocides that were consistently one order of magnitude higher than effect concentrations derived from the *Vibrio fischeri* and algae tests. The effect concentrations derived from the bacteria and algae assays ranged within the same order of magnitude when comparing each single biocide. For MIT and OIT the limnic green alga was found to be more sensitive (lower EC_{50} values) than the marine bacteria whereas for both chlorinated substances no significant differences in the EC_{50} values could be identified between *V. fischeri* and the algae.

Apart from the isothiazol-3-ones, for the Hep G2 cell culture a series of homologues alkyl alcohols - representing narcotically acting or baseline toxicants - was measured in the cell viability assay and the log EC_{50} values were calculated from the corresponding concentration-response data (Table 3). For the luminescence inhibition assay with *Vibrio fischeri* and the algae reproduction test the decadic logarithm of the EC_{50} values for the alkylalcohols were taken from literature references basisd on the same assay conditions as used in the present study and they are also shown in Table 4 together with their literature values for log $P_{O/W}$. For all three test systems - within the series of alkylalcohols - a decrease in the EC_{50} values and thus an increase in toxicity could be identified with increasing chain length.

Furthermore, linear regressions were performed by correlating the decadic logarithm of the octanol-water partition coefficients with the decadic logarithm of the EC_{50} values for the alkylalcohols. For all three test systems a good correlation between lipophilicity (log $P_{O/W}$) and toxicity could be found with R^2 values for the linear regressions ranging from 0.9933 to 0.9974 (Table 3).

Table 3: QSAR equations describing the baseline toxicity of a series of alkyl alcohols in each of the three test systems are presented together with the corresponding R^2 values for each regression. The corresponding graphs are shown in Figure 3.

Test system	Linear regression for baseline toxicity	R^2
Hep G2 cell culture (cell viability)	log EC_{50} (µM) = -0.829 X log $P_{O/W}$ + 5.396	0.9933
Vibrio fischeri (luminescence inhibition)	log EC_{50} (µM) = -1.252 X log $P_{O/W}$ + 5.392	0.9935
Scenedesmus vacuolatus (reproduction)	log EC_{50} (µM) = -0.866 X log $P_{O/W}$ + 5.197	0.9974

The slopes and the intercepts of the obtained equations revealed similar baseline toxicity QSARs for the human Hep G2 cell line and the limnic green alga, whereas the

marine bacterium *Vibrio fischeri* showing a steeper regression line was found to be slightly more sensitive towards the baseline toxicants (Figure 3).

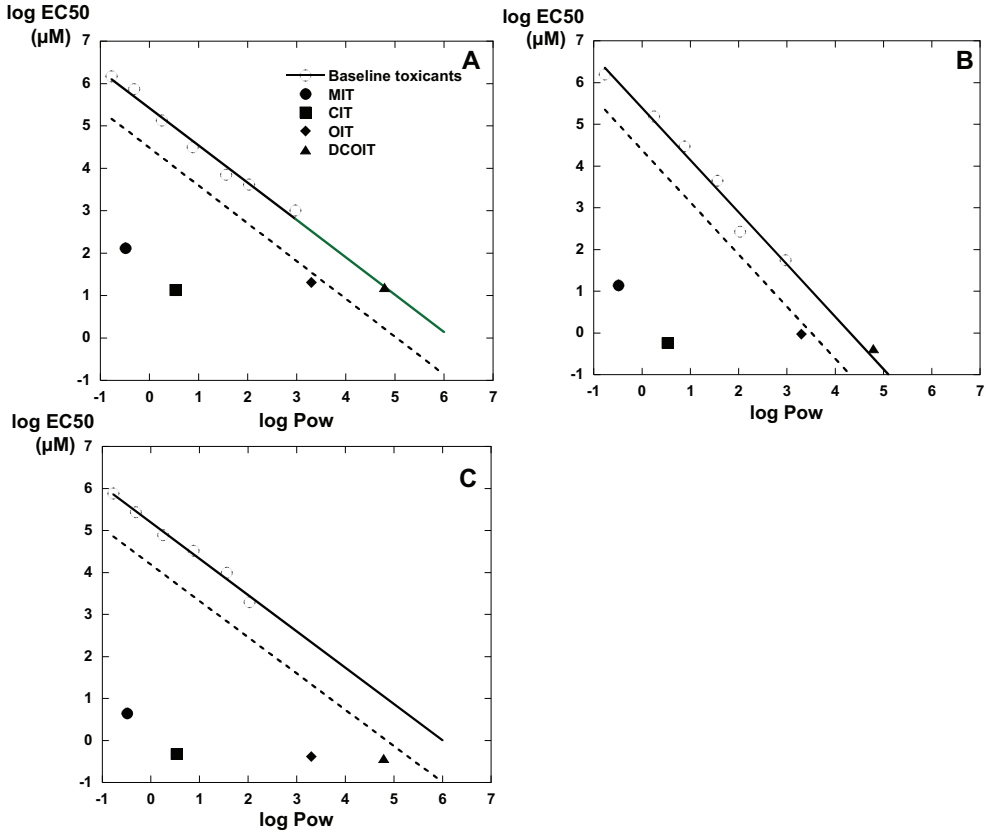

Figure 3 Toxic ratio illustration for each biocide measured with Hep G2 cells (A), *Vibrio fischeri* (B) and *Scenedesmus vacuolatus* (C). The dashed line represents the TR=10 benchmark and the solid regression refers to the series of alkyl alcohols taken as baseline toxic reference substances. The regression parameters for the derived baslinetoxicity QSAR equations for each test system and the corresponding R^2 are given in Table 3.

The toxic ratios (TR) for the isothiazol-3-one biocides for each test system were calculated due to the following equation:

$$TR = \frac{EC_{50bl.}}{EC_{50}}$$

With $EC_{50bl.}$ (µM) representing a calculated value derived from the previously obtained QSARs and describing the expected baseline toxicity of the biocides. In contrast, the EC_{50} (µM) in the denominator is the calculated effect concentration derived from the measured concentration-response data. Thus, the resulting ratio can be used to identify excess toxicities of the test chemicals in addition to the narcotic potential of every single substance. This excess toxicity is reflected graphically by the distance of the measured log EC50 values of the isothiazol-3-ones from the linear regression lines obtained for the baseline toxicants in each of the three test systems (Figure 3). A high

excess toxicity of the isothiazol-3-ones can readily be identified by their distance from the dashed line (Figure 3) representing the chosen benchmark for TR = 10. In the following – according to Maeder *et al.*[15] - we term substances with a TR ≥ 10 to exert excess toxicities. The log EC_{50bl}. values, the corresponding EC_{50bl}. data and the calculated TRs for the four isothiazol-3-ones in each test system are presented in Table 4.

Table 4 Decadic logarithm of the octanol-water partition coefficients (log $P_{O/W}$), measured EC_{50} (µM) values, calculated baslinetoxicity (EC_{50bl}. in µM) and the derived toxic ratio (TR) for the four isothiazol-3-ones in each of the three test systems. Additionally, the log $P_{O/W}$ values and the measured (Hep G2) or literature values for the EC_{50} (µM) of a series of narcotic acting alkyl alcohols are presented. **a** Value taken from Hansch *et al.* (1995), **b** Values taken from Russom *et al.* (1997), **c** Values taken from Kaiser and Palabrica (1991) **d** Values taken from Walter (2002)

Substance	Log $P_{O/W}$	Hep G2		
		EC_{50} (µM)	$EC_{50bl.}$ (µM)	TR
MIT	-0.49	132	634,978	4,817
CIT	0.53	13	90,589	6,715
OIT	3.30	20	458	22
DCOIT	4.79	16	27	2
Methanol	-0.77[a]	1,479,108		
Ethanol	-0.31[b]	741,310		
1-Propanol	0.25[b]	134,896		
1-Butanol	0.88[b]	31,623		
1-Pentanol	1.56[b]	7,079		
1-Hexanol	2.03[b]	4,169		
1-Octanol	2.97[b]	1,023		
Substance	**Log $P_{O/W}$**	***Vibrio fischeri***		
		EC_{50} (µM)	**$EC_{50bl.}$ (µM)**	**TR**
MIT	-0.49	13.8	1,012,236	73,330
CIT	0.53	0.6	53,511	92,992
OIT	3.30	0.9	18	20
DCOIT	4.79	0.4	0.2	1
Methanol	-0.77[a]	1,584,893[c]		
Ethanol	-0.31[b]	-		
1-Propanol	0.25[b]	154,882[c]		
1-Butanol	0.88[b]	29,512[c]		
1-Pentanol	1.56[b]	4,467[c]		
1-Hexanol	2.03[b]	269[c]		
1-Octanol	2.97[b]	56[c]		
Substance	**Log $P_{O/W}$**	***Scenedesmus vacuolatus***		
		EC_{50} (µM)	**$EC_{50bl.}$ (µM)**	**TR**
MIT	-0.49	4.5	418,016	93,582
CIT	0.53	0.5	54,724	114,335
OIT	3.30	0.4	219	525
DCOIT	4.79	0.4	11	30
Methanol	-0.77[a]	765,000[d]		
Ethanol	-0.31[b]	276,000[d]		
1-Propanol	0.25[b]	77,000[d]		
1-Butanol	0.88[b]	33,000[d]		
1-Pentanol	1.56[b]	10,000[d]		
1-Hexanol	2.03[b]	2,000[d]		
1-Octanol	2.97[b]	-		

Taking the TR as a measure to rank the excess toxicity of a chemical entity, it could be shown that the chlorinated CIT exhibited the highest excess toxicities (TR ranging from 6,000 to 114,000) in all three test systems. Also the non-chlorinated and hydrophilic MIT, which showed high excess toxicities (TR from 5,000 to 9,000). For the hydrophobic OIT still moderate excess toxicities (TR between 20 and 500) could be observed in all three assays, whereas for the highly hydrophobic DCOIT only in the reproduction test with *Scenedesmus vacuolatus* an excess toxicity (TR > 10) was found. For the Hep G2 culture and the marine bacterium the TRs for DCOIT were found to be in the same order of magnitude, as it would be expected for non-reactive baseline toxicants.

Comparing the three test systems on the basis of the obtained TRs, again it could be shown that the algae reproduction test was the most sensitive assay showing consistently the highest TR values for all isothiazol-3-ones tested. The *Vibrio fischeri* luminescence inhibition assay exhibited a higher sensitivity than the Hep G2 culture, which represented again the least sensitive test system.

Discussion

It was the aim of this study to analyse the toxic impact of four differently substituted isothiazol-3-one biocides on three different cellular test systems (human liver cell line, marine bacterium and limnic green alga) out of an (eco)toxicological test battery. In the following sections the obtained physicochemical data for the test chemicals, the T-SAR concept and the toxic-ratio approach are used to link the observed toxicities to substructural elements of the biocides and to identify possible modes of toxic action.

Physicochemical data

When dealing with electrophilic organic chemicals - like isothiazol-3-ones - in aqueous test media a problem arises owing to a significant hydrolysis of the test compounds during long incubation times (24-48 h). This can result in falsely high effect concentrations entailing an underestimation of the hazard potential of the corresponding substances[21]. Therefore, after purification of the four biocides, we performed the described standardised hydrolytical stability test. The obtained results showed that even for long incubation times - *i.e.* 48 h in the Hep G2 assay - all four isothiazol-3-ones were stable at 37 °C in PBS buffer (pH 7.4) with half-life times exceeding at least twice the incubation times. Hence, an underestimation of the cytotoxicity due to hydrolytic degradation of the biocides could be neglected. This fact is noteworthy, because the Hep G2 viability assay was performed at 37 °C, whereas the luminescence inhibition assay (15 °C) and the algae reproduction test (28 °C) were conducted at lower temperatures.

An accurately determined octanol-water partition coefficient of a chemical entity is an essential parameter in the field of toxicology and ecotoxicology. It is commonly used to describe the hydrophobicity of a compound and to estimate bioconcentration factors

(BCFs) of chemical substances[22]. In particular, the octanol-water partitioning is of high concern in the hazard assessment of chemicals since it governs bioavailabilities and internal effect concentrations[23]. By using the described HPLC-based method we obtained comparable log $P_{O/W}$ values for the set of isothiazol-3-ones tested. As expected from the T-SAR analysis the small and polar MIT molecule was found to be hydrophilic with a log $P_{O/W}$ of -0.49. The introduction of the hydrophobic chlorine substituent yielded an one order of magnitude increase in the hydrophobicity for CIT (log $P_{O/W}$ = 0.53). Additionally, the lipophilic octyl side chain increased the log $P_{O/W}$ as it could be shown by comparing MIT and OIT (log $P_{O/W}$ = 3.3). Finally, the double-chlorinated and octyl-substituted analogue DCOIT (log $P_{O/W}$ = 4.79) exhibited the highest hydrophobicity of the four isothiazol-3-ones.

In this study for the lipophilic OIT and DCOIT we found higher log $P_{O/W}$ values than literature references, which report for OIT a log $P_{O/W}$ of 2.5[24] and for DCOIT a value of log $P_{O/W}$ = 2.8[7]. The measured values for MIT and CIT are in good accordance with literature data of log $P_{O/W}$ = -0.5 for MIT and 0.4 for CIT[7], respectively.

Especially for DCOIT this difference between the published value and the log $P_{O/W}$ obtained in this study is enormous. Therefore, we confirmed the high log $P_{O/W}$ for DCOIT using a standardised shake flask method as a second experimental reference.

However, especially this high log $P_{O/W}$ value for DCOIT has (eco)toxicological consequences since chemicals exhibiting a log $P_{O/W} \geq 3$ are termed to be bioaccumulative and exhibit high BCFs[25]. Even if bioaccumulation in this case is of minor relevance owing to the high reactivity of the isothiazol-3-ones, interactions of these compounds with biological membranes and an elevated bioavailability at the intracellular target sites should be taken into higher consideration when assessing the hazard potential of OIT and DCOIT.

Toxicity data

The high biocidal activity of the isothiazol-3-ones is assumed to be mediated via the electrophilic reaction of these substances with essential intracellular thiol groups[9]. Especially the reaction of the isothiazol-3-ones with glutathione (GSH) can lead to a rapid breakdown of the cellular reduction potential, accompanied by apoptotic or necrotic cell death[13,14]. With respect to this, we could recently show that DCOIT and CIT react readily with GSH. Additionally, we found that the incubation of Hep G2 cells with both chlorinated isothiazol-3-one species leads to a drastic depletion of intracellular GSH and a concomitant increase in the amount of oxidised glutathione disulphide (GSSG). These chlorinated biocides were also found to be strong inhibitors of cellular glutathione reductase - one of the most important enzymes maintaining the cellular reduction potential - in Hep G2 cells[18]. Thus, GSH and subsequently the cellular reduction potential seem to be the main molecular targets for the toxic mode of action of the isothiazol-3-ones tested.

Structure-activity relationships and modes of toxic action

The observed strong influence of the chlorine substituents on the reactivity of CIT and DCOIT can be explained by quantum chemical calculations predicting an increased positive polarisation of the aromatic sulphur atom in the chlorinated isothia-zol-3-one ring systems[26] compared to non-chlorinated analogues. Furthermore, a chlorine substituent at the 5 ring-position (see Figure 1) enables the formation of a highly reactive thioacylchloride intermediate in the reaction pathway of DCOIT and CIT with thiol containing agents[27]. Hence, for isothiazol-3-one biocides a similar and specific mode of toxic action can be assumed, which is most likely mediated by the interaction of these substances with cellular GSH. Furthermore, it was expected from the T-SAR analysis that the different substitution pattern at the isothiazol-3-ones modulates this reactivity and thereby the toxicity of the corresponding substances.

Comparing toxicities between different species and different testing endpoints, it is important to consider that two general modes of toxic action of chemicals can be distinguished. Firstly, a large number of toxicants target highly conserved cellular structures and functions (*e.g.* biological membranes, cellular nucleophils like GSH, DNA or the cellular energy metabolism), which are universally present in nearly all organisms and tissues[23]. Thus, knowing these general cellular target sites allows for universally applicable predictive models. This assumption forms the basis of the so called "basal cytotoxicity concept" introduced by Ekwall *et al.*[28].

Secondly, apart from these basal targets, some chemicals can act via mechanisms that are specific for certain organisms or tissues. Among these mechanisms are *e.g.* the interactions of compounds with the photosynthesis system or the binding to specific receptor proteins that are solely expressed in certain tissues. Such highly specific toxic effects especially at the organism level are difficult to predict and can only be screened in special complex test systems. Thus, for a rapid and general hazard assessment of chemicals - as it is required under REACH - it is important to identify basal cellular molecular targets of toxicants. In this context, the obtained toxicity data for the human liver cell line, the marine bacterium and the limnic green alga are discussed in the following.

Cytotoxicity of the isothiazol-3-one biocides

Compared to other electrophilic toxicants, the isothiazol-3-ones exhibited in all three test systems a high toxicity. For example, for the most sensitive test system - the growth recovery assay with the green alga *Scenedesmus vacuolatus* - Niederer *et al.*[21] reported EC_{50} values of 340 μM and 500 μM for the strong electrophiles di-chloro-2-butene and benzyl chloride, respectively. In contrast, the EC_{50} values found for each of the four biocides (4.5 - 0.4 μM) were two to three orders of magnitude lower. As it was expected from the above-described structure-activity relationships, the non-chlorinated and hydrophilic MIT consistently exhibited the lowest toxicity. The chlorinated CIT and DCOIT species showed the highest toxicities with corresponding low effect concentrations in each of the three test systems. The hydrophobic but non-chlorinated

OIT exhibited toxicities shortly above or in the range of CIT and DCOIT. The obtained data for the aquatic test systems (bacteria and green algae) for DCOIT are in well accordance with previously published EC_{50} values ranging from 0.01 μM to 1.2 μM[29,30] for the 30 minutes *Vibrio fischeri* luminescence inhibition assay and an EC_{50} of 0.31 μM[31] for the green alga *Scenedesmus vacuolatus*. For the remaining three substances to our knowledge no toxicity data are reported for the test systems used.

Since for all three cellular test systems endpoints reflecting the general cell viability were measured, the consistent toxicity pattern of the isothiazol-3-ones is most likely due to the same basal mode of toxic action - *i.e.* the depletion of GSH and the breakdown of the cellular reduction potential. This hypothesis is further supported by the fact that even in the short luminescence inhibition assay (30 min), the isothiazol-3-ones exhibited drastic effects. With respect to this we could recently show that GSH depletion in Hep G2 cells reaches maximal effects after a 15 minutes incubation with CIT and DCOIT[18].

However, the measured EC_{50} values represent external aqueous effect concentrations. This makes it difficult to identify the intrinsic toxicity of the isothiazol-3-ones, since the hydrophobicity governed effects (uptake and baseline toxicity) cannot be seperated from reactivity mediated effects like GSH depletion. To approach this problem, internal effect concentrations – *i.e.* the concentrations present at the molecular target sites - can be used. Therefore, we used the toxic ratio (TR) concept to analyse the toxicity mechanisms of the isothiazol-3-ones in more detail and to compare the three different test systems. It could be shown by Maeder *et al.*[17] that the TR reflects internal effect concentrations and thus can be used to identify the intrinsic or excess toxicities of chemicals.

To identify these excess toxicities of the four isothiazol-3-ones, for each of the three test systems QSARs describing the baseline toxicity were derived. The obtained linear regressions for the log EC_{50} versus the log $P_{O/W}$ of a series of alkyl alcohols showed nearly the same slopes and intercepts in all test systems used. This phenomenon is typical for baseline toxicants since their internal effect concentrations in the lipidpools of cells or organisms are similar and largely independent of test protocols and incubation times[32]. Taking now the TRs of the isothiazol-3-ones to identify the excess toxicities of these substances, the most striking results were found for the DCOIT. Only for the green alga *Scenedesmus vacuolatus* a TR > 10 was observed, whereas for both remaining test systems the effect of DCOIT was in the range for a baseline toxicant (TR < 10). However, from the T-SAR analysis we predicted a high excess toxicity of DCOIT owing to the chlorine substituents and to the found dramatic cellular GSH depletion in Hep G2 cells[18]. For MIT and CIT the extremely high TRs indicated for high intrinsic toxicities. Here the T-SAR analysis could be confirmed, because the chlorinated CIT exhibited significantly higher TRs in each test system than the non-chlorinated MIT. In contrast to the pure effect concentrations, the TRs revealed that MIT and CIT exhibited the highest specific toxicity, whereas DCOIT - showing the lowest EC_{50} values - was identified to act as baseline toxicant. The same tendency was found for the hydrophobic but non-chlorinated OIT where the TRs were one to three orders of magnitude lower compared to the hydrophilic and also non-chlorinated MIT.

Hence, the here presented results show that for highly hydrophobic substances narcosis can mask further and more specific modes of toxic action. From this it might be concluded that for highly hydrophobic substances the QSARs for baseline toxicity are sufficient to predict the compound´s effects. But this conclusion only holds true for acute effects. For chronic or long term effects, especially the specific reactivity of a compound is of high relevance because even subtoxic internal concentrations can alter cellular processes via the interaction with essential biomolecules like GSH or DNA.

Comparing the three test systems at the TR basis, again the same pattern for the four isothiazol-3-ones was found. The green algae assay was found to be the most sensitive test system followed by the marine bacteria and the Hep G2 cell culture as the least sensitive species. But now the observed differences in sensitivity can be interpreted separately for MIT and CIT compared to OIT and DCOIT. The high TRs for MIT and CIT indicate that the toxicity of these two substances is most likely dominated by their specific reactivity with cellular thiol-containing targets, whereas for OIT and DCOIT baseline toxicity seems to be the dominating mode of toxic action. This assumption explains the small differences observed in the TRs of OIT and DCOIT between the three test systems. Only the reproduction inhibition test with the green alga exhibited relatively high TR values for OIT and DCOIT. In contrast, for MIT and CIT - where we assumed a high excess toxicity - the differences in sensitivity between the three test systems are larger than expected owing to *e.g.* differences in cellular GSH contents or variations in the activity of glutathione-S-transferases (GSTs) - an enzyme family that catalyses the reaction of a broad spectrum of electrophilic xenobiotics with GSH[33]. For example, for green algae it is known that they exhibit significantly higher GST activities than bacteria, which generally lack significant GST activities[34,35]. Thus, the observed high sensitivity of the green alga compared to the *Vibrio fischeri* assay can be explained by an enhanced enzyme catalysed reaction rate of the isothiazol-3-ones with GSH. Since Hep G2 cells represent metabolically active liver cells, they are generally well equipped with defence mechanisms to handle reactive xenobiotics. Hence, the observed low sensitivity of this test system towards the isothiazol-3-ones can be explained by these well developed defence mechanisms, the algae and prokaryotic bacteria are lacking.

CONCLUSION

Using three cellular test systems out of a flexible (eco)toxicological test battery, we could show for the first time that the four commonly used isothiazol-3-ones biocides MIT, CIT, OIT and DCOIT exhibited high acute cytotoxicities in each test system compared to other electrophilic toxicants.

Additionally, with a standardised HPLC method we could derive comparable log $P_{O/W}$ values for all tested iosthiazol-3-ones and thereby we were able to demonstrate that the hydrophobicities of OIT and DCOIT were so far underestimated entailing drastic consequences for the bioavailability, the acute cytotoxicity and the TR analysis of these two substances.

The T-SAR analysis of the expected reactivities of the substances due to a different substitution pattern at the isothiazol-3-one core structure could consistently be confirmed in each of the three test systems. The chlorinated biocides exhibited a significantly higher toxicity than their non-chlorinated analogues. Additionally, we could confirm the expected influence of the hydrophobicity on the toxicity, because the EC_{50} values decreased in each test system with increasing hydrophobicity of the substances. The detailed analysis of the possible modes of toxic action using the TR approach revealed that for the less lipophilic MIT and CIT substances the intrinsic reactivity towards cellular thiols is likely to be the predominant mode of toxic action, whereas for the lipophilic OIT and DCOIT baseline toxicity seems to dominate the observed effects.

In contrast, at least for DCOIT we could recently show that it exerts drastic effects on the cellular reduction potential in Hep G2 cells by rapidly depleting cellular GSH pools. Hence, taking the isothiazol-3-ones as an example for reactive electrophilic toxicants, we could show that for highly hydrophobic substances baseline toxicity may mask intrinsic and specific toxicities of a chemical entity. This is important to remember when assessing the hazard potential of hydrophobic substances, since their specific reactivities may contribute to long term effects. These effects are not covered when solely applying hydrophobicity based QSARs to predict a compound´s toxicity. Therefore, for an accurate hazard assessment of chemicals we suggest to combine the TR analysis with the T-SAR approach representing an useful tool to identify molecular substructures, which may exert toxic effects to basal cellular structures or functions.

EXPERIMENTAL

Chemicals

The four isothiazol-3-one biocides N-methylisothiazol-3-one (MIT), 5-chloro-N-methylisothiazol-3-one (CIT), N-octylisothiazol-3-one (OIT) and 4,5-dichloro-N-octylisothiazol-3-one (DCOIT) were purchased from the Rohm&Haas Ltd. (Coventry, UK). The obtained KATHON^{TM}, mixture of 5-chloro-N-methylisothiazol-3-one and N-methylisothiazol-3-one was separated and purified using the chromatographic method described below. Before use all biocides were recrystallised from a methanolic solution and the purity was determined to be > 99.8 % by HPLC and NMR analysis. Stock solutions (1 mM and 10 mM) of every single biocide in methanol were prepared freshly every day.

The RPMI 16/40 medium, fetal calf serum, and phosphate buffered saline (PBS) were purchased from GIBCO BRL Life Technologies (Eggenstein, Germany). Penicillin/streptomycin and L-glutamine as well as $NaHCO_3$ were obtained from PAA Laboratories (Cölbe, Germany).

All further chemicals were either be provided by the SIGMA-Aldrich Cooperation (Deisenhofen, Germany) or by the Merck KgaA (Darmstadt, Germany).

Separation and purification of the isothiazol-3-ones

Since for the structure-activity analysis all substances need to be tested as single and purified chemical entities the purchased KATHON™, mixture of 5-chloro-N-methylisothiazol-3-one (CIT) and N-methylisothiazol-3-one (MIT) needed to be separated and purified from stabilisers and co-solvents like polyethylene glycols. The following chromatographic method was developed to obtain the pure substances.

5 g of the aqueous solution of CIT and MIT was loaded on a preparative chromatographic column (50 mm ∅) filled with 100 g silica gel (size of the beads 10 μM). The analytes were eluted using a mobile phase consisting of CH_2Cl_2 and methanol in a 15:1 ratio. Fractions of 2 mL sample size were collected and the purity of each sample was checked via thin layer chromatography. Subsequently, the pure fractions of CIT and MIT were pooled and the mobile phase was evaporated under vacuum. The remaining crystals were redissolved at 40 °C in a small amount of CH_2Cl_2 and then recrystallised at room temperature over night in a hexane saturated atmosphere. Subsequently, the solvent was evaporated under vacuum and the obtained pure crystals of each substance were packed under an argon atmosphere and stored in the dark at 4 °C.

Cell culture

The Hep G2 cell line was cultured in 10 mL flasks in culture medium consisting of 90 % RPMI 16/40 medium and 10 % fetal calf serum supplemented with 2 mg/mL $NaHCO_3$, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2 and the culture medium was replaced every second day. The cells were trypsinated and seeded to new flasks when reaching 70 % - 80 % confluency.

For all cytotoxicity experiments viable cells were seeded at a density of 125,000 cells per well in 96-well tissue culture plates and incubated in culture medium for 48 h (at 37 °C and 5 % CO_2) to allow the cells to attach to the bottom of the wells.

Cell viability assay

The cytotoxicity assay using the WST-1 reagent was described in detail in Ranke *et al.*[36].

Briefly, human hepatocarcinoma cells from the Hep G2 cell line[37] were incubated in 96-well microtiter plates for 48 h (at 37 °C and 5 % CO_2) with a 1:1 dilution series of either the isothiazol-3-ones in culture medium (methanol content < 1 %). The concentration of the biocides ranged from 700 μM – 10 μM for MIT, from 200 μM – 1 μM for OIT and from 50 μM – 2 μM for CIT and DCOIT, respectively. Additionally, a series of alkylalcohols (methanol to octanol) and 2,4,5-trichlorotoluene were tested as baseline toxicants in a concentration range from 2000 mM to 1 μM. At the end of the 48 h period the cultures were incubated for 4 h with the tetrazolium dye 2-(4-iodophenyl)-3-(4-nitrophenyl)- 5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) and the change in the absorbance at 630 nm and 450 nm was measured

photometrically to monitor the remaining cell viability. Each plate contained blanks (no cells) and untreated controls. Each dose response curve was recorded for at least 9 parallel dilution series on three independent 96-well plates. The cell viability was expressed as percentage by relating the activity of the biocide-treated cells to the untreated controls. Positive controls using Carbendazim as a strong toxicant were checked in regular intervals to validate the used protocol.

Vibrio fischeri luminescence inhibition assay

The bioluminescence inhibition assay using the marine bacterium *Vibrio fischeri* is a highly standardised and widely used bioassay to monitor the aquatic toxicity of chemicals. The test was performed according to the DIN EN ISO 11348-2 guideline. The freeze-dried bacteria were purchased from Dr. Lange GmbH (Düsseldorf, Germany). All tests were carried out at least in triplicates for each substance. First a range-finding experiment was performed for each of the tested biocides with two replicates per dilution series. The obtained results were subsequently validated within a second concentration-response test series using three replicates for each concentration and substance. For the four isothiazol-3-ones the range finding yielded in the following concentration ranges for the concentration-response tests: MIT 300 µM – 0.03 µM, CIT 30 µM – 0.0023 µM, OIT 50 µM – 0.03 µM and DCOIT 10 µM – 0.005 µM. Within each test at least 4 controls (2 % NaCl solution, phosphate buffer 20 mM, pH 7.0) were used. To exclude pH-effects all selected isothiazol-3-ones were prepared as phosphate-buffered solutions (20 mM, pH 7.0, including 2 % sodium chloride) and the methanol content was < 1 %, which did not exhibit any effect on the assay conditions (data not shown). The tests were performed at 15 °C using thermostats (LUMIStherm, Dr. Lange GmbH, Düsseldorf, Germany). The luminescence was measured with a luminometer (LUMIStox 300, Dr. Lange GmbH, Düsseldorf, Germany).

The freeze-dried bacteria were re-hydrated according to the test protocol, then 500 mL aliquots of the bacteria solution were pre-incubated for 15 minutes at 15 °C. After measuring the initial luminescence 500 mL of the diluted samples were added to the pre-aliquoted bacteria. The bioluminescence was measured again after an incubation time of 30 minutes. The relative toxicity of the samples was expressed as percentage inhibition compared to the controls.

Reproduction inhibition assay with *Scenedesmus vacuolatus*

For this assay the unicellular limnic green algae *Scenedesmus vacuolatus*[38] (strain 211-15, SAG Culture Collection of Algae, Universität Göttingen, Göttingen, Ger-many) was used. All toxicity assays were performed using synchronised cultures. The stock culture was grown under photoautotrophical conditions at 28 °C (± 0.5 °C) in an inorganic, sterilized medium (pH 6.4) with saturating white light (intensity of 22 to 33 kilolux, Lumilux Daylight L 36 W-11 and Lumilux Interna L 36 W-41, Osram, Berlin, Germany). Cells were aerated with 1.5 Vol % CO_2 and synchronised by using a 14 h light and 10 h darkness cycle. The stock culture was diluted every day to a cell density of 5 X 105 cells/mL.

The used test protocol is a modified version of the assay described in Altenburger *et al.*[39] and the sensitivity is comparable to the standardised 72-h test (ISO, 1989).

The toxicity tests started with autospores (young algal cells at the beginning of growth cycle). Algae were exposed to the test substances for one growth cycle (24 h). Endpoint of this assay is inhibition of algal reproduction measured as inhibition of population growth. All cell numbers (stock culture and test tubes) were determined using a Coulter Counter Z2 (Beckmann, Nürnberg, Germany). The tests were performed in sterilized glass tubes (20 mL Pyrex tubes sealed with caps containing a gas tight Teflon membrane), algae were stirred over the whole test period of 24 h and the test conditions were the same as for the stock culture except for the CO_2 source. Here 150 ml of $NaHCO_3$ solution was added to each test tube. The methods for stock culturing and testing are described in detail in Faust *et al.*[38].

Laboratory facilities allowed parallel testing of up to 60 tubes. All substances were tested at least in duplicates: first a range finding was undertaken (4 concentrations, two replicates) and in a second test the results were verified with 8 concentrations per substance in two replicates. The range-finding identified the following optimised test concentration ranges of the isothiazol-3-ones: MIT 100 μM - 0.1 μM, CIT 10 μM - 0.01 μM and 50 μM - 0.05 μM for OIT and DCOIT. The growth inhibition was calculated using the cell counts of the treated samples in relation to the untreated controls (pure medium). For each assay at least six controls were used.

Determination of the octanol-water partition coefficient

The decadic logarithm of the octanol-water partition coefficient (log $P_{O/W}$) of the four isothiazol-3-ones was determined according to the OECD guideline No. 117 for a HPLC based method. This method using lipophilic stationary phases (RP-8 or RP-18) was developed to determine a broad range of the octanol-water partitioning (log $P_{O/W}$ from 0 to 6) of different chemical substances. Briefly, the measurements were performed on a Hewlett Packard HPLC system Series 1100, with gradient pump, online degasser, autosampler and a variable wavelength UV detector. The column used was a an endcapped LiChro Cart® 125-4 Purospher® Star RP-8 column with 5 μm particle size (Merck KgaA, Darmstadt, Germany). All measurements were performed at a column temperature of 25 °C. The hold-up time of the chromatographic system at a flow rate of 1 mL/min. was calculated from the isocratically measured total retention times of a series of homologues alkylketones (propan-2-one to octan-2-one) according to the method of Berendsen *et al.*[40]. For the isocratic determination of the log $P_{O/W}$ values of CIT, OIT and DCOIT a mobile phase consisting of 60 % methanol and 40 % water and a flow rate of 1 mL/min. was used. As reference set for the linear regression a mixture of propan-2-one, butan-2-one, pentan-2-one, hexan-2-one, heptan-2-one, pyridine, toluene, butylbenzene, 1,2-dichlorobenzene and triphenylamine was analysed under the same conditions.

Due to the expected low log $P_{O/W}$ of MIT, for this substance the mobile phase was changed to 30 % methanol and 70 % water. Additionally, the reference substances for

the linear regression were adopted to include low lipophilicities. Thus, the reference mixture for MIT was composed of adenosine, propan-2-one, butan-2-one, pentan-2-one, hexan-2-one and pyridine.

The literature values of the log $P_{O/W}$ of all reference substances are given in Table 5. To validate the method benzophenone and aniline were used as internal standards and all measurements were at least performed in triplicates.

To verify the HPLC-derived log $P_{O/W}$ for DCOIT, additionally the octanol-water parti-tioning of this substance was determined using the shake flask method according to OECD guideline No. 117. The chromatographic system to analyse the remaining fractions of DCOIT in both phases (octanol and water) was the same as described above. As column now a Lichrosorb®, RP-18 (Merck KgaA, Darmstadt, Germany) with 7 µM particle size was used. The analysis was performed at a flow rate of 1 mL/min using a mobile phase composed of 42 % methanol, 18 % acetonitrile and 40 % water. The log $P_{O/W}$ was then calculated from three independent experiments.

Table 5 HPLC reference substances for the determination of the log $P_{O/W}$ values of the isothiazol-3-ones. **a** Values taken from Hansch *et al.* (1995), **b** Values taken from Collander *et al.* (1951), **c** Values taken from Hansch and Anderson (1967), **d** Values taken from Iwasa *et al.* (1965), **e** Values taken from Tewari *et al.* (1982), **f** Values taken from de Brujin *et al.* (1989)

Substance	CASRN	Log $P_{O/W}$
Adenosine	58-61-7	-1.05[a]
Propan-2-one	67-64-1	-0.24[b]
Butan-2-one	78-93-3	0.29[c]
Pyridine	110-86-1	0.65[d]
Aniline	62-53-3	0.90[a]
Pentan-2-one	107-87-9	0.91[a]
Hexan-2-one	591-78-6	1.38[d]
Heptan-2-one	110-43-0	1.98[e]
Toluene	108-88-3	2.73[a]
Benzophenone	119-61-9	3.18[a]
1,2-Dichlorobenzene	95-50-1	3.43[f]
n-Butylbenzene	104-51-8	4.38[f]
Triphenylamine	603-34-9	5.74[a]

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Hydrolytical stability of the isothiazol-3-ones

To analyse the hydrolytical stability of the four biocides during the longest incubation time of 48 h (cell viability assay) a standardised degradation test was performed according to OECD guideline 111. Briefly, hydrolysis rate constants and half-life times at 37 °C were calculated measuring the degradation kinetics for each of the compounds in PBS (20 mM, pH 7.4) at 20 °C, 50 °C and 70 °C using the HPLC methods described above.

Statistical analysis

The normalised (0 to 100 % effect compared to the controls) concentration response curves for the Hep G2 cytotoxicity experiments, for the luminescence inhibition assay and for the green algae growth inhibition tests were fitted to the data with the statistical software environment R (R core team, 200X) using the probit or linlogit model for the relation of the observed effect to the decadic logarithm of the tested concentrations[41]. Confidence intervals (a = 0.05) of the estimated EC_{50} values were calculated with the R language as well as the parameters for the linear regression for the determination of the octanol-water partition coefficient of the four isothiazol-3-ones. The linear regressions and the R^2 values for the QSARs describing the baseline-toxicity in the three test systems were also performed with the R language.

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PAPER NO. 2:

STRUCTURE-ACTIVITY RELATIONSHIPS FOR THE IMPACT OF SELECTED ISOTHIAZOL-3-ONE BIOCIDES ON GLUTATHIONE METABOLISM AND GLUTATHIONE REDUCTASE OF THE HUMAN LIVER CELL LINE HEP G2

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Abstract

To investigate the toxic mode of action of isothiazol-3-one biocides the four compounds N-methylisothiazol-3-one (MIT), 5-chloro-N-methylisothiazol-3-one (CIT), N-octylisothiazol-3-one (OIT) and 4,5-dichloro-N-octylisothiazol-3-one (DCOIT) were purified and tested as single chemical entities for their effects on the human hepatoblastoma cell line Hep G2 and on isolated and cellular glutathione reductase GR). The two chlorinated substances CIT and DCOIT significantly decreased the amount of total cellular glutathione (GSx) in a dose and time dependent manner. Concomitantly, an increase in the level of oxidised glutathione (GSSG) was observed. The resulting shift in the GSH/GSSG ratio entailing the breakdown of the cellular thiol reduction potential was accompanied by necrotic morphological changes like swelling of the plasma membrane and subsequent lysis of the cells. Additionally, CIT and DCOIT were found to inhibit cellular GR in the cells in a concentration dependent manner. The T-SAR-based (thinking in terms of structure-activity relationships) comparison of the chlorine-substituted structures CIT and DCOIT with their non-chlorinated and less active analogues MIT and OIT identified the chlorine substituents and the resulting reaction mechanisms to be the key structural mediators of the observed toxic effects. Furthermore, differences in the activity of both chlorinated substances could be explained using the T-SAR approach to link the hydrophobicity and the intrinsic glutathione-reactivity of the compounds to the expected target site concentrations inside the cells.

Introduction

Especially since the new EU legislation for the registration, evaluation authorisation and restriction of chemical substances (REACH) was put into force in June 2006 efficient testing strategies in the field of toxicology and ecotoxicology are required to assess the hazard potential of chemicals and their products. With respect to this we use a T-SAR (thinking in terms of structure-activity relationships) guided strategy to systematically analyse the hazard potential of a chemical entity (Jastorff *et al.* 2007). To verify the identified possible modes of toxic action the substances of interest are subsequently tested in selected test systems out of a flexible toxicological and ecotoxicological test battery (Stolte *et al.* 2007). This tiered strategy allows for the identification of critical structural elements of a chemical compound, which govern its toxic mode of action. Such knowledge is essential for establishing mode of action-based quantitative structure activity relationships (QSARs), which represent a promising tool for an effective toxicity screening of large substance libraries (Bradbury 1994).

Following this strategy, in the present study four differently substituted isothiazol-3-one biocides (Figure 1) were systematically selected due to the test-kit concept (Jastorff *et al.* 2003) and their toxicity towards the human hepatoblastoma cell line Hep G2 and

towards isolated and cellular glutathione reductase (GR) was investigated to contribute to a deeper insight into the structure-activity relations mediating the toxicity of this substance class.

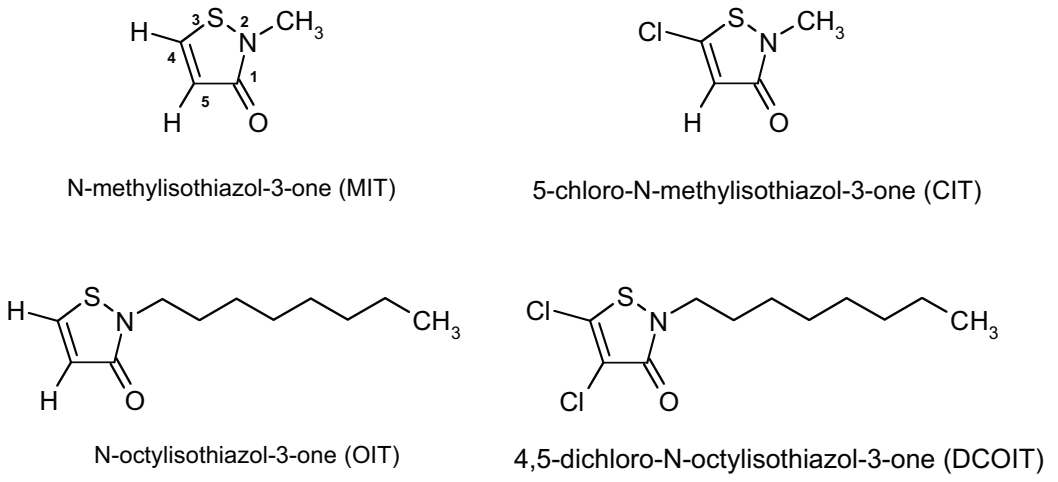

Figure 1 Structures of the selected isothiazol-3-one biocides. The chemical names are presented with the used acronyms in parenthesis.

Isothiazol-3-ones are well-known and highly reactive biocides, which find manifold commercial applications in wall paintings (OIT), antifouling paintings (DCOIT) and as stabilisers in cosmetics (MIT and CIT) (Jacobson and Williams 2000; Jacobson and Willingham 2000; Du *et al.* 2002). This group of biocides is assumed to exert its high toxicity towards microorganisms and fungi by diffusing through the cell membrane and subsequently reacting with essential intracellular sulfur-containing proteins or smaller biomolecules like glutathione (Collier *et al.* 1990; Chapman and Diehl 1995). Furthermore, isothiazol-3-ones were identified to act as strong skin-sensitisers in humans via the irreversible reaction with cysteine residues in proteins (Alvarez-Sanchez *et al.* 2003; Alvarez-Sanchez *et al.* 2004). Data from different cellular assays testing commercially available mixtures of MIT and CIT (*e.g.* KATHON®) indicate that the observed cytotoxicity is related to the interaction of the biocides with the cellular glutathione (GSH) metabolism leading to apoptotic or necrotic cell death (Du *et al.* 2002; Ettorre *et al.* 2003; Di Stefano *et al.* 2006). In this literature references it was also found that the chlorinated CIT species exerts higher effects compared to its non-chlorinated analogue MIT.

To get a deeper insight into this cytotoxic mode of action of isothiazol-3-ones and to identify substructural elements, which are responsible for the observed effects we used the four single and purified substances instead of the commonly applied mixtures to address the following questions:

iv. Is there a significant influence of the isothiazol-3-ones on the total cellular glutathione content (GSx) and on the ratio of GSSG to GSx and can this interaction be linked to cytotoxicity?

v. Can the predicted and measured intrinsic thiol-reactivity of the isothiazol-3-ones be verified in a molecular test system using GR as molecular target?

vi. Can the observed differences between the four isothiazol-3-one structures be rationalised with the T-SAR approach by taking into account the different substitution patterns and the intrinsic glutathione-reactivity of the substances?

As test system to answer these questions the Hep G2 cell line (Aden *et al.* 1979), which is broadly applied in toxicity studies (Dierickx 2005) was chosen as a human model system due to its high metabolic activity. As a second test system the GR was chosen as molecular target since this enzyme plays a pivotal role in maintaining the high GSH to GSSG ratio within the cell. Furthermore, the active enzyme contains in its reduced state a highly nucleophilic cysteine-thiolate at its active site (Picaud and Desbois 2002). This highly nucleophilic thiolate moiety should render the GR susceptible to the attack of the electrophilic isothiazol-3-one structures.

Our results obtained with these test systems showed that the chlorinated CIT and DCOIT biocides significantly depleted glutathione contents in the Hep G2 cells and concomitantly increased the GSSG content. This resulted in a severe shift of the cellular GSH/GSSG ratio which is likely to be the reason for the observed necrotic effects indicated by swelling of the plasma membrane of the cells. Additionally, CIT and DCOIT exhibited a strong inhibitory potential towards isolated yeast GR and GR in Hep G2 cells.

Since the non-chlorinated structural analogues MIT and OIT showed no or only weak effects in both test systems the chlorine substituents and the resulting reaction mechanisms could clearly be identified as mediators for the observed toxic mode of action. Furthermore, the different hydrophobicity of the isothiazol-3-ones was found to be a second parameter explaining the observed variation in the activity of the biocides by linking their intrinsic glutathione-reactivity to the assumed target site concentration of each tested substance inside the cells. Hence, using for the first time this test-kit of purified and isolated iosthiazol-3-ones we could identify substructural elements in the biocides responsible for the type and mechanism of cytotoxic action of this substance class.

Materials and Methods

Chemicals and materials

The four isothiazol-3-one biocides N-methylisothiazol-3-one (MIT), 5-chloro-N-methylisothiazol-3-one (CIT), N-octylisothiazol-3-one (OIT) and 4,5-dichloro-N-octylisothiazol-3-one (DCOIT) were purchased from the Rohm&Haas Ltd. (Coventry, UK). The obtained KATHON^{TM}, mixture of 5-chloro-N-methylisothiazol-3-one and N-methylisothiazol-3-one was separated and purified by column chromatography using a preparative chromatographic column (50 mm ∅) filled with 100 g silica gel (size of the beads 10 μM). The analytes were eluted using a mobile phase consisting of CH_2Cl_2 and methanol in a 15:1 ratio. Before use all biocides were recrystallised from a methanolic

solution and the purity was determined to be > 99.8 % by HPLC and NMR analysis. Stock solutions (1 mM and 10 mM) of every single biocide in methanol were prepared freshly every day.

The RPMI 16/40 medium, fetal calf serum, and phosphate buffered saline (PBS) were purchased from GIBCO BRL Life Technologies (Eggenstein, Germany). Penicillin/streptomycin and L-glutamine as well as $NaHCO_3$ were obtained from PAA Laboratories (Cölbe, Germany).

Yeast glutathione reductase, NADPH, glutathione (GSH), glutathione disulfide (GSSG) and all further chemicals were from SIGMA (Deisenhofen, Germany).

Sterile cell culture dishes and flasks were purchased from Sarstedt (Newton, USA) and from NUNC (Roskilde, Denmark), repectively.

Cell culture

Hep G2 cells were cultured in 10 mL flasks in culture medium consisting of 90 % RPMI 16/40 medium and 10 % fetal calf serum supplemented with 2 mg/mL $NaHCO_3$, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin sulphate. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and the culture medium was renewed every second day. The cells were trypsinised and seeded to new flasks when reaching 70 % - 80 % of confluency.

For all incubation experiments viable cells were seeded at a density of 300,000 cells per well in 24-well tissue culture plates and incubated in culture medium for 48 h before experiments were performed.

Incubation protocol

The culture medium was removed and the Hep G2 cells were washed twice with 1 mL of pre-warmed (37 °C) RPMI 16/40 medium. To determine the cellular contents of GSx and GSSG the cells were subsequently incubated up to 30 minutes in an incubator (37 °C and 5 % CO_2) with 2 mL pre-warmed RPMI 16/40 medium containing the isothiazol-3-one biocides in various concentrations (100 µM, 50 µM, 25 µM, 12.5 µM and 6.25 µM). The methanol concentration in the incubation mixture was < 0.5 % for all experiments. In this percentage methanol did not affect the cell viability, cellular GSx content and GSH/GSSG ratio (data not shown).

For measuring the specific activity of GR in cell lysates the Hep G2 cultures were incubated for 30 minutes with biocides in the indicated concentrations. For every series of incubation experiments untreated control samples were processed the same way as the biocide containing cell cultures.

Cell viability and protein content

The cell viability and the plasma membrane integrity after incubation with 100 µM of the isothiazol-3-on biocides for 15 and 30 minutes was assessed by measuring the

activity of lactate dehydrogenase (LDH) in the media and in cell lysates using a photometric method to determine the enzymatic activity of LDH (Dringen *et al.* 1998). The cellular protein content was determined according to the method of Lowry and co-workers (Lowry *et al.* 1951).

Glutathione content

The cellular contents of total glutathione (GSx = amount of GSH plus twice the amount of GSSG) and GSSG were determined from cell lysates in a microtiter plate assay as described previously (Dringen and Hamprecht 1996), using a modification of the method originally described by Tietze (1969).

The presence of the isothiazol-3-one biocides did not affect the assay (data not shown). For all calculations the cellular contents of GSx and GSSG were normalised using the total cellular protein content determined from identically treated replica wells.

Glutathione reductase activity

The specific activity of glutathione reductase (GR) in Hep G2 cell lysates after incubation with isothiazol-3-one biocides was determined using a modification of the as-say protocol described by Gutterer *et al.* (1999). Briefly, after 30 minutes of incuba-tion the cells were washed once with 500 µL ice cold PBS (10 mM potassium phos-phate buffer, 150 mM NaCl, pH 7.4) and subsequently lysed with 100 µL of hypotonic potassium phosphate buffer (20 mM, pH 7.0) for 10 minutes on ice. The lysates were collected and centrifuged at 15,000 g for 5 minutes at 4 °C. Samples of 50 µL of the resulting supernatants were tested for GR activity by measuring the decrease of absorbance at 340 nm due to the oxidation of NADPH in a total volume of 360 µL in 96-well microtiter plates in a microtiter plate reader (MRX TC, Dynex Technologies, Denkendorf, Germany). The reaction mixture contained 100 mM potassium phosphate buffer, 1 mM EDTA, 1 mM GSSG and 0.2 mM NADPH (pH 7.0, room temperature). The obtained data for lysates of biocide treated cells were normalised to those of untreated controls.

The biocide induced inhibition of yeast GR was determined using a modification of the above described microtiterplate assay. The enzyme (0.04 U/mL) was incubated with a 1:1 dilution series of the biocides in 100 mM potassium phosphate buffer (containing 1 mM EDTA and 4 mM NADPH, methanol content < 1 %). In the case of DCOIT the concentration range was chosen from 500 µM to 1.9 µM due to the low solubility of this substance. For all other isothiazol-3-ones the concentrations ranged from 1000 µM down to 1.9 µM. Then the activity of the GR was measured in a total volume of 360 µL either by direct addition of 180 µL of 2 mM GSSG in 100 mM potassium phosphate buffer (pH 7.0, room temperature) or after a 30 minutes incubation time at 37 °C. For the 30 minutes incubation experiments a series of identical measurements was performed but without adding the co-substrate NADPH before starting the incubation with the aim to analyse whether the biocides act directly at the active site of the enzyme. The concentration response curves were calculated from the measured decrease in the

absorbance at 340 nm. The obtained data for the biocide treated enzyme were normalised to those of untreated controls.

Reaction kinetics of the isothiazol-3-ones with glutathione

The observed initial rate constants for the pseudo first order reaction of the four isothiazol-3-one substances with L-glutathione were determined by an adaptation of the method recently described by Morley *et al.* (Morley *et al.* 2007). Briefly, 500 µL of pre-warmed (37 °C) solutions of the biocides (50 µM) in PBS were incubated at 37 °C in a quartz cuvette (10 mm path length) with 500 µL of pre-warmed 20 mM GSH dissolved in 10 mM PBS. The reaction was monitored spectrophotometrically over time by measuring the change of absorbance between the isothiazol-3-ones and the corresponding ring-opened biocide-glutathione adducts (*e.g.* shift from 270 nm for the CIT to 260 nm for the CIT-GSH species with the maximum change of absorbance observed at 256 nm).

Pseudo first order rate constants were calculated from the linear regression of $\ln A_{(t)}$ (change in the absorbance at a certain time point) versus the reaction time with $A_{(0)}$ representing the initial change in the absorbance. The observed reaction rate constant k_{obs} is then given by the slope of the following regression function:

$$\ln A_{(t)} = -k_{obs}*t + \ln A_{(0)}$$

The changes in the absorbance were recorded by using a Beckmann DU 640 UV/VIS spectrophotometer.

Statistical analysis

The cell culture experiments were carried out in independent duplicates with three replicates for each data point. All other measurements were at least conducted with three independent setups containing duplicates for each data point. The data are presented as means ± SD. Statistical analysis was performed by a one-way ANOVA using the Bonferroni multiple comparisons test from the SPSS software package. The level of statistical significance ($P<0.05$, $P<0.01$ and $P<0.001$) between controls and treated samples as well as between the different isothiazol-3-one substances is indicated in each figure using different marks.

The normalised (0 to 100 % enzyme activity) concentration response curves for the GR inhibition experiments were fitted to the data with the statistical software environment R (R core team, 200X) using the probit model for the relation of enzyme activity to the decadic logarithm of the tested concentrations (Ranke 2006). Confidence intervals ($a = 0.05$) of the calculated IC_{50} values were calculated with the R language as well as the parameters for the linear regression for the determination of the reaction rate constants.

RESULTS

In the following the results for the impact of the four differently substituted isothiazol-3-one moieties on the cellular glutathione metabolism and on isolated yeast glutathione reductase are presented. Reaction rate constants of the four isothiazol-3-ones with glutathione and the logarithm of the octanol/water partition coefficients (log $P_{O/W}$) for the biocides taken from Arning *et al.* (2007) are shown in Table 1.

Table 1 Reaction rate constants $k_{obs.}$ (s^{-1} for the pseudo first order reaction of the isothiazol-3-ones (50 µM) with GSH (20 mM) at 37 °C in PBS at pH 7.4 and the decadic logarithm of the measured octanol-water partition coefficients log $P_{O/W}$ of the biocides (values ± SD). The reaction of GSH with DCOIT was that fast that no rate constant could be calculated. **a** Values were taken from Arning *et al.* (2007).

Substance	$k_{obs.}$ (s^{-1})	log $P_{O/W}$
N-methylisothiazol-3-one (MIT)	14.8 X 10^{-3}	-0.49 ± 0.01[a]
5-chloro-N-methylisothiazol-3-one (CIT)	22.8 X 10^{-3}	0.53 ± 0.01[a]
N-octylisothiazol-3-one (OIT)	12.9 X 10^{-3}	3.30 ± 0.01[a]
4,5-dichloro-N-octylisothiazol-3-one (DCOIT)	not detectable	4.79 ± 0.01[a]

The four isothiazol-3-one species reacted readily with GSH at 37 °C and a neutral pH. Comparing the observed reaction rate constants it is obvious that both chlorinated biocide species (CIT and DCOIT) reacted significanttly faster (high $k_{obs.}$ value for CIT) with GSH than their non-chlorinated analogues. The double chlorinated DCOIT even reacted that fast that no reaction rate constant could be determined. The reaction rate constants for both non-chlorinated substances (MIT and OIT) were found to be closely related, with MIT (k_{obs} = 14.8 X 10^{-3}) reacting a little bit faster with glutathione than its octyl-substituted analogue OIT (k_{obs} = 12.9 X 10^{-3}) (Table 1).

The log $P_{O/W}$ values - representing a parameter to rank the compounds hydrophobicity - revealed that the investigated isothiazol-3-ones nearly cover five orders of magnitude showing log $P_{O/W}$ values ranging from -0.49 ± 0.01 for the non-chlorinated and methyl-substituted MIT up to 4.79 ± 0.01 for the double-chlorinated and octyl-substituted DCOIT (Table 1). Thus, the small and polar MIT was found to be hydrophilic whereas its chlorinated analogue CIT and the octyl-substituted OIT and DCOIT exhibited a hydrophobicity ranging from moderate (CIT) up to very high (DCOIT).

Cell viability and protein content

To test whether the applied isothiazol-3-one biocides caused any changes in cell viability or membrane integrity the Hep G2 cells were incubated with the highest test concentration (100 µM) of the biocides and the LDH release to the medium was measured as well as the remaining LDH activity in the cell lysates. After 15 and 30 minutes of incubation no increase in the extracellular LDH activity was observable for any of the biocides and concomitantly the intracellular LDH activity did not differ to

the level found in untreated controls (Figure 2). Thus, under these conditions the biocides did not compromise cell viability. The cellular protein content of the Hep G2 cells was determined to be 130 ± 7 µg per well (n = 20, obtained from five independent experiments) of a 24-well plate.

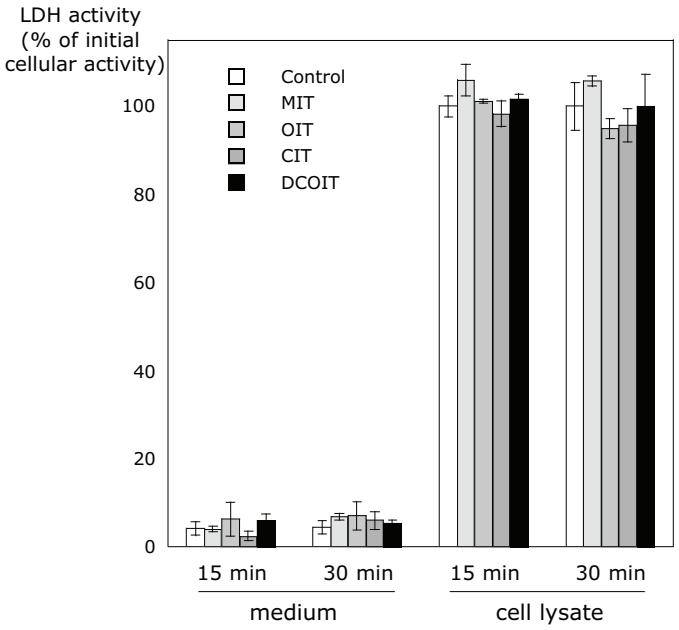

Figure 2 The LDH activity in the medium and the remaining LDH activity in the cell lysates are presented as a percentage of total initial LDH in untreated cultures. The cells were incubated for 15 and 30 minutes with 100 µM of each isothiazol-3-one biocide.

Intracellular glutathione content

To examine the impact of the four isothiazol-3-ones and their substructural variations on the total glutathione content (GSx = GSH + [2 x GSSG]) and on the GSSG levels of the cells, the Hep G2 cultures were incubated with 0 - 100 µM of biocides for up to 30 minutes (Figure 3). The GSx content of the untreated Hep G2 cells was determined to be 40.4 ± 3.6 nmol/mg protein (n = 30, obtained in 10 independent experiments) whereas the amount of GSSG in these samples was 2.6 ± 0.6 in nmol GSx/mg protein (n = 30) resulting in a GSH/GSSG ratio of roughly 30:1 for the untreated cells.

Incubating cells with the chlorine-substituted (CIT, DCOIT) isothiazol-3-ones resulted in severe changes in the intracellular GSx and GSSG contents.

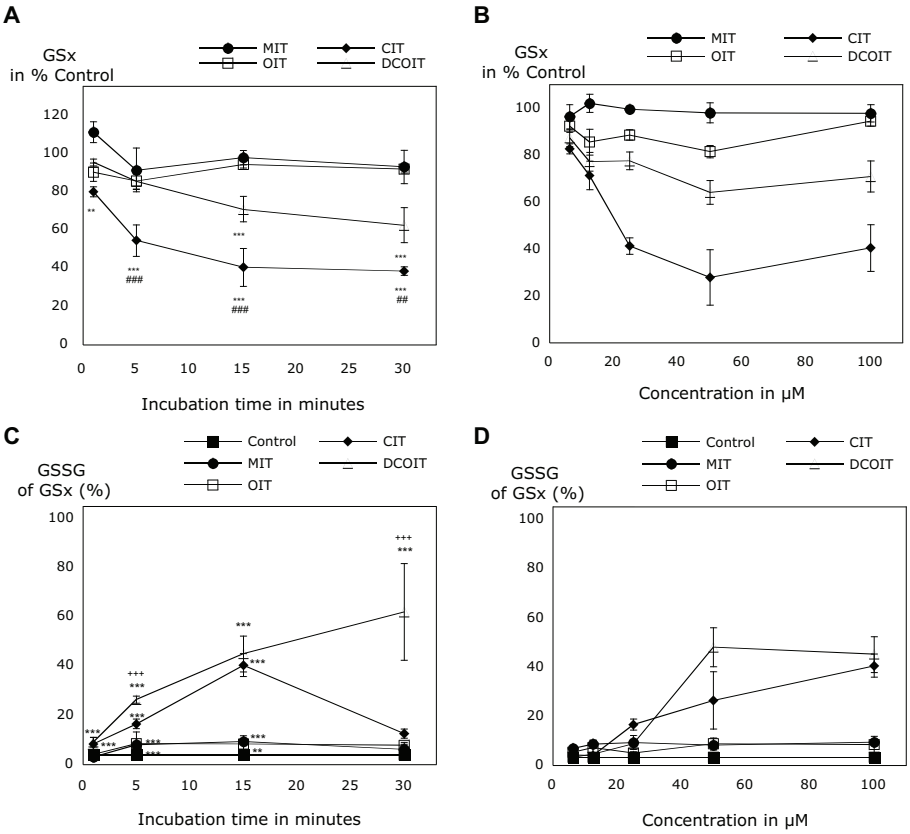

Figure 3 To demonstrate quantitative differences between the isothiazol-3-ones the GSx is presented as percentage of the untreated controls for each incubation (A,B) and the GSSG content is expressed as GSSG/GSx ratio for each well to compare the relative intracellular GSSG amount for each treatment (C,D). Cells were exposed to 100 µM biocide for the indicated time periods (A,C) or for 15 minutes with the indicated isothiazol-3-one concentrations (B,D). ** P < 0.01, *** P < 0.001 compared to the untreated controls; ## P < 0.01, ### P < 0.001 compared to DCOIT and +++ P < 0.001 compared to CIT.

The chlorinated substances CIT and DCOIT significantly decreased the amount of GSx compared to the untreated controls in a dose and time dependent manner, whereas the non-chlorinated isothiazol-3-ones exhibited no or only marginal effects (in the case of OIT for 15 minutes and concentrations of 25 µM and 50 µM) on the GSx content (Figure 3 A,B).

For CIT and DCOIT the maximal effects were observed after an incubation time of 15 minutes. These compounds reached in a concentration of 50 µM a maximal effect that did not differ significantly to the data obtained for respective 30 minutes incubation and the 100 µM concentration. CIT exhibited stronger effects than DCOIT, decreasing the GSx content to 27.9 ± 11.8 % of the control value ($P<0.001$) at the most effective concentration of 50 µM and after 15 minutes of incubation, whereas for the same conditions the decrease in the cellular GSx for DCOIT was only to 64.2 ± 5.1 % of the controls ($P<0.001$). This pattern was found consistently for all incubation times from 5 minutes and for concentrations starting from 12.5 µM. Highly significant differences between GSx levels of cells treated with CIT and DCOIT, between both substances and the control samples and between both chlorinated substances and the MIT and OIT

treated cells were already observed for incubation times of 5 minutes and for a concentration of 25 μM ($P<0.001$). In the lowest tested concentration of 6.25 μM the biocides did not cause any significant effects on the GSx levels under the conditions used ($P>0.05$).

All biocides significantly increased the amount of cellular GSSG in a dose and time dependent manner compared to untreated controls. Maximal effects were observed after an incubation time of 15 minutes and at concentrations of 100 μM of the isothia-zolo-3-one compounds. Comparing the four biocides at the 100 μM concentrations revealed significant but small increases for the non-chlorinated MIT and OIT after 5 minutes of incubation that remained during longer incubation times (8 – 9 % GSSG of total GSx, $P<0.001$). For the chlorinated CIT and DCOIT substances the pattern was more diverse. Both chemicals - applied in a concentration of 100 μM - strongly increased the amount of GSSG already within 1 minute of incubation ($P<0.001$), reaching maximum values of 45.1 ± 7.3 % GSSG for the DCOIT and 40.5 ± 4.6 % of GSSG for the CIT after 15 minutes. However, for the 30 minutes incubation the high GSSG level inside the DCOIT treated cells remained constant whereas a rapid and significant ($P<0.001$) drop in the GSSG content down to 12.7 ± 1.8 % GSSG was de-tected for the 100 μM CIT treatment compared to the 15 minutes incubation time.

The chlorinated isothiazol-3-ones CIT and DCOIT significantly decreased the GSx content and concomitantly strongly increased the level of GSSG in Hep G2 cells. These resulted in a severe shift of the cellular GSH/GSSG ratio from 30:1 for the untreated cells down to ratios of 3:1 for the CIT and 2.5:1 for the DCOIT at a concentra-tion of 100 μM after 15 minutes exposure. To detect morphological changes of such a treatment the Hep G2 cells were incubated up to 1.5 h with 100 μM of the biocides. After 1 h exposure, cells treated with the chlorinated compounds CIT and DCOIT showed strong morphological alterations with swelling of the plasma membrane and some cells were found to be detached from the bottom of the culture dishes (Figure 4). For the non-chlorinated substances MIT and OIT no saliences could be observed within the monitored time and concentration range (data not shown).

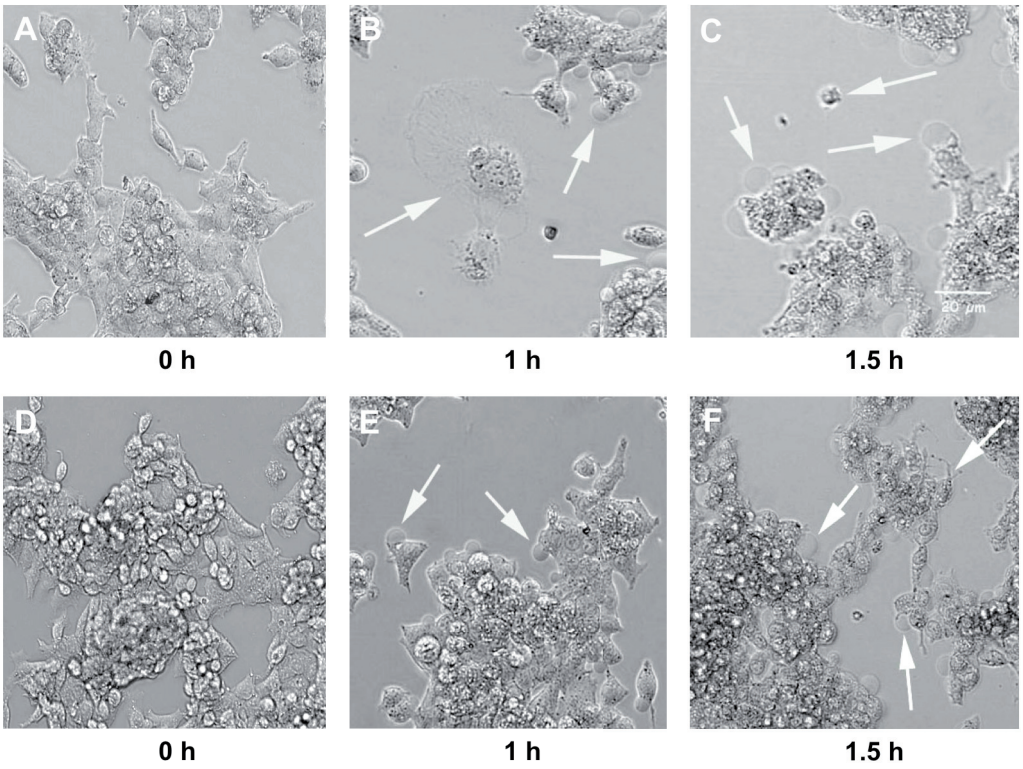

Figure 4 Morphological changes of the Hep G2 cells after incubation with 100 µM of CIT (A-C) and DCOIT (D-F) for the indicated time intervalls. The Arrows indicate swelling effects of the plasma membrane or lysed cells.

Inhibition of glutathione reductase

To examine the inhibition potential of isothiazol-3-one biocides towards GR and to get an insight into the mode of inhibition, yeast GR was incubated with different concentrations of MIT, CIT, OIT (0 - 1000 µM) and DCOIT (0 - 500 µM) with and without a 30 minutes preincubation period before measuring the enzyme activity. Addition-ally, these preincubations were performed with and without the co-substrate NADPH in the incubation mixture. The calculated IC_{50} (µM) values from the fitted concentration-response curves are summarised for all experimental conditions (Table 2). DCOIT and CIT strongly inhibited yeast GR when incubated for 30 minutes in the presence of NADPH with IC_{50} values of 7 µM for the CIT and 13 µM for the DCOIT, respectively. In contrast, no inhibitory effect on GR could be observed for the non-chlorinated MIT and OIT. For all incubation experiments lacking the co-substrate NADPH in the incubation mixture incomplete concentration-response curves were obtained. For each of the four biocides the GR activity was found to be above the 50 % benchmark even for the highest concentration tested.

Table 2 Calculated IC_{50} (µM) values for the isothiazol-3-ones in all performed inhibition experiments using yeast or cellular GR. If no complete concentration response curve was obtained in the enzyme inhibition asssay the IC_{50} value is indicated to be higher than the maximum test concentration (*e.g.* > 1000). The 95% confidence intervals of the IC_{50} (µM) values are given in parenthesis.

	IC_{50} (µM)				
Substance	**Isolated GR + 30 min. incubation**		**Isolated GR – 30 min. incubation**		**Cellular GR**
	- NADPH	+ NADPH	- NADPH	+ NADPH	
MIT	> 1000	> 1000	> 1000	> 1000	> 200
CIT	> 1000	7.0 (6.6 – 7.4)	> 1000	> 1000	9.0 (8.0 – 9.8)
OIT	> 1000	> 1000	> 1000	> 1000	> 200
DCOIT	> 500	13.2 (12.0 – 14.8)	> 500	81.3 (77.6 – 97.7)	14.1 (13.2 – 14.8)

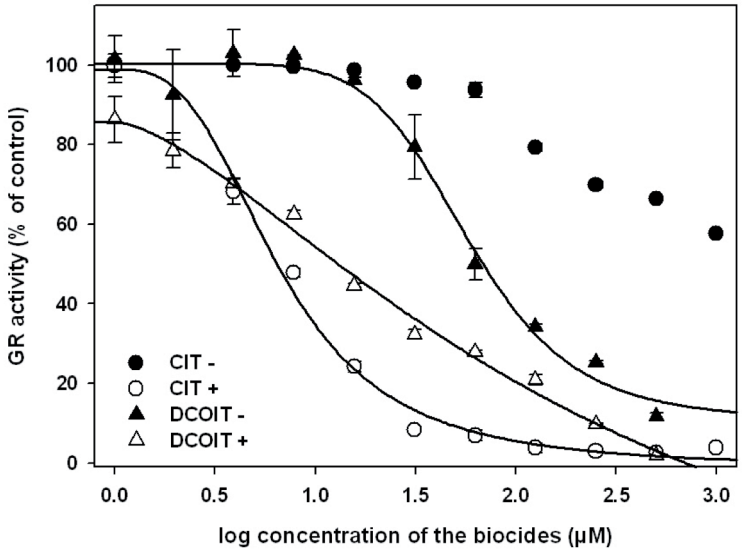

Figure 5 Inhibition of yeast GR by CIT and DCOIT. The enzyme was preincubated with the indicated concentrations of CIT and DCOIT for 0 min. (CIT -/DCOIT -) or 30 min. (CIT +/DCOIT +) in the presence of the co-substrate NADPH. The corresponding calculated IC_{50} values are presented in Table 2.

Comparing the two active chlorinated isothiazol-3-one species (Figure 5), the CIT and the DCOIT, for the 30 minutes preincubation containing NADPH the CIT was found to exhibit a significantly ($P<0.01$) stronger inhibitory potential and the slope of the dose response-curve was much steeper than for DCOIT. However, in the assay without the preincubation period only for DCOIT a concentration-response curve could be recorded and an IC_{50} value of 81 µM was calculated. For the CIT the GR activity remained over 50 % for the highest tested concentration of 1000 µM compared to the controls (Figure 5).

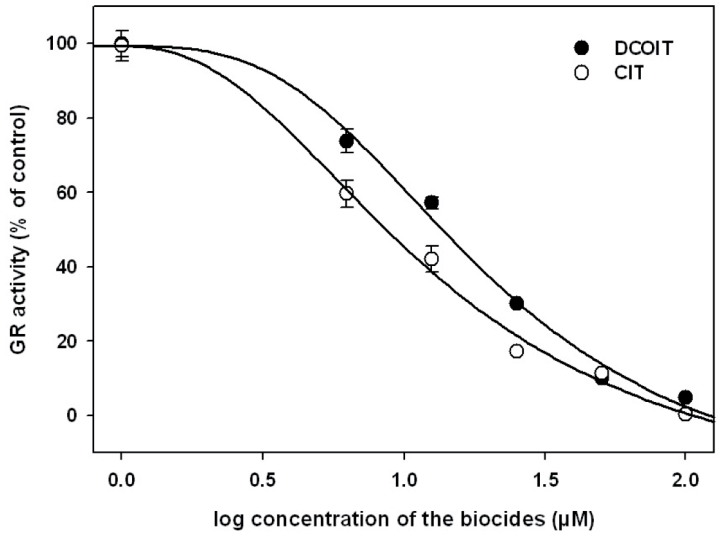

Figure 6 Inhibition of cellular GR after a 30 minutes incubation of Hep G2 cells with various concentrations (0 – 100 µM) of CIT and DCOIT. The corresponding calculated IC_{50} values are given in Table 2.

To test whether the observed inhibitory effects on purified yeast GR could be confirmed for GR in cells, the Hep G2 cells were incubated for 30 minutes with up to 100 µM of the biocides and subsequently the GR activity in the cell lysates was determined. Again the CIT and the DCOIT significantly blocked the cellular GR activity in a dose dependent manner (Figure 6). Incubation with 100 µM of the chlorinated isothiazol-3-one species completely inhibited cellular GR. Similar IC_{50} values of 9 and 14 µM were calculated for the inhibition of cellular GR by CIT and DCOIT, respectively. In contrast, MIT and OIT did not affect GR activity in the Hep G2 cells in concentrations up to 200 µM (data not shown).

Discussion

In the present study, human hepatocarcinoma cells were used as a model system to examine structure-activity based impacts of four differently substituted isothiazol-3-one biocides on the cellular glutathione metabolism. Additionally, the inhibitory potential of these biocides towards GR was investigated. The most important outcomes are: i.) The two chlorinated biocide species CIT and DCOIT drastically decreased the total cellular glutathione content in a concentration and time dependent manner and this effect is significantly larger for CIT. ii.) All investigated isothiazol-3-ones increased the cellular amount of GSSG in a concentration and time dependent manner. This increase was drastic for the chlorinated CIT and DCOIT species, whereas the non-chlorinated MIT and OIT substances only exhibited a moderate effect on the GSSG content. iii.) After treatment with CIT and DCOIT the Hep G2 cells showed rapid (after 1 h) morphological changes with swelling of the plasma membrane. iiii.) Both chlorinated biocides inhibited the cellular GR in a concentration dependent manner and the highest tested concentration (100 µM) completely blocked GR activity within the Hep G2 cells.

Furthermore, CIT and DCOIT offered a strong inhibitory potential towards purified yeast GR after an incubation time of 30 minutes in the presence of the co-substrate NADPH.

To discuss these effects and to explain the observed differences between the chlorinated and non-chlorinated isothiazol-3-one species the reaction mechanism be-tween these biocides and sulfhydryl groups is briefly presented. Quantum chemical calculations and *in vitro* reaction studies using thiol containing compounds (*e.g.* 2-methyl-2-propanethiol) provide strong hints that the first reaction step is based on a nucleophilic attack of a sulfhydryl group at the sulfur atom of the isothiazol-3-one ring structure (Morley *et al.* 2007). These results in the ring-opened amidodisulfide adduct of the biocides. In a second step this disulfide-bridged species is cleaved via a disulfide exchange reaction with a further thiol-containing agent. The resulting ring-opened mercapto-acrylamides in the case of MIT and OIT exhibit a strongly reduced reactivity towards thiol containing compounds. However, looking at the CIT and DCOIT species which both are substituted with a chlorine at the 5-position of the isothiazol-3-one ring system (see Figure 1), the cleavage of the disulfide leads to a highly reactive thioacyl-chloride intermediate which can react readily and irreversibly with thiol containing substances like GSH or cysteine residues in proteins (Morley *et al.* 1998). Additionally, thiol-containing compounds can be bound irreversibly to the chlorinated isothiazol-3-ones via a nucleophilic substitution reaction at the chlorine-carrying carbon atom in the ring system and the displacement of the chlorine moiety. Finally, the electron withdrawing effect of the chlorine substituents increases the positive polarisation of the sulfur atom and thereby facilitates the nucleophilic attack of the biocides by thiol containing agents (Morley and Charlton 1995). Thus, the substitution pattern of the isothiazol-3-ones seems to be the key factor dominating their reaction with thiol groups.

Considering these facts the order of reactivity for the four investigated biocides towards GSH was predicted to be DCOIT > CIT > MIT = OIT. Our *in vitro* results for the reaction of a 400 fold excess of GSH with the isothiazol-3-ones (Table 1) confirmed this assumption. The DCOIT even reacted that fast that a reaction rate constant could not be calculated. The mono-chlorinated CIT then exhibited the highest measurable reaction rate compared to MIT and OIT. The small differences in k_{obs} for MIT and OIT may be due to the sterically bulky octyl chain at the OIT which hindered the nucleophilic attack of GSH at the sulfur atom.

Beside the intrinsic reactivity of the biocides towards GSH the uptake into the cells via passive diffusion through the plasma membrane is a second pivotal parameter when looking at the intracellular impacts of the substances on the GSH content. Comparing the log $P_{O/W}$ values (Table 1) as a measure for the hydrophobicity of the four isothiazol-3-ones the highly hydrophobic DCOIT and OIT should easily cross the lipid bilayer of the plasma membrane. The CIT is still hydrophobic enough to enter the cell via passive diffusion but for the polar MIT (log $P_{O/W} < 0$) one would expect a reduced uptake into the cells. Combining now the intrinsic reactivity towards GSH and the hydrophobicity of the four compounds, DCOIT and CIT are likely to be rapidly taken up by cells and should react immediately and irreversibly with GSH. The OIT can also easily

accumulate inside the cells but due to the lower reactivity the effects on GSH should be weaker compared to CIT and DCOIT. For the MIT the weakest effects were predicted due to a presumably slow uptake into the cells and a reduced reactivity compared to the chlorinated species.

In the light of the above considerations the observed changes in the total glutathione content and the increase in GSSG in the biocide treated Hep G2 cells can be interpreted. Both chlorinated substances decreased the amount of GSx down to about 30 % (CIT, 50 μM) compared to the initial GSx values within the first 15 minutes of incubation most likely due to the irreversible formation of biocide-GSH adducts. This is in accordance with previously published results where a rapid GSH depletion is shown for HL60 cells after incubating them with a mixture consisting of MIT and CIT (Di Stefano *et al.* 2006). The here presented rapid intracellular GSx depletion after application of CIT and DCOIT was in the same order of magnitude as the effect of the well known GSH reagent monochlorobimane (Waak and Dringen 2006). Unexpectedly, the mono-chlorinated CIT exhibited a significantly larger effect compared to the double chlorine substituted DCOIT. This may be explained by the extremly high hydrophobic interaction potential of the DCOIT (log $P_{O/W}$ = 4.79) wich allows for a tight and unspecific binding to hydrophobic proteins and membranes resulting in a reduction of the freely accessible fraction of this substance. OIT showed little and MIT showed no significant influence on the GSx level for all tested concentrations and incubation times which is in accordance to the presumed slow uptake and the relatively low intrinsic reactivity towards GSH.

The observed maximal effects in GSx depletion for CIT and DCOIT (Figure 3 A,B) after 15 minutes exposure to 50 μM substance can be explained by assuming that the diffusion through the plasma membrane was the rate limiting step for the intra-cellular reaction with GSH since the time frame was too short for biosynthesis of fresh GSH. This diffusion step was saturated with 50 μM of each biocide and reached a steady state after 15 minutes.

Since the total glutathione content GSx (GSH + [2 × GSSG]) does not reflect changes in the ratio of GSH to GSSG, GSSG was measured for each GSx value separately. Each of the four isothiazol-3-ones increased the GSSG level significantly during 5 minutes incubation in a concentration of 100 μM. This can be explained by either a direct or a glutathion-S-transferase catalysed attack of the GSH at the sulfur atom of the biocides and a subsequent disulfide exchange reaction that led to an accumulation of GSSG. Alternatively, the reaction of the isothiazol-3-ones with other essential sulfhydryl groups inside the cell could have been caused oxidative stress and thereby led to an increase in the GSSG level.

In the case of MIT and OIT the disulfide exchange reaction yields the ring-opened and no longer active mercaptoacrylamides. Neither MIT nor OIT inhibited cellular GR. Thus, in this case the formed GSSG can partialy be recycled to GSH which led to the observed low and constant GSSG level for both non-chlorinated biocides (Figure 3 C,D). However, the weak but significant increase in the GSSG levels for MIT and OIT suggests that both chemicals reacted in the predicted and reversible manner with GSH.

The two chlorinated isothiazol-3-one species showed a strong inhibitory potential towards GR activity, which slowed down or even inhibited (for the 100 µM incubation) the GSH recycling mechanism, explaining the rapid increase in cellular GSSG levels. Surprisingly, DCOIT caused a stronger increase in the GSSG level than CIT and this high level remained constant for DCOIT wheras there was a significant drop in the GSSG content for the CIT between 15 and 30 minutes. These effects may be explained by differences in the reactivity of CIT and DCOIT. It is known that the chlorinated isothiazol-3-ones cannot only interact with GSH but it also reacts readily with other essential redox sensitive cellular thiols in a way that free radicals are formed (Chapman and Diehl 1995). This entails a general increase in cellular oxidative stress and in response to that an increase in the GSSG level which is likely to be stronger for the DCOIT due to its extremly fast reaction with GSH. Furthermore, due to its high hydrophobicity DCOIT is likely to represent a good substrate for cellular glutathione-S-transferases (GSTs) compared to the small and less hydrophobic CIT since GSTs are known to bind preferably hydrophobic substrates (Vos and Vanbladeren 1990). Hence, the first step - the formation of the ring-opened disulfide adduct - was predominantly catalysed with the highly hydrophobic DCOIT species. After the following disulfide exchange reaction the formed thioacylchloride of the DCOIT could have been bound unspecifically to other proteins due to its remaining high hydrophobicity. This is in accordance with the high and remaining GSSG level and the lower GSx depletion observed for DCOIT treated cells compared to the CIT incubations. CIT also caused a rapid increase in the GSSG content in the first step due to its high intrinsic reactivity towards GSH and then the formed polar thioacylchloride was freely accessible for further and irreversible reactions with GSH leading to the measured low cellular GSx levels. Nevertheless, the observed drop in the GSSG content for the 30 minute incubation and the highest tested concentration remains unclear and needs to be further investigated.

Taking together, the intracellular GSx depletion and the concomitant increase in the GSSG levels after application of CIT or DCOIT caused a dramatic shift in the GSH/GSSG ratio and led to the breakdown of the cellular thiol reduction potential which is likely to contribute to the cytotoxicity of the chlorinated isothiazol-3-ones. Since the GSH concentration is represented by a quadratic term within the Nernst-equation of the GSH/GSSG equilibrium reaction, especially the GSH depletion exerts a dramatic influence on the reduction potential (Jacob *et al.* 2003). This breakdown of the cellular reduction potential influences the redox state of essential proteins and the mitochondrial membrane potential and thereby is likely to be the reason for the observed morphological changes with swelling of the CIT and DCOIT treated cells after 1 h of incubation (Figure 4). In this context Vesce *et al.* recently reported that acute GSH depletion can lead to severe cell damage by decreasing cell respiration (Vesce *et al.* 2005). At least, for a mixture of MIT and CIT necrotic cell death was recently reported for HL 60 cells (Di Stefano *et al.* 2006).

As a second molecuar target for the toxic action of the investigated isothiazol-3-ones GR activity was examined. In its oxidised state this enzyme contains an essential

disulfide bridge between Cys-58 and Cys-63 in its active centre (Boese *et al.* 1997). In the first step of the catalytic cycle of GR the co-substrate NADPH is bound near the active site of the enzyme and the disulfide bridge is reduced via an electron transfer from the NADPH to the active centre (Picaud and Desbois 2002). This results in a highly nucleophilic thiolate anion at Cys-63, which then attacks the natural substrate GSSG. We assume that this thiolate anion might be attacked by the thioacylchloride species of the CIT and DCOIT isothiazol-3-ones resulting in an irreversible inhibition of the GR. The presented results support this hypothesis. The isolated yeast GR was significantly inhibited by CIT and DCOIT only after incubation with NADPH. Without this incubation procedure nearly no (in the case of CIT) or only a weakn inhibition (for DCOIT) of GR was found. The fact that the presence of the co-substrate NADPH is essential for the inhibition of GR through CIT and DCOIT (Table 2) strongly supports the hypothesis that the isothiazol-3-ones acted directly at the Cys-residues in the catalytic centre of the enzyme. Without the reduction equivalents donated by the NADPH the disulfide bridge masks the thiolate moieties in the active site of GR and the biocides are not able to attack them. The inhibitory potential found for the DCOIT in the experiments lacking the incubation step can be explained by the high reactivity of the sulfur atom due to the substitution with two electron withdrawing chlorine moieties.

Conclusion

Using the human hepatocarcinoma cell line Hep G2 as a metabolically active cellular test system and GR as a subcellular molecular target we could show that the isothiazol-3-one biocides CIT and DCOIT caused a strong decrease in cellular GSH levels and a concomitant increase in the amount of cellular GSSG. This resulted in a severe shift of the cellular thiol reduction potential which subsequently led to morphological changes of the cells providing evidence for necrotic cell death with swelling of the plasma membrane. For the two chlorine-substituted CIT and DCOIT structures this effect was amplified by their potential to inhibit cellular GR. Additionally, with isolated GR we obtained evidences that this inhibition might be due to an irreversible reaction of the biocides with the essential cystein residues at the active centre of the enzyme.

The observed differences between the four substances could be explained by a structure-activity based analysis. As predicted from the chemical structures and from the resulting reaction mechanisms the chlorinated and hydrophobic CIT and DCOIT compounds exhibited stronger effects than their non-chlorinated and less hydrophobic analogues MIT and OIT in both test systems. Furthermore, the results support the structure-activity derived hypothesis that GSH and the thiolate moiety at the active site of the GR should represent pivotal molecular targets for the toxic mode of action of isothiazol-3-ones. Hence, the T-SAR based analysis of possible modes of toxic action of chemical entities and the resulting selection of test systems represented a powerful tool for assessing the hazard potential of chemical substances.

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PAPER NO. 3:

EFFECTS OF DIFFERENT HEAD GROUPS AND FUNCTIONALISED SIDE CHAINS ON THE CYTOTOXICITY OF IONIC LIQUIDS

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Abstract

To enlarge the restricted knowledge about the hazard potentials of ionic liquids to man and the environment we have concentrated on analysing systematically the effects of 7 head groups, 10 side chains (mainly containing functional groups) and 4 anions on cytotoxicity. For our investigations, we used the promyelotic leukemia rat cell line IPC-81 as test system with the reduction of the WST-1 dye as an indicator of cell viability. Our results show that most of the tested 101 ionic liquids generally exhibit a low cytotoxicity compared to previously investigated ionic liquids in consequence of their polar ether, hydroxyl and nitrile functional groups within the side chains. Those functional groups hamper cellular uptake via membrane diffusion and reduce lipophilicity based interactions with the cell membrane. In general, a low influence of the head group and a clear anion effect of the $[(CF_3SO_2)_2N]^-$ anion could be observed. Furthermore, we could confirm the general dependency between ionic liquid cation lipophilicity and cytotoxicity using a HPLC derived lipophilicity parameter. For one head group and for one side chain deviations were obtained concerning this general dependency. This can be interpreted as a first hint for a more specific mode of action for these structural elements.

Introduction

Especially since the imminent intensification of the EU chemicals legislation (REACH project) increasing importance is attached to the knowledge of (eco)toxicological hazard potentials of chemical substances. This requires efficient testing strategies to generate data sets leading to a profound insight in modes of toxic action and target sites of chemicals. Regarding this issue we follow a T-SAR[1,2] (thinking in terms of structure activity relationships) guided strategy to:

i. Systematically select test compounds and structural elements according to the "testkit concept".[2,3]

ii. Test the selected substances in a flexible (eco)toxicological test battery at different levels of biological complexity (*e.g.* enzymes, cells, microorganisms and organisms).[3]

iii. Identify toxicophore substructures in chemicals and to use this knowledge in the prospective design of inherently safer chemical products.

iv. Improve the molecular understanding of (eco)toxicological results by relating them to physicochemical properties.[4]

Following this concept we are aiming to assess the hazard potential of ionic liquids. The interest in ionic liquids and in their promising physical and chemical properties is still growing rapidly. Diverse applications of ionic liquids in different fields have been recently described.[5-10] They are discussed as "green" or "sustainable" chemicals just based on their negligible vapour pressure, resulting in reduced inhalatory exposure and

the absence of flammability. However, the knowledge about their (eco)toxicity is still very basic and restricted to only a few chemical entities out of the enormous pool of available ionic liquids.

To handle this structural variability, we subdivide ionic liquids into the following sub-structural elements: the cationic head group, the side chain and the corresponding anion. Following this classification we systematically examine the (toxic) effects of these structural elements on different tests systems out of our flexible (eco)toxicological test battery. Recently we described the effects of the alkyl side chain length,[11] the cation lipophilicity[4] and the anion[12] inter alia on the cytotoxicity.

The test kit presented here was assembled to identify the impact of further head groups and functionalised side chains on biological effects.

To attain the goal of producing more sustainable ionic liquids, which imply an optimum of technical applicability on the one hand and a minimum of hazard potentials for man and the environment on the other hand, we formed a university-industry partnership. Sustainable chemicals cannot be developed in academia alone because of the need for knowledge on current industrial products and processes. The Merck KGaA synthesised a set of ionic liquids - guided by the test kit concept and T-SAR - with the result of 101 new ionic liquids which allows to investigate systematically the impact of 7 different head groups, 10 side chains and 4 anions in (eco)toxicological studies.

For our first screening investigations, we used the promyelotic leukemia rat cell line IPC-81[13] as test system with the reduction of the WST-1[14-16] dye as an indicator of cell viability. This test system has proven to provide reproducible results for measuring cytotoxicity of various industrial chemicals.[11,14,17-19]

Furthermore, we are aiming to correlate the obtained cytotoxicity data with a HPLC derived lipophilicity parameter, which is described in detail by Ranke *et al.*,[4] for some of the test kit compounds. In this previous study a good correlation between the lipophilicity parameter of the cation and the observed cytotoxicity for a set of approximately 70 ionic liquids was found.[4] In general, the lipophilicity of chemical substances is considered to be the mediator of non-specific toxic effects evoked by membrane interactions, termed narcosis (polar and non polar)[20-23] or baseline toxicity[24,25] when applied to mammals or in aquatic toxicology respectively.

In this context, several recent publications[4,11,12,26-35] outline that the acute toxic effects of ionic liquids can be attributed to side chain length or lipophilicity of the com-pounds as descriptive parameters. Due to this fact we suggest that in the absence of more specific modes of actions, a type of baseline toxicity similar to polar narcosis is assumed to be the predominant mode of toxic action for ionic liquid cations.

The testkit compounds

The test kit comprised three aromatic head groups (4-(dimethylamino)pyridinium, pyridinium and imidazolium), three non aromatic heterocycles (4-methylmorpholinium,

1-methylpiperidinium, 1-methylpyrrolidinium), and one noncyclic quaternary ammonium head group (N,N-dimethylethylammonium).

The seven head groups (Table 1, second row) are combined with eight different aliphatic side chains containing ether (in different positions), terminal hydroxyl and nitrile functions (Table 1, first column).

For all cationic head groups one halide (chloride, bromide, or iodide; Table 1) and the $[(CF_3SO_2)_2N]^-$ anion was tested (Table 2).

Acronyms for ionic liquids

A detailed description of the following system of acronyms will be published elsewhere. The cation is abbreviated according to the type of the head group as "Py-4NMe2" (dimethylamino)pyridinium, "Py" (pyridinium), "IM" (imidazolium), "Mor" (morpholinium), "Pip" (piperidinium), "Pyr" (pyrrolidinium) and as "N" (quaternary ammonium). The substituents at the nitrogen atom(s) of the head group are given as numbers corresponding to their alkyl chain length. For example the 1-butyl-3-methylimidazolium cation has the shorthand notation IM14. Ether containing side chains are indicated by splitting the chain in alkyl units with the symbol "O" for the oxygen in between (*e.g.* IM11O2 for 1-(ethoxymethyl)-3-methylimidazolium). Terminal hydroxyl or nitrile groups are shortened as OH (*e.g.* IM14OH is 1-(4-hydroxybutyl)-3-methyl-imidazolium) or CN (*e.g.* IM11CN is 1-cyanomethyl-3-methylimidazolium). The acronyms used for the halides are as in the periodic table. The bis(trifluoromethylsulfonyl)imide is written as $[(CF_3SO_2)_2N]^-$ according to its structural formular. The identifiers for the cation and for the anion - separated by a white space - give the whole acronym for an ionic liquid.

RESULTS

All fitted EC_{50} values are shown in Table 1 and Table 2 and are described in the following sections. Confidence intervals and a complete listing of the parameters of the fitted models are given in the Electronic Supplementary Information.

Table 1 Structures of the tested head groups, side chains and their abbreviations. List of all EC_{50} values as halides with the exact anion species in brackets.

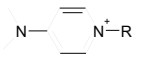
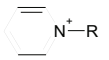
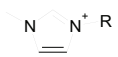
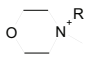
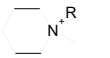
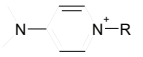
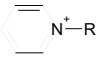
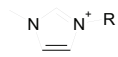
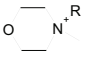
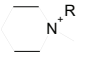
R	EC_{50} in µM (Anion)						
structure							
	"Py-4NMe2"	"Py"	"IM1"	"Mor1"	"Pip1"	"Pyr1"	"N112"
$-C_2H_5$	790 (Br)						
$-CH_2CH_2$-OH		14300 (I)	>20000 (I)	>20000 (I)	>20000 (I)	>20000 (I)	>20000 (I)
$-CH_2CN$		6100 (Cl)	>20000 (Cl)	>20000 (Cl)	>20000 (Cl)	17100 (Cl)	>20000 (Cl)
$-C_4H_9$	85 (Cl)	8000 (Br)	3600 (Cl)	>20000 (Br)	11000(Br)	>20000 (Cl)	>20000 (Cl)
$-CH_2CH_2CH_2$-OH		>20000 (Cl)	>20000 (Cl)	>20000 (Cl)	>20000 (Cl)	>20000 (Cl)	8600 (Cl)
$-CH_2$-O-CH_2CH_3		2100 (Cl)	4000 (Cl)	3300 (Cl)	17200 (Cl)	850 (Cl)	3900 (Cl)
$-CH_2CH_2$-O-CH_3		>20000 (Cl)	>20000 (Cl)	>20000 (Cl)	> 20000 (Br)	>20000 (Cl)	>20000 (Cl)
$-CH_2CH_2$-O-CH_2CH_3		17500 (Br)	13800 (Br)	>20000 (Br)	>20000	>20000 (Br)	>20000 (Cl)
$-CH_2CH_2CH_2$-O-CH_3		>20000 (Cl)	>20000 (Cl)	>20000 (Cl)	>20000 (Cl)	>20000 (Cl)	>20000 (Cl)
$-C_6H_{13}$	9 (Cl)						

Table 2 Structures of the tested head groups, side chains and their abbreviations. List of all EC_{50} values as $[(CF_3SO_2)_2N]^-$ anion.

R	EC_{50} in µM (Anion=$[CF_3SO_2)_2N]^-$)						
structure							
	"Py-4NMe2"	"Py"	"IM1"	"Mor1"	"Pip1"	"Pyr1"	"N112"
$-C_2H_5$	700						
$-CH_2CH_2$-OH		6200	5800	1500	4500	5200	6300
$-CH_2CN$		3200	8000	3400	10000	6400	7300
$-C_4H_9$	50		500	2700	2600	1000	2700
$-CH_2CH_2CH_2$-OH		3500	4600	3400	4200	4000	6700
$-CH_2$-O-CH_2CH_3		1300	1600	2300	2600	1800	6300
$-CH_2CH_2$-O-CH_3		1500	1800	6500	1900	2000	2000
$-CH_2CH_2$-O-CH_2CH_3		1800	1500	4900	2200	1600	2000
$-CH_2$-CH_2CH_2-O-CH_3		2400	2200	5900	1900	2500	6700
$-C_6H_{13}$	9						

Range of cytotoxicity

To illustrate the range of cytotoxicity covered by the test kit compounds, we composed a set of reference chemicals consisting of ionic liquids tested in earlier studies, antifouling biocides and conventional solvents (Table 3).

Looking at the cytotoxicity of the ionic liquid reference substances we can demonstrate that in general, EC_{50} values of ionic liquids cover five orders of magnitude. This range can be interpreted to result from the contributions of different head groups, side chain lengths and anions. We consider substances showing EC_{50} values lower than 100 µM to be significantly cytotoxic. As demonstrated earlier, ionic liquids can reach drastic cytotoxicities (EC_{50}<1 µM) comparable with commonly used and highly active biocides such as carbendazim and 4,5-dichloro-2-n-octyl-3(2H)-isothiazolone (DCOIT).

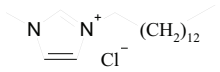
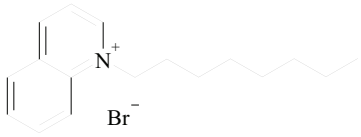
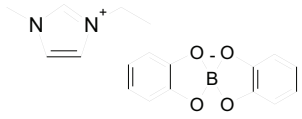
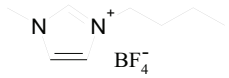
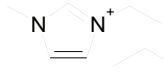
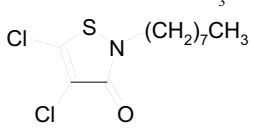
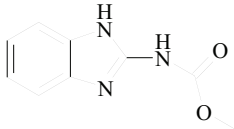
On the other hand the 1-ethyl-3-methyl-imidazolium ethyl sulphate is introduced in our lab as reference ionic liquid exhibiting a comparatively low cytotoxicity (EC_{50}~10000 µM) but which is still some orders of magnitude more cytotoxic than ordinary polar organic solvents, *e.g.* methanol, ethanol or propanol.

Midways of the above described cytotoxicity spectrum (EC_{50}~1000 µM) one can find ionic liquids such as the 1-butyl-3-methyl-imidazolium tetrafluoroborate showing neither a significant nor a negligible cytotoxicity.

Ranking our results for the test kit compounds (Table 1 and Table 2) due to this scheme, 45 of the 101 ionic liquids showed a lower cytotoxicity than the 1-ethyl-3-methyl-imidazolium ethyl sulphate reference for a low cytotoxicity. Furthermore, 52 out of the test substance pool are located around the 1-butyl-3-methyl-imidazolium tetrafluoroborate reference for moderate cytotoxicity. Significant cytotoxicity (EC_{50} < 100 µM) was only found for 4 compounds.

In the following sections the influence of the different structural features (anion, head group and side chain) on the observed biological effects are illustrated.

Table 3 Reference compounds to demonstrate the range of cytotoxicity of ionic liquids, biocides, and common solvents.

structure	name	EC_{50} [µM]
	1-Methyl-3-tetradecyl-imidazolium chloride	0,4
	1-Octyl-quinolinium bromide	1
	1-Ethyl-3-methyl-imidazolium [bis(1,2-benzendiolato)] borate	10
	1-Methyl-3-octyl-imidazolium chloride	100
	1-Butyl-3-methyl-imidazolium tetrafluoroborate	1300
	1-Ethyl-3-methyl-imidazolium ethyl sulfate	8500
	4,5-Dichloro-2-n-octyl-3(2H)-isothiazolone (DCOIT)	1
	Carbendazim	10

	Propanol	100.000
	Ethanol	700.000
CH_3OH	Methanol	1.600.000

Influence of the anion

The halides (chloride, bromide and iodide) do not exhibit an intrinsic anion effect up to a concentration range of 5000 μM.[12] Thus, it was concluded that all observed cytotoxic effects for compounds with these anions (Table 1) can be exclusively attributed to the cation.

In a recent paper from our group, the intrinsic cytotoxic effect of the $[(CF_3SO_2)_2N]^-$ anion is described.[12] Comparing the EC_{50} values of the halides (Table 1) with the cytotoxicity data of the corresponding $[(CF_3SO_2)_2N]^-$ anion (Table 2) this observation could be confirmed for nearly all investigated ionic liquids (with the exception of Py6-4NMe2, Pyr11O2 and N1121O2).

Influence of the head group

The influence of the 7 investigated head groups on cytotoxicity is analysed by comparing the results obtained with ionic liquids containing the same fixed side chain and halides as counter ions (Table1).

As mentioned above, most of the ionic liquids tested in this study have a relatively low cytotoxicity (high EC_{50} values). There are no clear differences in the EC_{50} values within the rows (Table 1) indicating a generally low influence of the head group itself on cytotoxicity. Only for the butyl substituted head groups and for two further compounds (Py1CN Cl and N1123OH Cl) one can observe a more general pattern in the EC_{50} values. By comparing the different head groups with the butyl side chain a clear head group effect on cytotoxicity can be found for the 4-(dimethylamino)pyridinium cations. For this head group, no functionalised side chains were available. Thus, the test kit was supplemented by the ethyl and the hexyl side chain for a comparison with the corresponding imidazolium moieties. For all three side chains the 4-(dimethylamino)pyridinium cation shows a remarkably lower EC_{50} value (by 1-2 orders of magnitude) than the corresponding imidazolium head group. Additionally the well known side chain length effect (decrease in EC_{50} values with elongation of the alkyl side chain) could also be confirmed for the 4-(dimethylamino)pyridinium cation (Figure 1).

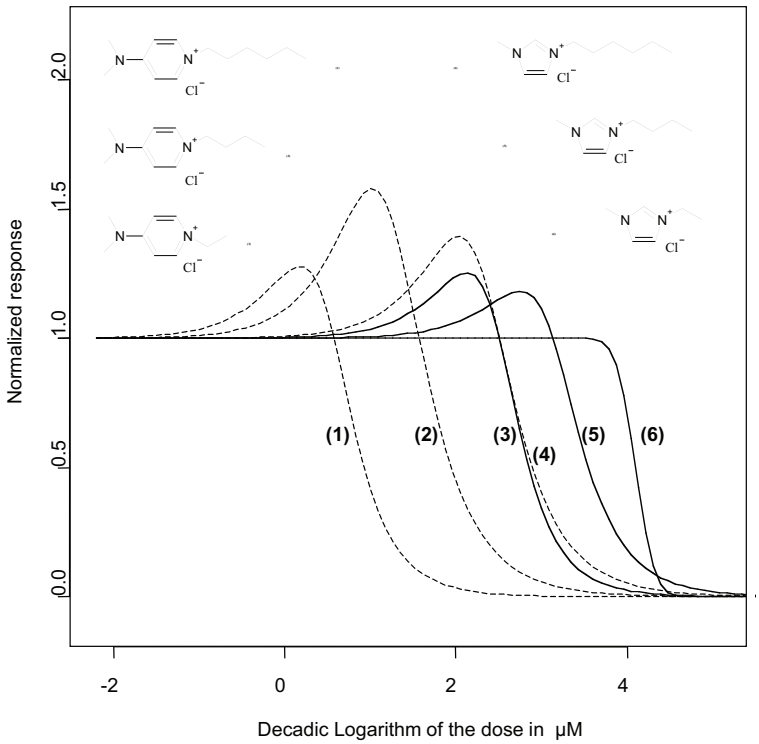


Figure 1 Dose response curves of the 4-(dimethylamino)pyridinium (dashed lines) and imidazolium (bold line) head group with different alkyl chain length. Most of the dose-response-curves show a hormetic effect (response >1) to which no significance is attributed in the context of the present study.

For the cations with the $[(CF_3SO_2)_2N]^-$ anion (Table 2) the general pattern observed for the halides is still present but higher deviations within the head groups can be found. However, a consistently higher cytotoxicity for one head group over the other - except for the 4-(dimethylamino)-pyridinium compounds was not found.

Influence of the side chain

The impact of the side chains on the cytotoxicity of an ionic liquid can be analysed by comparing the results for one head group and one anion, only varying within the ten side chains.

For the ionic liquids with the halides in general, a low cytotoxicity was observable. However, the ethoxymethyl ($-CH_2OCH_2CH_3$) side chain clearly increases the cytotoxicity for all head groups with exception of the piperidinium cation (Table 1) as compared to the isomeric methoxyethyl ($-CH_2CH_2OCH_3$) side chain. This finding could also be confirmed for most of the ionic liquids containing the $[(CF_3SO_2)_2N]^-$ an-ion but the effect is negligibly low (Table 2).

Furthermore, the increased cytotoxicity of the ionic liquids with the $[(CF_3SO_2)_2N]^-$ anion allows for a more differentiated interpretation of the observed effects and the influence of the side chains. Generally the butyl side chain has the strongest cytotoxic influence on all head groups except for the morpholinium head group. Taking a closer look at the imidazolium compounds, it can be found that the introduction of oxygen into the side chain results in a lower cytotoxicity (Figure 2). In particular the terminal

hydroxyl group and the nitrile function reduce the cytotoxicity by about one order of magnitude.

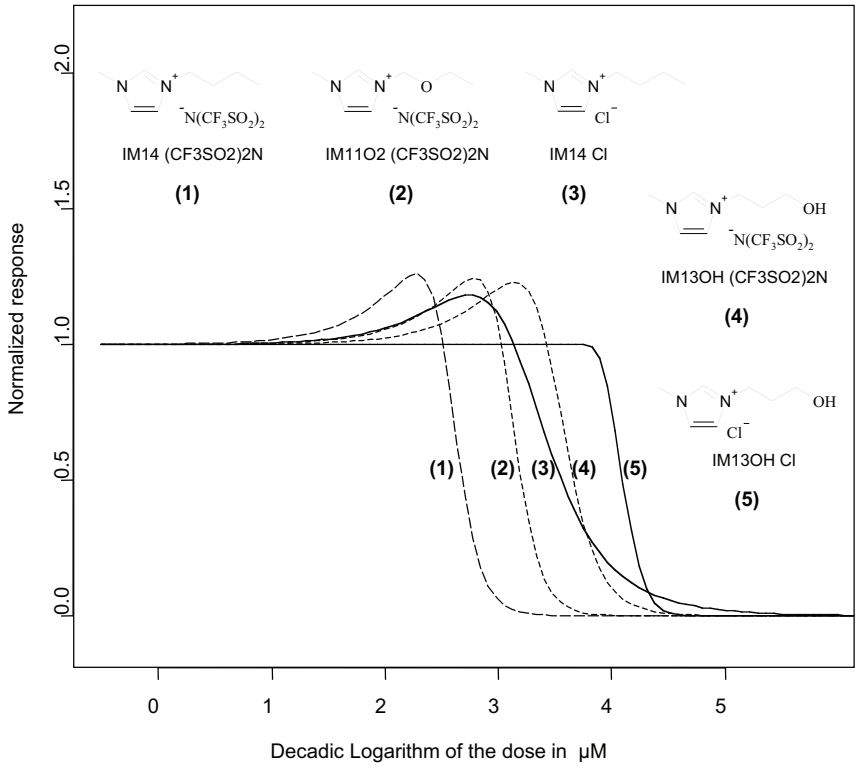


Figure 2 Dose response curves of imidazolium ionic liquids to demonstrate the shift in EC_{50} values for the functionalised side chains compared to the alkylside chain. Furthermore, the anion effect of the $[(CF_3SO_2)_2N]^-$ species (dashed lines) is demonstrated by comparison with the chlorides (bold line). The presented curves illustrate that cations containing terminal hydroxy functions in their side chain are able to equalise the anion effect of the $[(CF_3SO_2)_2N]^-$ moiety.

For the other head groups, a resembling trend is perceptible but not consistent for all compounds (*e.g.* Mor12OH $[(CF_3SO_2)_2N]^-$).

Correlation of cytotoxicity with HPLC lipophilicity data

First of all the results from the HPLC based lipophilicity determination of selected ionic liquid cations are presented. As expected, one can find increasing log k_0 values (increasing lipophilicity) with elongation of the alkyl chain (Table 4). For the functionalised side chains the pattern is similar. The short and polar $–CH_2CN$ side chain exhibits the lowest log k_0 whereas for the long and relatively non polar $–CH_2$-CH_2-O-CH_2-CH_3 ether side chain the highest log k_0 value can be found. Additionally, the terminal hydroxyl groups are to be found more polar than the ether functions (*e.g.* IM13OH versus IM12O1) as predicted from T-SAR. One can find even significant differences in the log k_0 values of IM12O1 and IM11O2. Responsible for this observation are electronical and sterical effects due to the vicinity of the oxygen atom to the positively charged imidazolium core which increases the lipophilicty of the $–CH_2$-O-CH_2-CH_3 chain.

Focusing on the cationic head groups with the butyl side chain, the 4-(dimethylamino)pyridinium moiety exhibits the highest lipophilicity (log k_0=1.08) due to the broadly delocalised positive charge and the lipophilicity of the dimethylamino group. On the other hand the non aromatic and oxygen containing morpholinium head group is found to be the most polar moiety with a log k_0 of 0.18. The remaining cations are located with their log k_0 in between the polar morpholinium and lipophilic 4-(dimethylamino)pyridinium moiety (Table 4).

Plotting the logarithm of the EC_{50} values derived from the cytotoxicity assay versus the logarithm of the HPLC derived lipophilicity parameter k_0 reveals that ionic liquid cations in general span several orders of magnitude considering their lipophilicity and cytotoxicity (recently published data[4] supplemented with results from this study, Figure 3).

Taking a closer look at the results obtained in this study for the ionic liquids containing the $[(CF_3SO_2)_2N]^-$ anion versus those with the small halides (with the same cation) an anion effect becomes obvious (Figure 3). The halides can be described by a good linear correlation between cytotoxicity and lipophilicity, whereas for the corresponding compounds containing the $[(CF_3SO_2)_2N]^-$ anion an increasing deviation to higher cytotoxicities from this linear correlation is observed with decreasing lipophilicity of the ionic liquid cation. For the 4-(dimethylamino)pyridinium compounds combined with $[(CF_3SO_2)_2N]^-$ no, or just a small anion effect was observable.

However, it must be noted that for the ionic liquids cations with low log k_0 values no EC_{50} data are presented in Figure 2 for the halides because no cytotoxic effects were detectable up to concentrations of 20 mM.

Additionally, four data points (IM11O2 Cl, Py2-4NMe2 Cl, Py4-4NMe2 Cl and Py6-4NMe2 Br) for the halides exhibit a significant deviation to higher cytotoxicities from the above mentioned linear correlation.

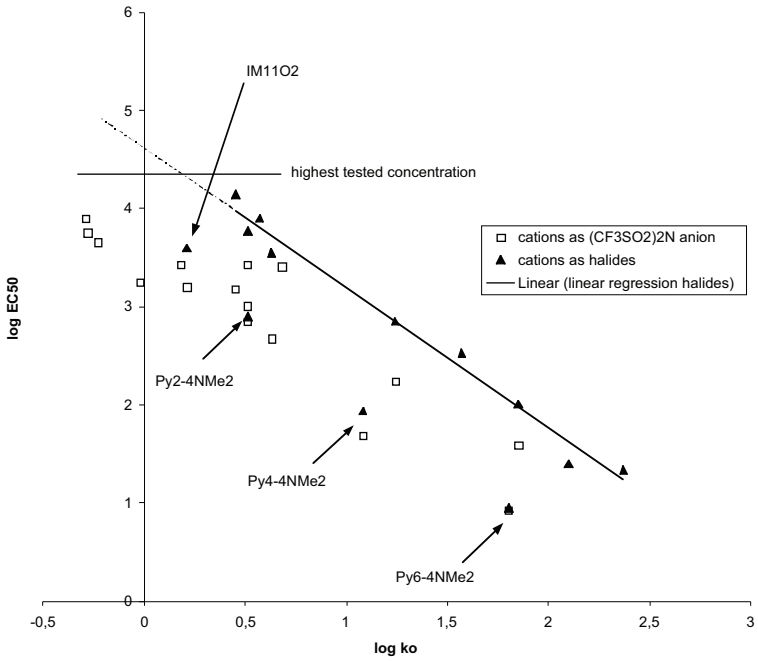


Figure 3 The correlation between lipophilicity log k_o and cytotoxicity log EC_{50} is demonstrated for the cations as halides (closed symbols) and for the $[(CF_3SO_2)_2N]^-$ anions (open symbols). The linear regression (R^2= 0.9825) relates to all cations with halides as anion (except of IM11O2 Cl, Py2-4NMe2 Cl, Py4-4NMe2 Cl and Py6-4NMe2 Br).

Table 4 Lipophilicity parameter log k_0 and log EC_{50} values for all compounds shown in Figure 3. Previously published data are marked with an asterisk.

Compound	log k_o	log EC_{50}	
		Halides	$[(CF_3SO_2)_2N^-]$
IM11CN	-0,29	>4,3	3,9
IM12OH	-0,28	>4,3	3,76
IM13OH	-0,23	>4,3	3,66
IM12O1	-0,02	>4,3	3,25
Mor14	0,18	>4,3	3,48
IM11O2	0,21	3,6	3,2
IM12O2	0,45	4,14	3,18
Pyr14*	0,51	3,77	3,01
Py4*	0,57	3,9	-
IM14	0,63	3,55	2,68
Pip14	0,68	>4,3	3,41
N1124	0,51	>4,3	3,43
Py2-4NMe2	0,51	2,9	2,85
Py4-4NMe2	1,08	1,94	1,85
Py6-4NMe2	1,8	0,95	1
IM13*	0,42	>4,3	-
IM14*	0,63	3,55	2,68
IM15*	0,92	>3	-

IM16*	1,24	2,85	2,24
IM17*	1,57	2,53	-
IM18*	1,85	2,01	1,59
IM19*	2,1	1,4	-
IM1-10*	2,37	1,34	-

Discussion

The correlation of our cytotoxicity data with the HPLC derived lipophilicity parameter gives a more detailed view on the effect of the substructural elements on cytotoxicity and leads to the following inferences.

A good linear correlation (R^2=0.9825) for all halides (different head groups and side chains) from Table 4 can be found which demonstrates the interdependency of lipophilicity and cytotoxicity for ionic liquids cations. Only for one head group (4-(dimethylamino)pyridinium) and one side chain (-CH_2-O-CH_2-CH_3) an obvious deviation from this regression is observed. These compounds exhibit a higher cytotoxicity than it could be expected from the measured lipophilicity parameter log k_0. In general the -CH_2-O-CH_2-CH_3 side chain (with the sole exception of the piperidinium head group) and the 4-(dimethylamino)pyridinium head group show this relative decrease in their EC_{50} values. In consequence, beside the lipophilicity based toxic interaction with membranes the possibility of further and more specific modes of toxic action (*i.e.* reactive interactions, inhibition of certain enzymes, interaction with signal transduction pathways) has to be taken into account.

The lipophilicity and concomitantly the cytotoxicity of the investigated ionic liquid cations are mainly affected by the polarity of the side chain (log k_0 in the range of –0.29 to 2.37). Thus, the side chains of the ionic liquid cations should be considered as one of the key structural elements when identifying toxicophore substructures.

In support of this thesis we could show that the structure of the cationic head group itself - apart from the 4-(dimethylamino)pyridinium moiety - plays only an inferior role in altering the lipophilicity/cytotoxicity of the entire cation (log k_0 from 0.18 to 0.68).

The data points for the cations combined with the $[(CF_3SO_2)_2N]^-$ anion show two trends. For high log k_0 and for the 4-(dimethylamino)pyridinium compounds the cation is dominating the cytotoxicity of the ionic liquid, which can be demonstrated by an approximation of the EC_{50} values for the ionic liquids with either the $[(CF_3SO_2)_2N]^-$ anion and the halides. On the other hand the intrinsic cytotoxicity of the $[(CF_3SO_2)_2N]^-$ anion represents the minimal cytotoxicity for ionic liquids containing cations with low log k_0 values (low cytotoxicity). It is proposed that the intrinsic (cyto)toxicity of this anion species is also based on its lipophilicity (log k_0>2, unpublished data). More detailed studies of toxic effects of the $[(CF_3SO_2)_2N]^-$ on microorganisms, aquatic and terrestrial plants and on terrestrial invertebrates are described by Matzke *et al.*[36] and Juffernholz and co-workers[37].

CONCLUSION

We used the WST-1 cytotoxicity assay for a preselection of toxicologically favourable structural elements for a large number of ionic liquids. Therefore we analysed 101 ionic liquids with different head groups, side chains and anions. We could confirm a previously found linear correlation between ionic liquid cation lipophilicity and cytotoxicity using a HPLC derived lipophilicity parameter.

Considering the structural design of ionic liquids cations, the side chain is the main effector to alter cytotoxicity. According to their relatively low lipophilicity short functionalised side chains can diminish the observed cytotoxcity compared to non polar alkyl chains.

Our HPLC data revealed that the head groups are of minor concern – compared to the side chain - regarding their influence on the lipophilicity and therefore on the ionic liquid cytotoxicity.

Thus, our investigations support the general hypothesis that the cytotoxic effects of ionic liquids can be attributed to lipophilic interactions with cell membranes and cellular proteins leading to disruption of membrane or protein function. Furthermore, the uptake rates of ionic liquids into the cells and thereby their intracellular effect concentrations are closely related to the lipophilicity of the compounds.

However, the 4-(dimethylamino)pyridinium head group in general and the ethoxymethyl ($-CH_2OCH_2CH_3$) side chain exhibit a significant deviation to higher cytotoxicities from the above mentioned linear correlation. This can be interpreted as a first hint for a more specific mode of action not only based on lipophilicity.

Furthermore, the clear influence of the $[(CF_3SO_2)_2N]^-$ anion on cytotoxcity could be verified. However, combined with a polar cationic species this anion effect is shifted to a moderate cytotoxicity.

Nevertheless, the following arising questions concerning the structural design and hazard potentials of ionic liquids are still unanswered and need additional investigations:

i. Is the design with respect to beneficial toxicological properties of ionic liquids compatible to their technical applicability?
ii. Can the observation that the anion effect of the $[(CF_3SO_2)_2N]^-$ is reduced by choosing a cation with neglectible cytotoxicity be expanded to other intrinsically toxic anions?
iii. Can the toxic effects be verified in higher level test systems (*e.g.* plants, animals)?

In general, the used acute cell viability assay and the tested IPC-81 cell line provide a useful tool for the screening of a large number of compounds allowing the identification of possible toxicophore substructures. However, since *in vitro* assays are lacking toxicokinetic, toxicodynamic parameters and some major metabolic pathways, for a detailed (eco)toxicological risk assessment further testing with different organisms and endpoints is required.

EXPERIMENTAL

Cell viability assay

The cytotoxicity assay using the WST-1 reagent was described in detail in[11]. Briefly, promyelocytic rat cells from the IPC-81 cell line are incubated for 4 hours in 96-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)- 5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) reagent. Each plate contained blanks (no cells) and controls (no toxicant). The cell viability assays were generally carried out for a 1:1 dilution series. Each dose response curve was recorded for at least 9 parallel dilution series on three different 96-well plates. Positive controls with Carbendazim were checked in regular intervals.

Dose-response curve parameters and plots were obtained using the drfit package (version 0.05-86) for the R language and environment for statistical computing (www.r-project.org[39]).

The HPLC system

The HPLC system used for deriving the lipophilicity parameters was a Hewlett Packard system Series 1100, with gradient pump, online degasser, autosampler and a Bruker esquire ESI-MS ion trap detector. The column was a MetaChem Polaris ether bridged RP-18 column with 150 mm length, 3 mm inner diameter and 3 μm particle size. A guard column with octadecylsilica material was also used (both Varian, Inc.). The eluent was composed of 0.25 % acetic acid (p.a.) in Milipore (TM) water (pH = 3.2), mixed with gradient grade acetonitrile. The column dead time to was calculated from retention time difference of thiourea with and without column. The equipment dwell volume t_D was quantified by switching from water to 0.1 mM $NaNO_3$ in 10 minutes. Cation retention times from a single gradient run with a gradient time t_G of 10 min were obtained for all substances listed in Table 4. The theoretical background and the calculation of the log k_o values were recently described in detail[4].

Chemicals

All tested ionic liquids were received by Merck KGaA (Darmstadt, Germany) with the exception of 1-ethyl-3-methyl-imidazolium ethyl sulphate, which was obtained from Solvent Innovation (Köln, Germany) and 1-octyl-quinolinium bromide, which was prepared at the ITUC in Jena, Germany. The 4,5-dichloro-2-n-octyl-3(2H)-isothiazolone (DCOIT) was donated by Rohm and Haas (Philadelphia, USA). Carbendazim, acetic acid, acetonitrile, methanol, ethanol, propanol and dimethylsulfoxide were purchased from the Sigma-Aldrich Cooperation (Germany).

Cell culture media, sera, and phosphate buffer were purchased from GIBCO BRL Life Technologies (Eggenstein, Germany). Antibiotics and glutamine were obtained from PAA Laboratories (Cölbe, Germany), and the WST-1 reagent was purchased from Roche Diagnostics (Mannheim, Germany).

Acknowledgements

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PAPER NO. 4:

QUALITATIVE AND QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIPS FOR THE INHIBITORY EFFECTS OF CATIONIC HEAD GROUPS, FUNCTIONALISED SIDE CHAINS AND ANIONS OF IONIC LIQUIDS ON ACETYLCHOLINESTERASE

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Abstract

To contribute to a deeper insight into the hazard potential of ionic liquids to man and the environment an acetylcholinesterase (AchE) inhibition screening assay was used to identify toxicophore substructures and interaction potentials mediating enzyme inhibition.

The positively charged nitrogen atom, a widely delocalised aromatic system and the hydrophobicity of the side chains connected to the cationic head groups can be identified as the key structural elements in binding to the enzymes active site. With respect to this the dimethylaminopyridinium, the quinolinium and the pyridinium head groups exhibit a very strong inhibitory potential to the enzyme with IC_{50} values around 10 μM. In contrast, the polar and non-aromatic morpholinium head group is found to be only weakly inhibiting the enzyme activity with IC_{50} values > 500 μM.

The introduction of polar hydroxy, ether or nitrile functions into the alkyl side chain is shown to be a potent structural alteration to shift the corresponding ionic liquids to a lower inhibitory potential. Supporting this fact, for a series of imidazolium cations a QSAR correlation was set up by the linear regression of the log IC_{50} versus the logarithm of the HPLC-derived hydrophobicity parameter k_0.

Additionally, a broad set of anion species (inorganic, organic and complex borate anions) commonly used as ionic liquid counterions was tested and the vast majority exhibited no effect on AchE. Only the fluoride and fluoride containing anion species, which readily undergo hydrolytic cleavage, can be identified to act as AchE inhibitors.

Introduction

Especially since the imminent intensification of the new EU chemicals legislation REACH (which was put into force in June 2006)[1] the knowledge of (eco)toxicological hazard potentials of chemical substances is attached to increasing importance.

This requires efficient testing strategies, similar to those applied in the field of pharmacological drug design, to generate valid datasets for the registration procedure under REACH and to get a profound insight into mode of toxic action and possible target sites of industrial chemicals. Regarding this issue we trace a T-SAR[2,3] (thinking in terms of structure activity relationships) guided and tiered strategy to:

- Systematically select test compounds and structural elements according to the "testkit concept"[3,4].
- Test the selected substances in a flexible (eco)toxicological test battery at different levels of biological complexity (*e.g.* enzymes, cells, microorganisms and organisms)[5].
- Improve the molecular understanding of (eco)toxicological results by relating them to physicochemical properties[6].

- Identify toxicophore substructures in chemicals and to use this knowledge in the prospective design of inherently safer chemical products.

Following this concept, we are aiming to assess the hazard potential for a set of 79 ionic liquids at the molecular level using an enzyme inhibition test.

The interest in ionic liquids and in their promising physical and chemical properties is still growing rapidly. Diverse applications of this heterogeneous substance class in different fields have been recently described[7-12].

Regarding toxicological issues they are predominantly discussed as "green" or "sustainable" chemicals just based on their negligible vapour pressure, resulting in reduced inhalatory exposure and the absence of flammability.

However, the knowledge about their (eco)toxicological impacts on men and the environment is still very basic and restricted to only a few chemical entities out of the enormous pool of available ionic liquids.[4] Our attempt to fill this gap of information is to test the ionic liquids systematically in different test systems out of our flexible test battery.[13-16]

By applying the above mentioned T-SAR based strategy we subdivide ionic liquids into the cationic head group, the side chain and the corresponding anion to handle this structural variability and to identify how these individual structural variables may evoke inhibitory effects on the enzyme acetylcholinesterase.

The enzyme acetylcholinesterase catalyses the rapid degradation of the neurotransmitter acetylcholine in the synaptic cleft - one of the key mechanisms in neurotransmission in nearly all higher organisms including humans.[17,18] Thus, an inhibition of acetylcholinesterase leads to various adverse effects in neuronal processes such as heart diseases or myasthenia in humans.[19,20] Furthermore, the acetylcholinesterase represented the main target in the development of potent insecticides based on phosphoric acid esters (*e.g.* Parathion) and carbamates (*e.g.* Carbendazim) and therefore the activity pattern of this enzyme in different biological matrices and tissues is used as an established biomarker to monitor the pesticide burden in non-target species.[17,21,22]

We considered the enzyme acetylcholinesterase to be an (eco)toxicologically relevant molecular target for a broad toxicity screening assay with ionic liquids based on several considerations.

vii. The acetylcholinesterase can be found in nearly all higher organisms with a highly conserved and well known active site region which allows for detailed structure activity analysis.[18,23]

viii. The enzyme is a crucial target in the development of insecticides and in human drug design and thus the structure activity relationships leading to enzyme inhibition have been intensively studied for a variety of chemical entities.[24-26]

ix. The amino acid residues with their specific interaction potentials building up the catalytic site and the substrate binding pocket are well known from detailed X-ray studies.[27]

x. As key features for the inhibitory potential a positively charged quaternary nitrogen atom, an electron-deficient aromatic system as well as a certain lipo-

philicity could be identified in all potent reversibly acting inhibitors of the enzyme.[24]

xi. The inhibition assay can be performed in a microtiter plate format and thus represents a fast and cost effective screening tool for an early toxicity testing of industrial chemicals.

With respect to this we could recently show that the nitrogen containing imidazolium and pyridinium head groups in ionic liquids act as potent inhibitors of electric eel acetylcholinesterase. In addition, a correlation between an increasing chain length of the side chains connected to the cationic head groups and an enhanced inhibitory potential of the ionic liquids was found.[28]

The here presented test-kits consisting of a larger variety of head groups, functionalised side chains, anions and substitution patterns are combined to identify the acetylcholinesterase inhibitory potential of ionic liquids in more detail. Furthermore, the necessity of certain molecular interaction potentials in the tested substances to interact specifically with the enzyme is demonstrated by comparing a potent imidazolium based inhibitor ionic liquid with structural analogues lacking the positively charged nitrogen moiety. And finally, the previously described side chain effect[14,28] could be confirmed in the enzymatic test system with a quantitative structure activity correlation.

Characterising such structural features responsible for the toxic mode of action of chemical substances, the so called toxicophores, can help to achieve the goal of a sustainable design of new chemical products by reducing or eliminating toxic or harmful structural elements of an industrial chemical. Additionally, such structural in-sights provide useful knowledge to concurrently maintain or even to improve the de-sired technical features of a substance, described by the so called technicopohres, and to reduce the toxicity of the resulting product.

The test-kit compounds

To investigate the influence of the cationic head groups on the inhibitory potential of the corresponding ionic liquid thirteen different commonly used aromatic, heterocyclic and non-cyclic quaternary nitrogen containing structures were selected (Figure 1). Additionally, the results for a quaternary phosphonium head group are presented for comparison.

To test whether the anion species frequently used in ionic liquids exhibit any intrinsic inhibitory effect on the acetylcholinesterase a selection of the sodium or lithium salts (depending on their availability) of different anions (Figure 2) was investigated in the enzyme assay and the results are presented.

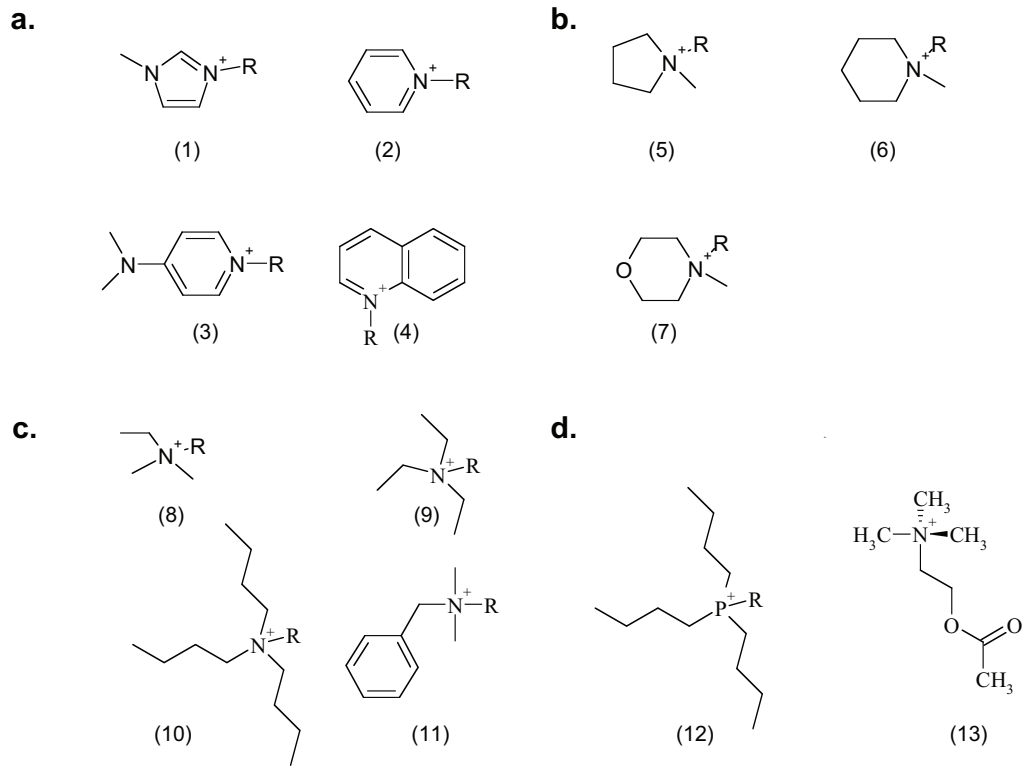


Figure 1 All tested cationic head group structures of ionic liquids and the natural substrate of the enzyme are presented grouped by their specific core elements. The side chain is replaced by the "R" marker. **(a)** Aromatic quaternary ammonium compounds: (1) imidazolium, (2) pyridinium, (3) dimethylaminopyridinium and (4) quinolinium; **(b)** Heterocyclic quaternary ammonium compounds: (5) pyrrolidinium, (6) piperidinium and (7) morpholinium; **(c)** Non-cyclic quatenary ammonium compounds: (8) ethyl-dimethyl-ammonium, (9) triethylammonium, (10) tributylammonium and (11) dimethylbenzylammonium; **(d)** Quaternary phosphonium compounds: (12) tributylphosphonium; **(e)** The natural substrate of acetylcholinesterase: (13) acetylcholine

Additionally, a test-kit comprising five cationic head groups and eleven alkyl and functionalised alkyl side chains containing ether (in varying positions), terminal hydroxy and nitrile functions (Table 2) was set up to demonstrate the impact of the side chain on the enzyme inhibitory potential of the ionic liquids.

Besides, for a series of 1-butylpyridinium ionic liquids the side chain was kept constant and the methyl substitution pattern at the aromatic core structure was altered to elucidate regioselective impacts on the inhibitory potential of the pyridinium containing ionic liquids (see Table 4a).

To demonstrate the need of certain molecular interaction potentials to bind to the ac-tive site of electric eel acetylcholinesterase a test-kit of three substances was arranged containing the potent inhibitor 1-methyl-3-octylimidazolium and two un-charged structural related octyl compounds (see Table 4b).

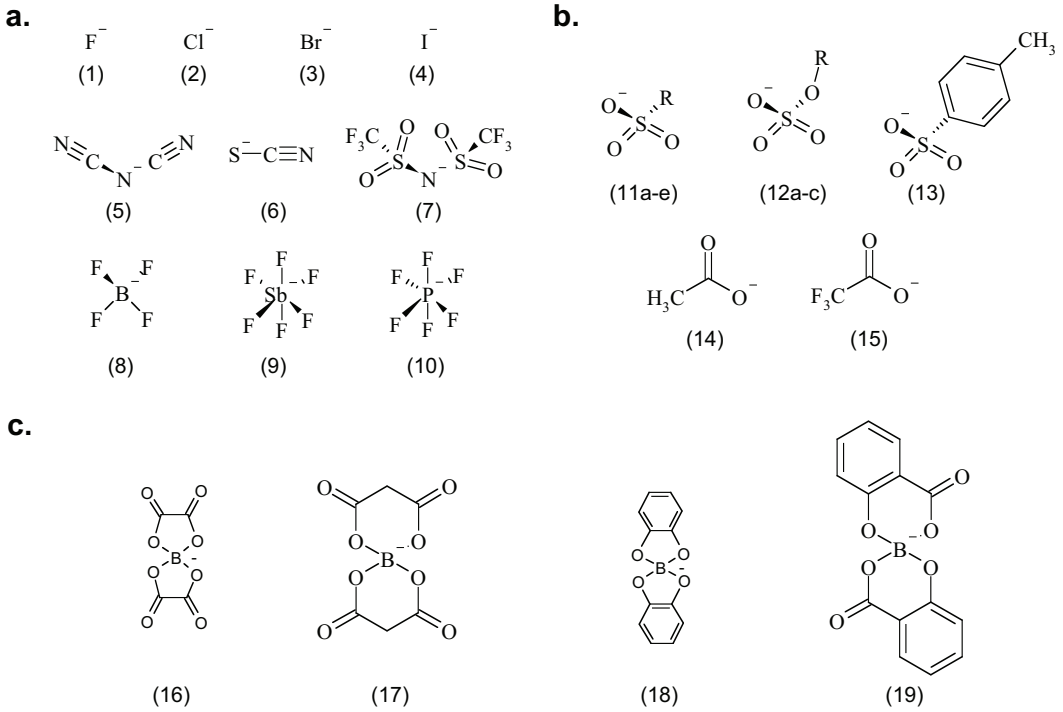


Figure 2 The structures or element symbols of all investigated anion species are shown grouped into inorganic **(a)**, organic **(b)** and complex borate **(c)** anions. The "R" marker replaces different side chains. The names and the corresponding log IC_{50} (μM) values of the tested sodium or lithium salts of the anion species together with the 95% confidence intervals in parenthesis are listed in Table 1.

Acronyms for the ionic liquids

The following system of acronyms is used to facilitate the notation of the ionic liquids. The cation is abbreviated according to the type of the head group as "Py-4NMe2" (dimethylamino)pyridinium, "Py" (pyridinium), "IM" (imidazolium), "Mor" (morpholinium), "Pip" (piperidinium), "Pyr" (pyrrolidinium) and as "N" (quaternary ammonium). The substituents at the nitrogen atom(s) of the head group are given as numbers corresponding to their alkyl chain length. For example, the 1-butyl-3-methylimidazolium cation has the shorthand notation IM14. Ether containing side chains are indicated by splitting the chain in alkyl units with the symbol "O" for the oxygen in between (*e.g.* IM11O2 for 1-(ethoxymethyl)-3-methylimidazolium). Terminal hydroxy or nitrile groups are shortened as OH (*e.g.* IM14OH is 1-(4-hydroxybutyl)-3-methyl-imidazolium) or CN (*e.g.* IM11CN is 1-cyanomethyl-3-methylimidazolium, see also Table 3). The acronyms used for the halides are as in the periodic table.

The identifiers for the cation and for the anion separated by a white space represent the complete acronym for an ionic liquid.

Statistics and effect data modelling

All enzyme inhibition experiments were carried out at least in triplicates with three replicates in each. The normalised (0 to 100 % enzyme activity) concentration response curves were fitted to the multinominal data with the R language and the environment for statistic computing using the probit model for the relation of enzyme activity to the decadic logarithm of the tested concentrations.[29] Confidence intervals (a = 0.05) of the calculated IC_{50} values and the linear regression parameters of the logarithm of the IC_{50} values versus the logarithm of the hydrophobicity parameter k_0 were calculated with the R language as well.

RESULTS

The data for the tested anion species are summarised in Table 1. All calculated IC_{50} values for the ionic liquids and the corresponding confidence intervals are presented in Table 2. In the following subsections the results obtained for the influence of anions, head groups, side chains and regioselective effects on the enzyme inhibitory potential of ionic liquids are systematically presented.

A summary of all acetylcholinesterase inhibition data of ionic liquids generated in our test battery has recently been published in Ranke *et al.*[30]. All relevant results discussed in the following sections are summarised in Table 2.

Influence of the anion species

For the vast majority of the tested anion species no influence on the activity of the acetylcholinesterase was detectable up to the highest tested concentration range of 1000 µM. Only for fluoride and the fluoride containing hexafluoroantimonate and hexafluorophosphate a significant inhibition was found with IC_{50} values of 575 µM (F^-), 219 µM (SbF_6^-) and 145 µM (PF_6^-) respectively (Table 1). The concentration response curves demonstrate the range of the inhibitory potential of the three substances with the PF_6^- anion acting as the strongest inhibitor and the fluoride ion at the upper end of the scale (Figure 3).

Table 1 The anion species are listed due to their numbers referring to Figure 2. The decadic logarithm of the IC_{50} values in µM is presented with the 95% confidence interval in parenthesis. If no complete concentration response curve was obtained in the enzyme inhibition assay the IC_{50} (µM) value is indicated to be higher than the decadic logarithm of the maximum test concentration (*e.g.* >3).

Number	Name	log IC_{50} (µM) AchE
1	fluoride	2.76 (2.69 – 2.82)
2	chloride	> 3
3	bromide	> 3
4	iodide	> 3
5	dicyanamide	> 3
6	thiocyanate	> 3
7	bis(trifluoromethylsulfonyl)amide	> 3
8	tetrafluoroborate	> 3

9	hexafluoroantimonate	2.34 (2.28 – 2.39)
10	hexafluorophosphate	2.16 (2.12 – 2.19)
11a	1-methanesulfonate	> 3
11b	trifluoromethanesulfonate	> 3
11c	1-butanesulfonate	> 3
11d	1-hexanesulfonate	> 3
11e	1-octanesulfonate	> 3
12a	1-methylsulphate	> 3
12b	1-octylsulphate	> 3
12c	1-dodecylsulphate	2.96 (2.91 – 3.00)
13	toluene-4-sulfonate	> 3
14	acetate	> 3
15	trifluoroacetate	> 3
16	bis[oxalato(2-)]-borate	> 3
17	bis[malonato(2-)]-borate	> 3
18	bis[1,2-benzenediolato(2-)-O1,O2]borate	> 3
19	bis[2-hydroxybenzoato(2-)-O1,O2]borate	> 3

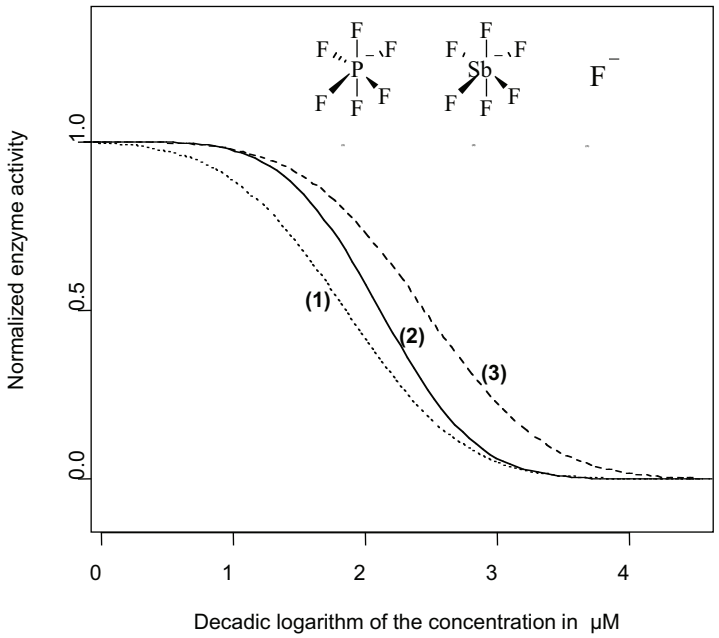


Figure 3 The fitted concentration response curves for the fluoride and two fluoride containing anion species exhibiting an inhibitory effect on the acetylcholinesterase are shown. The exact log IC_{50} (µM) values and the corresponding 95% confidence intervals are given in Table 1.

Additionally, the 1-dodecylsulphate anion was found to be a weak (IC_{50} = 912 µM) inhibitor of the enzymes activity. Since all cationic head groups discussed in the following sections were exclusively tested with either halides (chloride, bromide or iodide) or with the non-inhibiting tetrafluoroborate as counterions, it was concluded that all observed inhibitory effects on the enzyme can be exclusively attributed to the cationic moiety.

Influence of the head group

In general, it could be shown that all investigated cationic head groups containing the butyl side chain affected the activity of electric eel acetylcholinesterase in an inhibitory manner. Furthermore, the two ammonium based cations tetraethylammonium and

decylbenzyldimethylammonium were found to inhibit the enzymes activity (Table 2). To investigate this influence of the cations on the enzyme inhibitory potential of ionic liquids in more detail a subset of different core structures (Figure 4) was combined all carrying the butyl side chain as a reference standard.

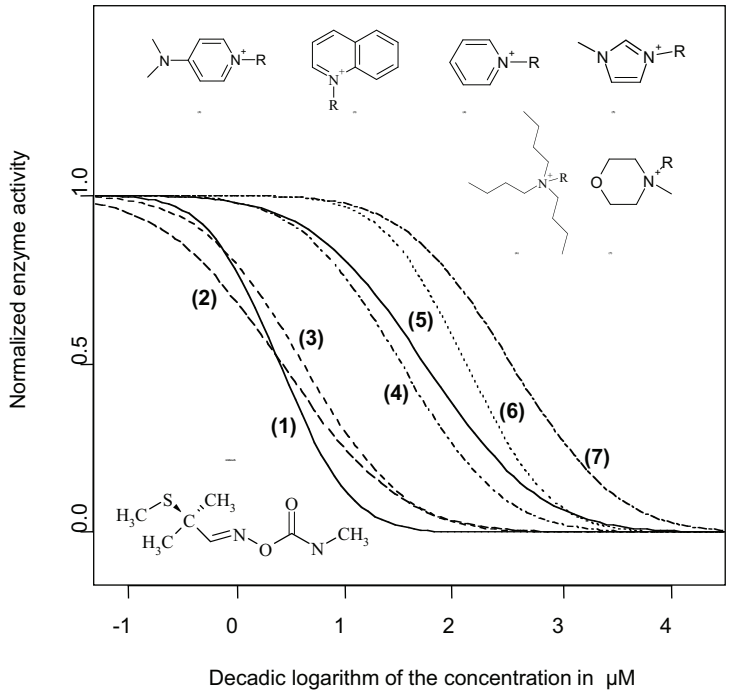


Figure 4 To demonstrate that the cationic head group acts as key structural element in the interaction with the active site of the acetylcholinesterase the fitted concentration response curves of some butyl ("R"= C_4H_9) cations are presented. For comparison the concentration response curve for the strong carbamatype acetylcholinesterase inhibitor Aldicarb, (2-methyl-2-(methylthio)propionaldehyde-O-methylcarbamoyloxime) was added. As counterions for the cationic head groups the halides chloride, bromide and iodide were used. The exact log IC_{50} (µM) data with the corresponding confidence intervals are listed in Table 2.

Table 2 All tested and discussed ionic liquids are listed in alphabetical order. The decadic logarithm of the IC_{50} (µM) is given with the 95% confidence interval in parenthesis. For the substances where no complete concentration response curve was obtained in the enzyme inhibition assay the decadic logarithm of the highest tested concentration is shown (*e.g.* >3). The decadic logarithm of the k_0 values are presented for all imidazolium compounds building up the correlation in Figure 6.

Name	log IC_{50} (µM) AchE	log K_0
1-(2-ethoxyethyl)-1-methylpiperidinium bromide	2.6 (2.58 - 2.63)	
1-(2-ethoxyethyl)-1-methylpyrrolidinium bromide	2.6 (2.58 - 2.63)	
1-(2-ethoxyethyl)-3-methylimidazolium bromide	2.27 (2.25 - 2.29)	0.45
1-(2-ethoxyethyl)pyridinium bromide	1.55 (1.53 - 1.57)	
1-(2-hydroxyethyl)-1-methylpiperidinium iodide	2.34 (2.31 - 2.38)	
1-(2-hydroxyethyl)-1-methylpyrrolidinium iodide	2.63 (2.61 - 2.65)	
1-(2-hydroxyethyl)-3-methylimidazolium iodide	2.96 (2.93 - 2.99)	-0.28
1-(2-hydroxyethyl)pyridinium iodide	2.69 (2.67 - 2.71)	
1-(2-methoxyethyl)-1-methylpiperidinium bromide	2.06 (2.03 - 2.09)	
1-(2-methoxyethyl)-1-methylpyrrolidinium chloride	2.38 (2.36 - 2.39)	
1-(2-methoxyethyl)-3-methylimidazolium chloride	2.58 (2.55 - 2.61)	-0.02
1-(2-methoxyethyl)pyridinium chloride	2.07 (2.05 - 2.09)	
1-(3-hydroxypropyl)-1-methylpiperidinium chloride	2.53 (2.51 - 2.56)	

1-(3-hydroxypropyl)-1-methylpyrrolidinium chloride	2.86 (2.84 - 2.89)	
1-(3-hydroxypropyl)-3-methylimidazolium chloride	2.99 (2.94 - 3.04)	-0.23
1-(3-hydroxypropyl)pyridinium chloride	2.65 (2.62 - 2.68)	
1-(3-methoxypropyl)-1-methylpiperidinium chloride	2.2 (2.17 - 2.23)	
1-(3-methoxypropyl)-1-methylpyrrolidinium chloride	2.74 (2.71 - 2.77)	
1-(3-methoxypropyl)-3-methylimidazolium chloride	2.61 (2.58 - 2.64)	
1-(3-methoxypropyl)pyridinium chloride	2.15 (2.11 - 2.18)	
1-(4-hydroxybutyl)-3-methylimidazolium chloride	2.74 (2.69 - 2.8)	-0.06
1-(8-hydroxyoctyl)-3-methylimidazolium bromide	1.28 (1.22 - 1.33)	0.90
1-(cyanomethyl)-1-methylpiperidinium chloride	2.43 (2.4 - 2.46)	
1-(cyanomethyl)-1-methylpyrrolidinium chloride	2.88 (2.86 - 2.91)	
1-(cyanomethyl)-3-methylimidazolium chloride	2.89 (2.86 - 2.92)	-0.29
1-(cyanomethyl)pyridinium chloride	2.47 (2.45 – 2.49)	
1-(ethoxymethyl)-1-methylpiperidinium chloride	2.14 (2.12 - 2.17)	
1-(ethoxymethyl)-1-methylpyrrolidinium chloride	1.86 (1.84 - 1.87)	
1-(ethoxymethyl)-3-methylimidazolium chloride	2.61 (2.59 - 2.63)	0.21
1-(ethoxymethyl)pyridinium chloride	2.06 (2.02 - 2.11)	
1,3-diethylimidazolium bromide	2.08 (2.02 - 2.13)	0.09
1-butyl-1-methylpiperidinium bromide	1.83 (1.81 - 1.85)	
1-butyl-1-methylpyrrolidinium chloride	1.92 (1.87 - 1.96)	
1-butyl-2-methylpyridinium chloride	0.7 (0.66 - 0.75)	
1-butyl-3-methylimidazolium chloride	1.91 (1.88 - 1.95)	0.63
1-butyl-3-methylpyridinium chloride	1.15 (1.13 - 1.17)	
1-butyl-4-methylpyridinium chloride	1.44 (1.42 - 1.46)	
1-butylpyridinium chloride	1.7 (1.68 - 1.71)	
1-butylquinolinium bromide	0.79 (0.77 - 0.82)	
1-decyl-3-methylimidazolium chloride	1.09 (1.04 - 1.13)	2.37
1-ethyl-3-methylimidazolium chloride	2.06 (2.02 - 2.1)	
1-ethyl-3-propylimidazolium bromide	2.21 (2.17 - 2.25)	0.56
1-ethylpyridinium chloride	2.1 (2.08 - 2.11)	
1-heptyl-3-methylimidazolium chloride	2.07 (2.04 - 2.11)	1.57
1-hexadecyl-3-methylimidazolium chloride	0.68 (0.66 - 0.71)	
1-hexyl-3-methylimidazolium chloride	1.92 (1.88 - 1.96)	1.24
1-hexylpyridinium chloride	1.72 (1.7 - 1.74)	
1-hexylquinolinium tetrafluoroborate	0.48 (0.46 - 0.5)	
1-methyl-1-octylpyrrolidinium chloride	2.36 (2.32 - 2.4)	
1-methyl-3-(2-phenylethyl)imidazolium chloride	1.91 (1.88 - 1.94)	1.01
1-methyl-3-(3-oxobutyl)imidazolium bromide	2.79 (2.75 - 2.84)	0.00
1-methyl-3-(phenylmethyl)imidazolium chloride	2.04 (1.97 - 2.11)	0.83
1-methyl-3-[(4-methylphenyl)methyl]imidazolium chloride	1.86 (1.81 - 1.91)	1.12
1-methyl-3-nonylimidazolium chloride	1.36 (1.31 - 1.42)	2.10
1-methyl-3-octadecylimidazolium chloride	0.96 (0.85 - 1.07)	
1-methyl-3-octylimidazolium chloride	1.6 (1.56 - 1.63)	1.85
1-methyl-3-pentylimidazolium chloride	1.96 (1.94 - 1.99)	0.92
1-methyl-3-propylimidazolium chloride	2.27 (2.24 - 2.3)	0.42
1-octylimidazol	>3	
1-octylpyridinium chloride	1.6 (1.57 - 1.64)	
1-octylquinolinium bromide	< 0	
1-pentylpyridinium bromide	1.52 (1.5 - 1.54)	
1-propylpyridinium bromide	2.22 (2.19 - 2.24)	
4-(2-ethoxyethyl)-4-methylmorpholinium bromide	> 3	
4-(2-hydroxyethyl)-4-methylmorpholinium iodide	2.96 (2.93 - 3)	
4-(2-methoxyethyl)-4-methylmorpholinium chloride	2.98 (2.95 - 3.02)	
4-(3-hydroxypropyl)-4-methylmorpholinium chloride	> 3	
4-(3-methoxypropyl)-4-methylmorpholinium chloride	> 3	
4-(cyanomethyl)-4-methylmorpholinium chloride	> 3	
4-(dimethylamino)-1-butylpyridinium chloride	0.6 (0.57 - 0.62)	
4-(dimethylamino)-1-ethylpyridinium bromide	0.99 (0.97 - 1.01)	
4-(dimethylamino)-1-hexylpyridinium chloride	0.5 (0.48 - 0.52)	
4-(ethoxymethyl)-4-methylmorpholinium chloride	2.96 (2.93 - 3)	
4-butyl-4-methylmorpholinium bromide	2.71 (2.69 - 2.73)	
Aldicarb® (2-methyl-2-(methylthio)propionaldehyde-O-methylcarbamoyloxime)	0.69 (0.63 – 0.75)	
butylethyldimethylammonium chloride	2.06 (2.04 - 2.08)	

decylbenzyldimethylammonium chloride	0.73	(0.68 - 0.77)
tetrabutylammonium bromide	2.3	(2.25 - 2.35)
tetrabutylphosphonium bromide	2.61	(2.58 - 2.64)
tetraethylammonium chloride	2.8	(2.74 - 2.87)

Comparing all these tested butyl-containing head groups the range of the measured IC_{50} values spans nearly three orders of magnitude (Figure 4) within which the most striking inhibitory effect could be detected for the N-dimethylaminopyridinium and the quinolinium head groups where IC_{50} values of 4 μM and 6 μM respectively were calculated from the concentration response data. These values are in the same range as the IC_{50} of the strong and specific carbamate-type acetylcholinesterase inhibitor Aldicarb, (IC_{50} = 5 μM) which was used as positive standard in our assay. Thus, the N-dimethylaminopyridinium and the quinolinium moiety exhibited an inhibitory potential one or even two orders of magnitude higher than that for all other tested butyl-containing ionic liquid head groups in this study.

Looking at the other end of the scale one could find that the polar and non-aromatic morpholinium head group as well as the sterically bulky tetrabutylammonium cation exhibited the lowest inhibitory potential to the enzyme corresponding to IC_{50} values of 513 μM and 197 μM, respectively.

Grouping the remaining cations into that range of enzyme inhibition potential one could find the aromatic pyridinium and imidazolium head groups as well as the heterocyclic but non-aromatic piperidinium and pyrollidinium moieties to show lower IC_{50} values lying closely together in the range from 50 μM ("Py") to 83 μM ("Pyr"). The butylethyldimethylammonium head group which is structurally closely related to the quaternary ammonium moiety in the natural substrate acetylcholine (see Figure 1) exhibited a middle inhibitory potential (IC_{50} = 115 μM) ranging significantly lower than those of the pyridinium (IC_{50} = 50 μM) and imidazolium (IC_{50} = 82 μM) cations. Looking at the solely tested phosphonium based head group one could observe that the tetrabutylphosphonium cation was significantly less active with an IC_{50} of 411 μM than its structural quaternary nitrogen analogue the tetrabutylammonium head group (Table 2).

Comparing the tetraethylammonium cation (IC_{50} = 637 μM) with the imidazolium, pyridinium and dimethylaminopyridinium head groups carrying the ethyl side chain (see Table 2), it is noticeable that the relatively small non-cyclic quaternary ammonium cation exhibited a very weak inhibitory potential towards the acetylcholinesterase.

In contrast, the decylbenzyldimethylammonium cation (IC_{50} = 5 μM) exhibited a slightly stronger effect on the enzyme activity compared to the aromatic imidazolium cation IM1-10 (IC_{50} = 12 μM).

Influence of the side chain

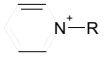
When first looking at the alkyl side chains the previously reported side chain effect could generally be confirmed for the imidazolium cations (IM12 - IM1-18) and for the pyridinium moiety (Py2 - Py8, see Table 2). Remarkably, the butyl side chain seemed to

represent a local minimum in the series of the IC_{50} values. The side chain effect for the imidazolium cations is analysed in more detail in the following.

Furthermore, it could be demonstrated that for the strong inhibiting dimethylamino-pyridinium (Py2-4NMe2 – Py6-4NMe2) and quinolinium (Quin4 – Quin8) head groups the side chain effect was only marginal compared to the less inhibitory imidazolium (IM12 – IM18) and pyridinium (Py2 – Py8, see Table 2) cations. That means that the strong effect on the enzyme activity for the dimethylaminopyridinium and the quinolinium cations is mainly dominated by the cationic core structure whereas for the less active head groups the hydrophobicity of the side chain is the dominating factor mediating the inhibitory potential.

Focusing now on the influence of functionalised side chains on the enzyme activity the results for the ethyl, propyl, butyl and pentyl side chains are presented together with their structurally related (with respect to the chain length) terminal hydroxy, ether and nitrile analogues (Table 3).

Table 3 The IC_{50} values in µM for a selection of different cationic head groups (see Figure 1) combined with varying alkyl side chains and their functionalised analogues (R) are presented to demonstrate the influence of a side chain modification on the enzyme inhibitory potential of the corresponding ionic liquid (for confidence intervals see Table 2). The abbreviations of the side chains and head groups are indicated. If no IC_{50} value could be calculated the highest tested concentration is given (*e.g.* >1000 µM) in the table. The alkyl side chains and the corresponding IC_{50} (µM) values are marked in bold serving as benchmark for the modified analogues with the same atom number in the chain. For all presented cations the halides chloride, bromide or iodide served as counterions.

Structure		**IC_{50} (µM) AchE**				
	head group					
		"Py"	"IM1"	"Mor1"	"Pip1"	"Pyr1"
R						
-C_2H_5	2	**125**	**115**			
-C_3H_7	3	**164**	**185**			
-CH_2CH_2-OH	2OH		913	919	221	430
-CH_2CN	1CN		776	>1000	267	767
-C_4H_9	4		**82**	**513**	**68**	**83**
-$CH_2CH_2CH_2$-OH	3OH		990	>1000	342	731
-CH_2-O-CH_2CH_3	1O2		407	920	139	72
-CH_2CH_2-O-CH_3	2O1		379	999	114	239
-C_5H_{11}	**5**		**92**			
-CH_2CH_2-O-CH_2CH_3	2O2		187	>1000	401	400
-$CH_2CH_2CH_2$-O-CH_3	3O1		405	>1000	158	545

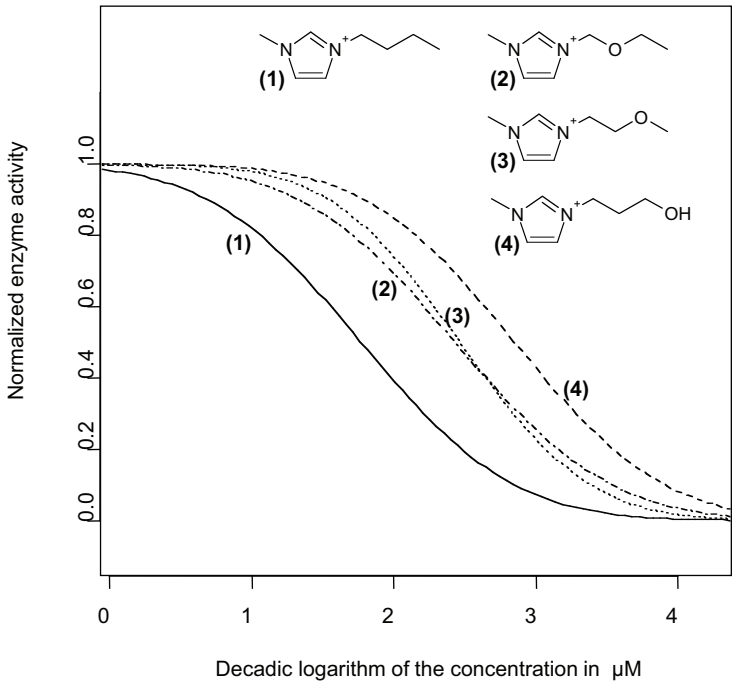


Figure 5 Fitted concentration response curves for 1-butyl-3-methylimidazolium and three 3-methylimidazolium headgroups containing functionalised butyl side chain analogues (ether (2), (3) and hydroxy (4) functions). The chloride serves as counterion for all shown cations. For the exact IC_{50} values in µM see Table 3.

In general, one could find a consistent pattern in which the more polar functionalised side chains exhibited a lower inhibitory potential than their hydrophobic alkyl references. The hydroxy-functionalised side chains, which provide a donor and an acceptor potential for hydrogen bonding - and thus are the most polar derivatives tested – showed the weakest inhibitory potential compared to the less polar (only hydrogen bonding acceptor potential) ether analogues. The short and polar nitrile side chain showed for all tested head groups relatively high IC_{50} values comparable to those for the other polar oxygen containing ether and hydroxy residues.

For the imidazolium head group connected to a four-atom-containing side chain it could be shown that the introduction of a hydroxy function into the alkyl side chain was able to shift the IC_{50} one order of magnitude to the side of lower enzyme inhibition. The ether-containing side chains are lying in between the highly polar hydroxy side chain and the butyl reference and no significant regioselective effect with respect to the position of the ether bridge was observable (Figure 5).

The above presented results for the alkyl and functionalised side chains reinforce the assumption that the hydrophobicity of the side chain for one cationic head group is a key parameter in predicting the acetylcholinesterase inhibitory potential of the corresponding ionic liquid when looking at a relatively weak inhibiting head group (*e.g.* imidazolium, pyrrolidinium or morpholinium). With respect to this for a series of imidazolium head groups connected to different alkyl and functionalised side chains a quantitative structure activity relationship was derived by the linear regression of the log

IC_{50} values versus the logarithm of the HPLC-derived hydrophobicity parameter k0 of the ionic liquids cations (Figure 6). A good correlation (r^2 = 0.79) with three outliers could be found for a decrease in the IC_{50} values with increasing hydrophobicity of the side chain.

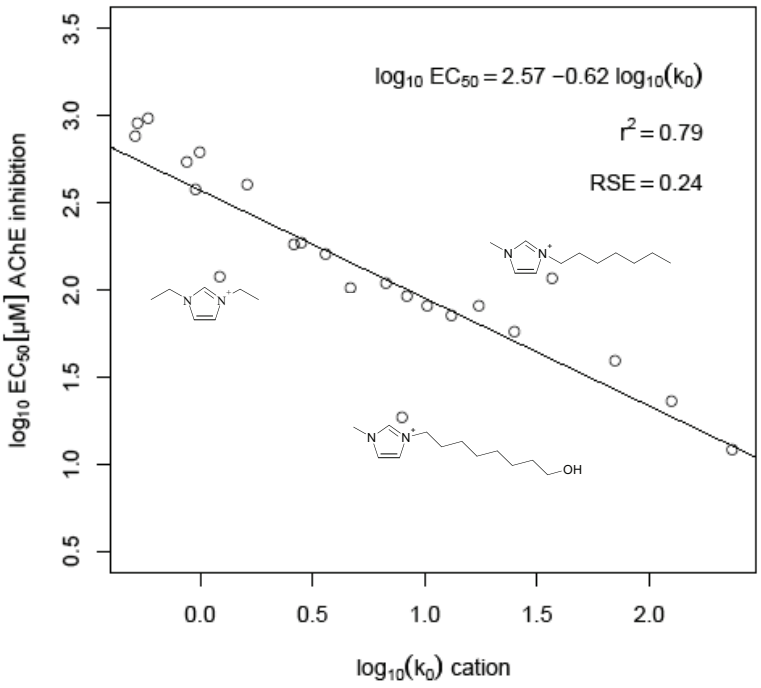
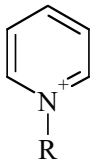
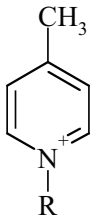
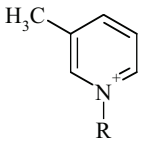
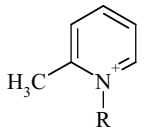
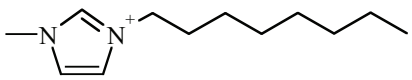
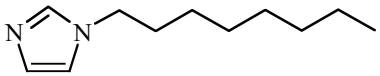
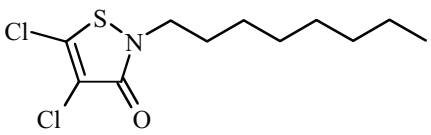


Figure 6 Quantitative structure activity relationship between the decadic logarithm of the hydrophobicity parameter k_0 and the log IC_{50} values (µM) of a series of imidazolium headgroups (see Table 2). The three prominent outliers are indicated by their structural formular. The calculated regression function is specified together with the corresponding quadratic correlation coefficient (r^2) and the residual standard error (RSE).

Regioselective and general structural considerations

To demonstrate the strong influence a simple structural alteration in a chemical entity can exert on the interaction with a biomolecule the IC_{50} values for a series of 1-butylmethylpyridinium cations are presented (Table 4a). The introduction of a methyl substituent at the aromatic core structure significantly increased the inhibitory potential of the 1-butylpyridinium cation. Additionally, it could be observed that the IC_{50} values decreased when going from the linear 1-butyl-4-methylpyridinium to the angled 1-butyl-2-methylpyridinium configuration showing more structural analogy to the natural substrate acetylcholine. Looking at the toxicity range of the substituted pyridinium type ionic liquids one could state that they all exhibited a rather high acetylcholinesterase inhibitory potential with a subtle regioselective influence.

Table 4 **a)** To demonstrate regioselective impacts on the enzyme inhibitory potential three methyl substituted 1-butylpyridinium cations with their corresponding IC_{50} values in µM are presented. The unsubstituted 1-butylpyridinium serves as reference. The 95% confidence intervals are given in parenthesis and for all cations the chloride serves as counterion. **b)** To show the necessity of certain molecular interaction potentials of a substance to interfere with the active site of the AchE the potent inhibitor 1-methyl-3-octylimidazolium chloride is compared with its mainly uncharged (pKs~6 and pH 8.0 of the testbuffer) analogue 1-octylimidazol and with a fivemembered aromatic heterocycle containing and highly reactive 2-octyl-isothiazol-3-one biocide. The IC_{50} value in µM of the imidazolium compound is given with the 95% confidence interval in parenthesis. For the two remaining substances the highest tested concentration in the enzym ihnhibition assay is presented.

	structure	name	IC_{50} (µM) AchE
a)		1-butylpyridinium	50.0 (48.3 – 51.5)
		1-butyl-4-methylpyridinium	27.4 (26.3 – 28.6)
		1-butyl-3-methylpyridinium	14.1 (13.5 – 14.8)
		1-butyl-2-methylpyridinium	5.1 (4.55 – 5.59)
b)		1-methyl-3-octylimidazolium	39.4 (36.7 – 42.3)
		1-octylimidazol	> 2000
		4,5-dichloro-2-octylisothiazol-3-one	> 1000

However, comparing the observed IC_{50} values for the positively charged aromatic 1-methyl-3-octylimidazolium, the uncharged (under the assay conditions) 1-octylimidazol and the aromatic, electron-deficient and highly reactive 4,5-dichloro-2-octylisothiazol-3-one the necessity of certain molecular interaction potentials for the binding to the active site of acetylcholinesterase becomes obvious (Table 4b). Neither the structurally closely related but uncharged 1-octylimidazol nor the octyl side chain containing and heteroaromatic isothiazolone structure exhibited any inhibitory effect in the tested concentration range. Thus, for a strong specific interaction of a compound with the active site of acetylcholinesterase the positively charged nitrogen atom could be identified to act as the key molecular interaction potential. The hydrophobicity of the

side chain and the presence or absence of an aromatic ring system modulated the strength of the resulting inhibitory potential.

Discussion

It is the aim of this study to get a deeper insight into the (eco)toxicological impacts of structural variations in ionic liquids substructural elements, built up by the positively charged head group substituted with one or more different side chains and the corresponding anionic species, in an (eco)toxicologically relevant molecular test system.

To discuss the above presented results the catalytic cycle of the enzyme and the essential amino acid residues involved in substrate binding are shortly presented. The active site of acetylcholinesterase is located at the bottom of a narrow gorge. The gorge is lined with hydrophobic aromatic amino acid residues and the entrance is built up with negatively charged residues. The active centre can be divided into the catalytic esteratic site where the acetyl group of the substrate is bound and the anionic site where the quaternary ammonium moiety of the acetylcholine is stabilised via a cation-p interaction with the essential tryptophane residue Trp 84.[31] Additionally, a peripheral anionic site (PAS) could be identified at the entrance of the narrow gorge where the substrate acetylcholine is bound to Trp 279 again via cation-p interactions and is thereby orientated towards the active centre.[32] The catalytic cycle of the enzyme can be described in three steps. At first the substrate is attracted by the negative potential surrounding the entrance of the gorge and binds to Trp 279. The so orientated substrate molecule is subsequently transferred through the hydrophobic gorge and bound to the active centre with the positively charged nitrogen moiety interacting with the Trp 84 and the acetyl group lying at the esteratic site. The ester bond is hydrolysed and the resulting choline moiety leaves the catalytic site via the gorge. In the last step a water molecule regenerates the acetylated enzyme and the acetate anion is expelled via the channel formed by the hydrophobic gorge.[33]

Thus, competitive inhibitors of acetylcholinesterase can act via two distinct mechanisms. They can either bind directly to the active site and thereby inhibit the cleavage of the natural substrate or inhibitors can bind to the PAS and block substrate traffic into and out of the catalytic centre by steric interference or allosteric alteration of the enzymes active centre.[32]

With respect to this the identified molecular interaction potentials found for the inhibiting ionic liquids can be interpreted. The cationic head groups are attracted by the negative surface potential of the enzyme and bind via the positively charged nitrogen atom in a competitive manner to the essential tryptophane residues at the catalytic site or the PAS. Especially the quinolinium and dimethylaminopyridinium head groups can bind strongly via p-p interactions to the Trp 279 at the PAS due to their large aromatic systems. The natural substrate acetylcholine or the acetylthiocholine used in our assay are only weakly bound to the PAS, which explains the strong inhibiting effect of this two aromatic ionic liquid head groups. The aromatic stacking interactions for the

quinolinium and the dimethylaminopyridinium head groups are that strong, that the side chain effect is negligible.

For the remaining aromatic head groups - the pyridinium and imidazolium cations - the side chain enforces the weaker p-p interactions of the smaller aromatic systems by hydrophobic interactions with the amino acid residues lining the narrow gorge. Thus, the correlation of increasing side chain hydrophobicity and decreasing IC_{50} values for the different head groups can be explained and even quantified in a QSAR correlation in the case of the imidazolium cations. The most prominent outlier in the linear regression the IM18OH may be explained by an additional interaction at an allosteric subsite far away from the active site.

The morpholinium head group is lacking the aromatic p-p interaction potential and provides the lowest hydrophobic interaction potential compared to all other tested cations and therefore exhibits the observed low inhibitory potential. The introduced free electron pairs at the oxygen atom of the morpholinium head group are only weak donors or acceptors for p-p interactions. It is more likely that they are involved into strong hydrogen bonding interactions to water molecules making the morpholinium head group even more hydrophilic.

The quaternary ammonium head groups as well as the remaining heterocyclic cations are also able to bind only via cation-p and lipohilic interactions to the tryptophane residues and the aromatic gorge. The significance of a p-p interaction for a tight binding to the enzyme can also be demonstrated when comparing the non-aromatic quaternary ammonium compound decylbenzyldimethylammonium with the 1-decyl-3-methylimidazolium cation. The benzyl residue connected to the ammonium moiety interacts slightly stronger with the tryptophane ring system than the smaller aromatic imidazolium system.

The tested phosphonium head group is more bulky and subsequently the positive charge density is decreased compared to its nitrogen containing analogue. Thus, the interactions with the tryptophane residues in the enzyme are smaller resulting in a higher IC_{50} for the tetrabutylphosphonium cation.

The local maximum in the inhibitory potential for the butyl side chain observed within the series of imidazolium and pyridinium ionic liquids may be explained by a strong interaction at the catalytic site in addition to the binding at the PAS. The butyl side chain is short enough to fit in the active centre whereas the longer side chains only allow for binding at the PAS.

Additionally, the observed regioselective effects can be interpreted by the fact that the 1-butylpyridinium compounds can bind directly at the active site. The angled configuration is able to interact stronger with the Trp 84 then the more stretched isomers due to its structural homology to the choline moiety. This angled configuration allows for an optimised orientation of the 2-methylpyridinium moiety towards the anionic subside in the active centre of the AchE.

Considering the negative surface potential at the entrance to the catalytic centre one would expect all anion species to exert no effect on the enzyme activity. Our results

confirm this presumption with the only exception of the fluoride anion and the fluoride containing SbF_6^- and PF_6^- species. Both species are known to readily undergo hydrolysis in aqueous media[34-36] and thus the fluoride seems to be the active compound and acetylcholinesterase inhibition by F^- has already been described in the literature.[37,38] The relatively low IC_{50} values for the SbF_6^- and PF_6^- anions compared to the fluoride are due to the fact that per mole SbF_6^- or PF_6^- six moles of fluoride may theoretically be released.

The very weak inhibitory potential observed for the 1-dodecylsulphate anion is presumably due to non-specific detergent like inactivation of the acetylcholinesterase.

CONCLUSION

Using different test-kits of anion species, cationic head groups and functionalised side chains connected to this head groups we were able to identify three molecular key interaction potentials for the inhibitory effect of a broad variety of ionic liquid species on the enzyme acetylcholinesterase. Considering these interaction potentials and the molecular interaction potentials provided by the catalytic centre of the enzyme the observed structure activity relationships of the tested substances can qualitatively and quantitatively be described. Thus, the applied enzyme inhibition screening assay with the electric eel acetylcholinesterase seems to be a valid and useful tool in a flexible (eco)toxicological test battery to analyse the impact of structural elements on the toxic mode of action of chemical substances. That implies, that the AchE inhibition assay can be used to identify toxicophore substructures in a chemical entity and thereby is able to support the design of new inherently safer and hence sustainable chemical products.

In detail, for the ionic liquids the positively charged nitrogen atom, a broad delocalised aromatic ring system and a certain hydrophobicity could be shown to be the mediators for the acetylcholinesterase inhibition potential. With respect to this the dimethylaminopyridinium and the quinolinium head groups were identified to be very strong inhibitors of the enzyme. This trend has also been recently described for the cytotoxicity of this two head groups[6,14] and thus the dimethylaminopyridinium and the quinolinium cations should be avoided when aiming at the design of non-toxic ionic liquids. The results obtained for the pyridinium and methylated pyridinium head groups confirm our previous results[28] marking these cations also as strong AchE inhibitors. In contrast, the morpholinium head group was found to be only weakly inhibiting or even inactive depending on the connected side chain. Again this is in well accordance with recently published cytotoxicity data generated in our flexible test battery.[14]

Furthermore, the well-known side chain effect[28] is confirmed for the imidazolium and pyridinium cations and could even be described by a QSAR correlation for a series of imidazolium ionic liquids. Additionally, the hydrophobicity of the side chain was identified to be a potent structural element to alter the enzyme inhibitory potential of a broad spectrum of ionic liquid head groups. For example, the IC_{50} of IM14 is decreased

one order of magnitude by the introduction of a hydroxy function into the side chain resulting in the IM13OH cation.

Since the vast majority of the tested anion species exhibited no inhibiting effect to the enzyme this structural element can be used to tune and improve the technicophore properties of the ionic liquids. Only fluoride or fluoride containing anions, which readily undergo hydrolytic cleavage, should be avoided.

Putting together our results, we have found a set of structural elements, which allows for the rough and fine-tuning of the molecular toxicity towards the electric eel acetyl cholinesterase. The inherent head group effect can be modulated to lower inhibitory potentials by choosing polar, non-aromatic head groups or incorporating polar hydroxy, ether or nitrile functions into the side chains connected to the cationic core structure. The anion species represents the most promising structural element to tune the technical properties of the ionic liquids because a big pool of different anions (inorganic, organic and complex borate species) was shown to be inactive in the AchE inhibition assay.

However, it should be mentioned that the design of inherently safer chemical products with optimised technological and economical features is not an easy task and often leads to goal conflicts. These conflicts can only be overcome in a close cooperation between industry and academic research groups.

EXPERIMENTAL

Chemicals

All tested ionic liquids, the 1-octylimidazol and the sodium or lithium salts of the investigated anion species were received by the Merck KGaA (Darmstadt, Germany) with the exception of 1-octyl-quinolinium bromide, which was prepared at the ITUC in Jena, Germany.

The 4,5-dichloro-2-octylisothiazol-3-one was donated by Rohm and Haas (Philadelphia, USA). Stock solutions of all test substances were prepared in methanol or dimethylsulfoxide depending on their solubility.

2-methyl-2-(methylthio)propionaldehyde-O-methylcarbamoyloxime (Aldicarb®), acetic acid, acetonitrile, methanol and dimethylsulfoxide as well as bovine serum albumin, and sodium hydrogen phosphate were purchased from the Sigma-Aldrich Cooperation (Steinheim,Germany).

Sodium hydrogen carbonate was purchased from GIBCO BRL Life technologies (Eggenstein, Germany) and acetylthiocholine iodide was provided by 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 mg Protein^{-1}.

Acetylcholinesterase inhibition assay

The inhibition of the acetylcholinesterase was measured using a colorimetric assay based on the 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 assay is described in detail in Stock *et al.*[28] Briefly, a dilution series of the test substances in phosphate buffer (0.02 M, pH 8.0) containing max. 1 % methanol was prepared directly in the wells of a 96-well microtiter plate. DTNB (2 mM, 0.185 mg mL^{-1} $NaHCO_3$ in phosphate buffer pH 8.0) and the enzyme (0.2 U mL^{-1}, 0.25 mg mL^{-1} bovine serum albumin in phosphate buffer pH 8.0) were added to each well. The reaction was started by the addition of acetylthiocholine iodide (2 mM in phosphate buffer). The final testconcentrations were 0.5 mM of DTNB and acetylthiocholine iodide and 0.05 U mL^{-1} acetylcholinesterase, respectively.

Enzyme kinetics were measured at 405 nm in 30 seconds intervals in a microplate-reader (MRX Dynatech) for a time period of 5 minutes. The enzyme activity was expressed as OD min^{-1} from a linear regression. To avoid false positive results in preliminary tests it was shown that none of the test substances interacts with the formed thiocholine during the assay (data not shown).

Determination of the hydrophobicity parameter k_0

The hydrophobicity parameter k_0 of the ionic liquids was derived using a gradient run HPLC method. The method, the theoretical background and the calculation of the log k_0 values were recently described in Ranke *et al.*[6] Briefly, the HPLC system used for deriving the hydrophobicity parameters was a Hewlett Packard system Series 1100, with gradient pump, online degasser, autosampler and a Bruker esquire ESI-MS ion trap detector. The column used was a MetaChem Polaris Ether bridged RP-18 column with 150 mm length, 3 mm inner diameter and 3 µm particle size. A guard column with octadecylsilica material was also used (both Varian, Inc.). The eluent was composed of 0.25 % acetic acid (p.a.) in Milipore (TM) water (pH = 3.2), mixed with gradient grade acetonitrile. The column dead time to was calculated from retention time difference of thiourea with and without column. The equipment dwell volume t_D was quantified by switching from water to 0.1 mM $NaNO_3$ in 10 minutes. Cation retention times from a single gradient run with a gradient time t_G of 10 min were obtained for all substances listed in Table 2.

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PART III:

FINDINGS OF THE CASE STUDIES AND OUTLOOK

1 Discussion

In the following, the results obtained from the two case studies of isothiazol-3-one biocides and selected ionic liquids will be summarised and discussed. The question whether and how the presented T-SAR based and tiered testing strategy can contribute to a mode-of-action-based hazard assessment will be addressed. Furthermore, taking the ionic liquids as an example for a technologically promising substance class, some remarks will be made on how a sustainable design of new chemicals can benefit from a molecular T-SAR guided hazard assessment

The case studies - forming the central component of this thesis - presented in part II were performed with respect to the following purposes:

- To extend the pool of existing test systems of the flexible test battery at the UFT to cellular and molecular test systems that are able to screen the toxicity of electrophilic organic chemicals.
- To validate these test systems using isothiazol-3-one biocides as highly electrophilic and environmentally relevant reference substances.
- To identify structure-activity relationships for a refined hazard assessment of the isothiazol-3-ones aiming for a better description of the risk indicator "uncertainty" for these biocides.
- To use established cellular and molecular test systems to identify possible modes of toxic action of ionic liquid structures. Especially, the question of how the different substructural elements (head group, side chain and anion) resembling an ionic liquid modulate the observed toxic effects was addressed.

1.1 Isothiazol-3-ones

The four isothiazol-3-ones (see Figure 1) N-methylisothiazol-3-one (MIT), 5-chloro-N-methylisothiazol-3-one (CIT), N-octylisothiazol-3-one (OIT) and 4,5-dichloro-N-methylisothiazol-3-one (DCOIT) were tested in three different cellular test systems (Paper No.1) and three molecular test systems (Paper No. 2). The following subsections discuss the results of these integral and molecular assays. Also, it is shown how both levels can be combined to form a detailed picture of the hazard potential of these biocides.

1.1.1 Cellular test systems

The results of the cellular assays showed that all of the four biocides exhibited the same toxicity pattern - *i.e.* the same order of EC_{50} values - in human hepatocytes (Hep G2), in the marine bacterium *Vibrio fischeri* and in the limnic green alga *Scenedesmus vacuolatus*. As EC_{50} values are a measure of absolute toxicity, it was consistently found

that the chlorinated derivatives CIT and DCOIT showed a significantly higher toxicity than their non-chlorinated analogues MIT and OIT. Furthermore, results indicate that the hydrophobicity of the compounds seems to be an important parameter influencing the toxicity of the isothiazol-3-ones. It was found that OIT (log $P_{O/W}$ = 3.3) was consistently more toxic than MIT (log $P_{O/W}$ = -0.49). The same holds true for the comparison of the chlorine substituted derivatives DCOIT (log $P_{O/W}$ = 4.79) and CIT (log $P_{O/W}$ = 0.53) of which DCOIT exhibited the lower EC_{50} values in each of the three test systems corresponding to a higher toxicity.

Comparing the test systems revealed that the human liver cell line Hep G2 was the least sensitive species followed by the marine luminescence bacterium *Vibrio fischeri*. The limnic green alga *Scenedesmus vacuolatus* was found to be the most sensitive species for each of the four isothiazol-3-ones. This is in line with published findings that identified this alga to respond very sensitively to cations and anions of various ionic liquids (Matzke *et al.*, 2007; Stolte *et al.*, 2007b). Also, algae have been identified as highly sensitive test species for a broad range of xenobiotics (Arrhenius *et al.*, 2006; Backhaus *et al.*, 2004; Niederer *et al.*, 2004). The data presented in paper No. 1 provides further evidence, that the 24 h reproduction inhibition assay with *Scenedesmus vacuolatus* constitutes the most sensitive test system out of the flexible test battery. This implies that this assay has to be given top priority in the screening of chemicals to identify toxic effects of chemicals on the cellular level.

Although the human liver cells even had been incubated for 48 h with the biocides, this test system exhibited the lowest sensitivity. The liver represents the main target organ for the detoxification and processing of toxicants so that liver cells are naturally well equipped with mature defence systems to handle toxic substances. This is likely to be the reason for the observed insensitivity of the Hep G2 cell culture compared to the other test species. That means, if excess toxicities can be observed in the Hep G2 assay, drastic specific effects have to be taken into considertaion for the hazard assessment of the corresponding chemicals. Thus, this test system can be used to identify chemicals with high hazard potential that need to be tested in more complex test systems (*e.g.* animal testings). It has already been proven that human acute toxicities of various substances can be predicted from Hep G2 EC_{50} data (Dierickx, 2005). Besides, since Hep G2 cells express active cytochrome P450 monooxygenase systems, they can be used to identify the bioactivation of substances owing to the formation of toxic metabolites during the phase I metabolism (Hewitt and Hewitt, 2004).

However, the fact that all isothiazol-3-ones tested exhibited consistently the same order of toxicity in each of the three test systems suggests that their modes of toxic action target basal cellular structures and functions. As described in detail in paper No. 1, the toxic ratio approach introduced by Lipnick *et al.* (1987) was used to identify whether the observed cytotoxicities of the isothiazol-3-one biocides can be explained by a narcotic mode of action (*i.e.* baseline toxicity) or if an excess toxicity indicates a more specific mode of action. Since it normalises these absolute cytotoxicities to the expected baseline toxicities of the corresponding isothiazol-3-ones, the toxic ratio allows for a more differentiated analysis of the observed toxic effects than solely measuring EC_{50}

values. Calculating the toxic ratios for each biocide revealed that only CIT and MIT exhibited a significant excess toxicity (TR>>10) in all three test systems, whereas the toxic ratios for the hydrophobic OIT and DCOIT were found to be in the range (TR≤10) of solely narcoticly acting chemicals. Hence, from the toxic ratio analysis of the cellular assays two modes of toxic action could be identified for the tested isothiazol-3-one biocides. The small and polar molecules MIT and CIT seem to act predominantly via a specific mode of toxic action most likely originating from their electrophilic reactivity. In contrast, the acute toxicity of the hydrophobic OIT and DCOIT seems to be dominated by a narcotic mechanism. To identify the molecular details of these modes of action the isothiazol-3-ones were tested in three molecular test systems.

1.1.2 Molecular test systems

Since the isothiazol-3-ones are known to be strong electrophiles, the most abundant cellular nucleophile glutathione (cellular concentration around 20 mM) was chosen as target to analyse the toxic mechanisms of the biocides. As described in paper No. 2 the reaction rate of the isothiazol-3-ones with glutathione, the inhibition of isolated and cellular glutathione reductase (GR) activity and the cellular GSH/GSSG ratio were selected as molecular endpoints.

The pseudo first order rate constants for the reaction of the biocides with an excess of GSH revealed that the chlorine substitution at the aromatic core structure dramatically increased the intrinsic reactivity of these compounds. The double chlorinated DCOIT even reacted that fast (within seconds) that it was impossible to record a reaction kinetic with the method used. For the mono-chlorinated CIT a reaction rate constant could be calculated that was significantly higher than those obtained for the non-chlorinated derivatives MIT and OIT. Independently from the enormous difference in their hydrophobicities, MIT and OIT showed nearly the same reactivity towards GSH. Thus, this test system confirms the order of reactivity predicted by the T-SAR analysis as well as by quantum chemical calculations and proposed reaction mechanisms (Morley *et al.*, 1998; Morley *et al.*, 2007), which also identify the chlorine substitution in isothiazol-3-ones to be a key structural parameter dominating their intrinsic electrophilicity.

This structure-activity relationship for the thiol reactivity of the isothiazol-3-ones could also be confirmed in the remaining test systems. It could be shown that both chlorinated biocide species completely inhibited isolated and cellular GR activity most likely owing to an irreversible reaction with the essential thiolate moiety at the active site of the enzyme. In contrast, the non-chlorinated analogues MIT and OIT exhibited no inhibitory potential on the enzyme´s activity. Finally, using the Hep G2 cells it was demonstrated that CIT and DCOIT dramatically depleted the total cellular glutathione content in a dose and time dependent manner. Especially, DCOIT increased the amount of cellular GSSG entailing a severe shift in the GSH/GSSG ratio and the breakdown of the cellular reduction potential. Again, the effects of the non-chlorinated MIT and OIT on the cellular GSH/GSSG ratio were negligible.

Taking together the results from the cellular toxicity assays and from the molecular test systems, the excess toxicities observed for CIT and MIT on the cellular level can be explained by their intrinsic reactivity towards GSH and other essential sulfhydryl groups in proteins. Furthermore, the observed difference in the excess toxicities of MIT and CIT (CIT exhibited a significantly higher excess toxicity than MIT in each of the three cellular test systems) fits in with the predicted and measured higher intrinsic electrophilic reactivity of the chlorinated isothiazol-3-one structures.

However, the dramatic effects of the double-chlorinated DCOIT observed in the molecular test systems suggest a high excess toxicity of this substance in the cellular test systems. Nevertheless, according to the obtained results from cellular assays DCOIT must be classified as a baseline toxicant with the alga reproduction assay being the only exception where a small excess toxicity (TR=30) was observable. The same tendency was found for OIT where also a high toxic ratio (but a lower one than for DCOIT) was predicted due to the measured intrinsic GSH reactivity, but only a low excess toxicity could be found in the cellular test systems. This discrepancy between the integral cellular test systems and the molecular assays is of high importance for the use of the toxic ratio approach in ecotoxicological research, since it suggests that for hydrophobic and at the same time reactive organic chemicals baseline toxicity is able to mask other, more specific modes of toxic action in acute cellular toxicity assays. As a consequence, these chemicals might erroneously be classified to act exclusively via baseline toxicity. This bears the trap to overlook other basal but more specific modes of toxic action that might lead to severe chronic or long-term effects. Thus, hazard evaluation schemes that exclusively employ classification models of toxicants based on excess toxicity - as proposed by von der Ohe *et al.* (2005) – bear the uncertainty to ignore hazard potentials of chemicals that would be revealed by a mode-of-action-based classification model.

1.1.3 Implications for the hazard assessment

The example of the isothiazol-3-one biocides demonstrates the necessity of an integrated testing strategy to get a complete picture of the hazard potential of chemical substances. Apart from the hydrophobic interaction potential, the used T-SAR approach was also able to identify structural features that can lead to specific adverse effects in addition to baseline toxicity. Furthermore, it could be shown that on the basis of T-SAR analysis, molecular test systems can specifically be selected to verify modes of toxic action that might be masked in integral cellular test systems.

The cellular test systems selected from the flexible test battery were capable to detect the presumed toxicity of the isothiazol-3-one biocides and its modulation by certain substructural elements. Additionally, a detailed analysis of these cytotoxicities using the toxic ratio approach identified two possible modes of toxic action that had been predicted before from the T-SAR analysis of the isothiazol-3-one test kit.

The molecular test systems that had been selected in a second step to monitor the electrophilic reactivity were found to be highly sensitive allowing for a detailed differentiation between the substitution patterns of the biocides. Using these test systems, the cellular GSH metabolism could be identified as an important target for the

biocides explaining the observed drastic excess toxicities. The significant effects of DCOIT in these molecular test systems revealed that the observed cytotoxicity of this compound cannot only be explained by baseline toxic effects. This implies that special care is needed when assessing the hazard potential of highly hydrophobic and at the same time reactive organic chemicals.

Summing up, one can state that the selection of the four biocide structures based on the test kit concept led to the identification of the chlorine substituents and the hydrophobic side chain as the key structural elements for the observed toxic effects. Thus, the T-SAR and mode-of-action-based approach was found to be a useful tool to detect structural alerts in the isothiazol-3-ones.

However, what do the obtained results mean for the hazard assessment of isothiazol-3-one biocides? The identified high reactivity towards GSH of the chlorinated isothiazol-3-ones can be extended to other thiol groups in proteins as it could be demonstrated by the inhibition of GR activity. Therefore, this case study supports the assumption that especially the chlorinated isothiazol-3-ones act as strong skin sensitisers and allergens by coupling to proteins in epithel cells via cysteine and histidine residues (Alvarez-Sanchez *et al.*, 2003; Alvarez-Sanchez *et al.*, 2004). The exposure of man to chlorinated isothiazol-3-one biocides is quite considerable, because a mixture of MIT and CIT (*e.g.* KATHON®) is used as a common preservative in cosmetics, household products, wall paintings and in the leather industry. Also, especially CIT evaporates quickly from wall paintings and significant concentrations of this biocide in the compartment air were measured (Binder *et al.*, 2001). Hence, the risk for a severe contact dermatitis or allergenic response in the respiratory tract is high, especially for workers handling large amounts of CIT containing preservatives. According to current regulation a mixture of MIT and CIT may be added to products up to a concentration of 15 ppm. For comparison, in Germany the maximum acceptable concentration of methyl isocyanate (also a strong electrophile and allergen) a worker may be exposed to should not exceed 0.01 ppm. Accordingly, CIT should be replaced in the commercially available mixtures by non-chorinated isothiazol-3-ones - *e.g.* benzoisothiazol-3-one - to reduce the risk of severe health defects for workers and consumers.

More than the other isothiazol-3-ones, DCOIT is of high ecotoxicological concern since it is predominantly applied as marine antifouling biocide in ship coatings. Several studies identified DCOIT burdens in marine sediments and coastal waters (Larsen *et al.*, 2003; Lambropoulou *et al.*, 2003; Martinez and Barcelo, 2001). Thus, the high toxicity of DCOIT against various algae species (Arrhenius *et al.*, 2006; deNys *et al.*, 1996) is of high relevance for assessing a possible risk of this biocide for non-target organisms. Additionally, so far DCOIT has not been classified as being bioaccumulative, because the producing industry published a log $P_{O/W}$ value < 3 (Jacobson and Williams, 2000). In contrast, the high log $P_{O/W}$ value of 4.79 found in the present case study can lead to a rapid uptake resulting in unexpected high internal concentrations of DCOIT in higher marine organisms. Combined with this accumulation potential, the measured high reactivity of DCOIT against cellular thiol groups from biomolecules represents an additional hazard potential that should definitely be taken into consideration. If possible,

from an ecotoxicological point of view the double chlorinated DCOIT should be replaced by the non-chlorinated OIT. Even though this compound is also highly hydrophobic (log $P_{O/W}$ = 3.3), it is lacking the chlorine moieties responsible for the high toxicity of DCOIT against higher organisms. Thus, OIT is an example for an inherently safer substitute of DCOIT.

1.2 Ionic liquids

As ionic liquids are termed to be non-reactive and chemically stable – in contrast to isothiazol-3-ones - a selection of ionic liquids was integrated into the case study. However, the knowledge about their hazard potential for man and the environment is still very limited. Therefore, the related risk indicator "uncertainty" must be reduced by generating ecotoxicological data that allow for a detailed insight into structure-activity relationships considering the highly variable substructural elements of an ionic liquid - *i.e.* the large number of head groups, side chains and anions. To contribute to a refined hazard assessment of ionic liquid structures, again the test kit concept was used to analyse the influence of these substructural elements in a cellular test system (paper No. 3) and in a molecular assay (paper No.4).

1.2.1 Cellular test system

The sensitive and little differentiated rat leukaemia cell line IPC-81 was used to screen the toxic effects of seven head groups, 10 side chains (mainly containing hydroxy, ether and nitrile functions) and four anion species. By correlating the measured cytotoxicities with a HPLC-derived hydrophobicity parameter it could be shown that the vast majority of the 100 tested ionic liquid structures is likely to act via a narcotic mode of action. Only the N,N-dimethylaminopyridinium head group deviated from the expected cytotoxicity-hydrophobicity correlation (this correlation is comparable to the toxic ratio approach applied to classify the isothiazol-3-ones) pointing to other more specific modes of toxic action. Additionally, the $[(CF_3SO_2)_2]^-$ anion was identified to exert a significant cytotoxicity compared to the tested halides.

The test kit of ionic liquids revealed that the side chain is the dominating structural element influencing the hydrophobicity and hence the toxicity of the corresponding cation. Various head groups connected to the same side chain exhibited only a weak influence on the hydrophobic interaction potential. In line with these findings, it could be demonstrated that the introduction of polar functional groups into hydrophobic alkyl side chains can significantly decrease the cytotoxicity of the corresponding ionic liquid. For example, replacing the butyl side chain at the imidazolium head group by a 3-hydroxypropyl chain leads to a more than 10 fold increase of the EC_{50} value. Using other test systems out of the flexible test battery this general tendency could recently be confirmed in the luminescence inhibition assay with the marine bacterium *Vibrio fischeri* as well as in the 24 h reproduction inhibition assay with the limnic green alga *Scenedesmus vacuolatus* (Stolte *et al.*, 2007b). Here, apart from the N,N-dimethylaminopyridinium head group for the majority of ionic liquids no excess toxicity could be identified in the integral cellular test systems. This supports the T-SAR based hypothesis that ionic liquid

structures act predominantly via a narcotic mode of action owing to their structural similarity (positively charged head group and hydrophobic side chains) to common surfactants.

However, the results of the inhibition of acetylcholinesterase by ionic liquids cations that will be discussed in the following section demonstrate that the T-SAR based testing strategy can reveal molecular mechanisms of toxic action that are disguised in integral cellular test systems.

1.2.2 Acetylcholinesterase inhibition by ionic liquids

Based on a previous study by Stock *et al.* (2004) indicating that imidazolium and pyridinium containing head groups inhibit acetylcholinesterase, paper No. 4 deals with a refined analysis of the structural features influencing these inhibitory effects. In contrast to the cytotoxicity assay, this molecular test system helped to identify the cationic head groups as the decisive factor for the inhibitory potential of the corresponding ionic liquids. The quinolinium, the N,N-dimethylaminopyridinium and the pyridinium head groups exhibited the highest inhibitory potential towards acetylcholinesterase whereas the polar and non-aromatic morpholinium head group was found to be the least active head group.

Additionally, the vast majority of ionic liquid anion species was found to be inactive in the enzyme inhibition assay. The only exception is represented by the fluoride anion and fluoride containing anion species (*e.g.* PF_6^- and BF_4^-), which readily undergo hydrolytic cleavage and were found to act as inhibitors towards acetylcholinesterase. This is in line with earlier published data that report on the inhibitory potential of fluoride anions on acetylcholinesterase (Heilbronn, 1965; Krupka, 1966).

However, taking a certain head group, the hydrophobicity of the connected side chain changed the inhibitory potential of the cation significantly. In accordance to the cytotoxicity study, it could be shown that the introduction of polar functional groups into hydrophobic alkyl side chains is able to shift the IC_{50} to higher values corresponding to a reduced inhibitory potential. For the imidazolium based ionic liquids even a QSAR correlation between the above mentioned HPLC-derived parameter describing the cation´s hydrophobicity and the measured IC_{50} values could be derived.

1.2.3 Implications for a sustainable design of ionic liquids

The results obtained for the selected ionic liquid structures further demonstrate that a tiered and T-SAR based approach in combination with the test kit concept is a valuable tool to identify structural alerts in chemical entities. Combining these tools also helps to efficiently uncover the impacts of these structural alerts on certain toxic effects. The presumed narcotic mode of action of ionic liquids could be confirmed in the IPC-81 cell line with the only exception of the N,N-dimethylaminopyridinium head group that also should be investigated for some more specific modes of action.

The structural alert “possible interaction with the active site of acetylcholinesterase due to a quaternary nitrogen and aromatic ring systems” could be verified in the

molecular enzyme inhibition assay. Thus, this example shows that non-reactive chemicals can exert strong specific effects owing to certain molecular interaction potentials. These effects may be missed, if exclusively basal integral test systems - that are lacking specific proteins or structures like *e.g.* acetylcholinesterase - are used to screen the toxicity of chemical substances. Besides, since acetylcholinesetrase is an extracellular enzyme bound to the plasma membrane of certain cells in the central nervous system of invertebrates, the presented case study demonstrates that even organ- or tissue-specific effects of toxicants can be predicted at the T-SAR level provided that the molecular interaction potentials at the biological target sites are known.

This identification of structural alerts and the detailed knowledge of how substructural elements can be used to modulate the toxicities are essential for the development of new and sustainable ionic liquids with a reduced hazard potential. Considering the results presented here, from an ecotoxicological point of view such inherently safer ionic liquids should be composed of head groups carrying short and polar side chains. The N,N-dimethylaminopyridinium and the quinolinium head group should be avoided as well as the $[(CF_3SO_2)_2]^-$ anion and other fluoride containing anions. A detailed overview on how the results of this case study and further investigations using the flexible test battery can contribute to the process of sustainable design is reviewed in the article "*Design of Sustainable Chemical Products – The Example of Ionic Liquids*" published by Ranke *et al.* (2007). This publication also shows how the generated data lead to a reduction of the risk indicator "uncertainty" by filling the existing gaps in the indicators "biological activity" and "bioaccumulation".

However, Stolte (2007) showed that especially for the case of ionic liquids the design of a genuinely sustainable compound is not an easy task, because toxicological and technological needs often prove to be counterparts. For example, many technological applications of ionic liquids are based on their imiscibillity with water, which requires highly hydrophobic cations and anions. But as it has been stated earlier, especially these properties are responsible for the cytotoxic and enzyme activity inhibiting effects of ionic liquids. Furthermore, a trade-off between different environmentally relevant properties of ionic liquids was discovered. Hence, for a ready biodegradability a certain hydrophobicity of the ionic liquids is needed (Stolte *et al.*, 2007a), which is in conflict with a low cytotoxicity of the compounds. That means that apart from the technological needs, for a sustainable design of ionic liquids one has to balance the acute toxicity of the compounds with their potential to be persistent in the environment.

The example of ionic liquids clearly demonstrates that understanding molecular structure-activity relationships do not lead to clear "reject or accept" decisions but that they are a powerful tool to support a balanced judgement in a weight of evidence approach. For example, if ionic liquids will be used predominantly in their technosphere of application in closed systems and since they exhibit a neglectable vapor pressure, the exposure of man and the environment presumably will be low. Combined with the fact that the acute toxicity of ionic liquids is mainly dominated by a narcotic mode of action, the risk of severe acute toxic effects of ionic liquids to man and the environment is relatively low. Thus, the potential of persistence in the environment should be of major

concern in the hazard assessment of ionic liquids and strong efforts should be made to enhance the ready biodegradability of these structures.

In contrast, the potential of isothiazol-3-one biocides to be persistent in the environment is relatively low owing to their high reactivity against biological nucleophiles. But the exposure of man and the environment will be high, if the biocides are released in the environment in large amounts and they can easily be taken up by organisms via skin contact or inhalation. Therefore, the hazard assessment here should be focused on the drastic acute excess toxicities of these substances towards selected model organisms like *e.g.* rabbits. In the case of isothiazol-3-ones, strategies to reduce the intrinsic reactivity of the compounds are needed to reach the goal of inherently safer chemicals with *e.g.* a reduced allergenic potential.

2 Conclusion and Outlook

"... if effects on the ecosystem are to be predicted and understood, it is necessary to identify the effects of a toxicant on lower levels of biological organisation, such as the subcellular level. Specific and sensitive biochemical methods can, therefore, serve as early warning indicators and adverse effects on the ecosystem can be avoided by taking protective measures." (Haux and Förlin, 1988)

The presented case studies on selected isothiazol-3-one biocides and ionic liquid structures showed that a tiered and mode-of-action-based testing strategy was successful to identify substructural elements in these two substance classes, which are responsible for specific modes of toxic action– the so called structural alerts.

The applied cellular test systems and their integral endpoints were found to be valuable methods for a screening of the basal cytotoxicity of the two investigated substance classes. From these cellular screening assays hints for possible modes of toxic action were derived that could subsequently be verified using more specific molecular and subcellular test systems as it could be demonstrated for the excess toxicities of isothiazol-3-one biocides. Thus, the theoretically derived structural alerts seem to reflect the general hazard potential of the substances tested as no false negative results - leading to an underestimation of the hazard potential at the T-SAR level - could be detected.

However, the cytotoxicity studies of the electrophilic isothiazol-3-ones also revealed that the toxic ratio approach - commonly used to identify excess toxicities of environmentally relevant chemicals - can fail to fully classify reactive and at the same time highly hydrophobic organic chemicals. This leads to the conclusion that cellular screening assays always need to be accompanied by a T-SAR analysis to identify structural alerts pointing to modes of action that might be missed using integral cellular testing endpoints. The T-SAR guided identification of certain ionic liquid structures as strong acetylcholinesterase inhibitors supports this conclusion, because in the cellular assays this toxic mechanism could not be uncovered owing to the fact that the enzyme is located extracellularly.

The examples show that a mode-of-action-based strategy for the hazard assessment of chemicals can reduce the risk indicator "uncertainty", since the T-SAR approach is able to identify structural alerts in chemicals and concomitantly the corresponding biological targets that allow for a precise verification of the presumed hazard potentials.

Furthermore, it could be demonstrated that this T-SAR based selection of molecular test systems represents a powerful tool to monitor structural variations within one substance class that influence toxic effects. For example, the modulation of the acetylcholinesterase inhibition potential of ionic liquids by various head groups and side chains could be described and even be quantified for a series of imidazolium based ionic liquids in the selected enzyme inhibition assay.

Especially the challenging high structural variability of ionic liquids qualified the applied mode-of-action-based testing strategy and the flexible test battery as complementary "hardware" to be a sucessful tool to systematically select lead structures and to screen their hazard potentials. Thus, this testing approach can be used to efficiently reduce and focus the amount of necessary animal testing *e.g.* under REACH. Additionally, the growing pool of known structure-activity relationships on the molecular level provides guidelines for the design of new and inherently safer chemical products. The so created reduction of "uncertainty" in the field of possible hazards of chemicals supports a weight of evidence risk assessment and decision-making as it is favoured by the European Commission and the US EPA.

However, cellular and molecular mode-of-action-based test systems will not be able to replace tests with organisms or populations since toxicokinetic and chronic lethal or sublethal effects cannot be modelled precisely by in vitro test systems. Especially the complex non-linear dynamics between different species need special ecotoxicological methods to predict the impact of toxicants on the ecosystem or biosphere level.

But mode-of-action-based strategies can help to predict toxic effects of so far untested and even "unsynthesised" chemicals and therefore they should be integrated in the industrial development process in order to facilitate the sustainable design of chemicals. These strategies provide a rational framework that helps to select, to reduce and to evaluate the testing of chemicals in an iterative process.

To fulfil these tasks as sound as possible, in the future more basal molecular target sites in cells, organs and tissues need to be identified and characterised to extend the pool of test systems and hence the amount of possible "answers" to molecular, structure-activity based "questions". In view of the tremendous amount of chemials that need to be tested in the near future, the assay development should be focused on rapid screening tests that allow for the high throughput testing of substances. Thus, the time and cost consuming accuracy of pharmacological assays is often not necessary in the field of ecotoxicology, but the test systems should be designed as simple as possible to uncover basal mechanisms and modes of toxic action.

However, ecotoxicological efforts alone will not lead to inherently safer and sustainable chemical products and processes. The shift in industrial paradigms towards sustainability and more responsibility is the essential prerequisite to meet the goals formulated in the Rio Declaration and the Agenda 21.

Thus, we need both, the science to provide efficient and reliable testing strategies and the wisdom of the industry to make use of them.

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Zhao,H. **(2006)**. Innovative applications of ionic liquids as "green" engineering liquids. *Chem Eng Commun* 193, 1660-1677

LIST OF PUBLICATIONS

In the following all publications and contributions - including the presented manuscripts in Part II - are listed, which were authored during the work for this thesis:

1. **Arning, J**., Stolte, S., Böschen, A., Stock, F., Pitner, W. R., Welz-Biermann, U., Jastorff, B., and Ranke, J. (2008). *Qualitative and quantitative structure activity relationships for the inhibitory effects of cationic head groups, functionalised side chains and anions of ionic liquids on acetylcholinesterase*. **Green Chemistry**, DOI: 10.1039/b712109a.
2. **Arning, J**., Stolte, S., Ranke, J., Bottin-Weber, U., and Jastorff, B. (2006). *Cytotoxicological effects of isothiazolon biocides and ionic fluids - Identification of molecular impact mechanisms with the help of the "toxic-ratio-concept"*. **Naunyn-Schmiedebergs Archives of Pharmacology** 372, 136.
3. **Arning, J**. (2005). *Nachhaltigkeit in der Chemie*. **Nachhaltigkeitsbericht der Universität Bremen**, 94-95.
4. **Matzke, M**., Stolte, S., Thiele, K., Juffernholz, T., Arning, J., Ranke, J., Welz-Biermann, U., and Jastorff, B. (2007). *The influence of anion species on the toxicity of 1-alkyl-3-methylimidazolium ionic liquids observed in an (eco)toxicological test battery*. **Green Chemistry** 9, 1198-1207.
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9. **Stolte, S**., Matzke, M., Arning, J., Böschen, A., Pitner, W. R., Welz-Biermann, U., Jastorff, B., and Ranke, J. (2007b). *Effects of different head groups and functionalised side chains on the aquatic toxicity of ionic liquids*. **Green Chemistry** 9(11), 1170-1179.
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Submitted manuscripts:

1. **Arning, J**., Dringen, R., Schmidt, M., Thiessen, A., Stolte, S., Matzke, M., Bottin-Weber, U., Caesar-Geertz, B., Jastorff, B., and Ranke, J. (2007a). *Structure-activity relationships for the impact of selected isothiazol-3-one biocides on glutathione metabolism and glutathione reductase of the human liver cell line Hep G2* . Submitted to **Toxicology**.
2. **Arning, J**., Matzke, M., Stolte, S., Nehen, F., Bottin-Weber, U., Böschen, A., Abdulkarim, S., Jastorff, B., and Ranke, J. (2007b*). Analysing cytotoxic effects of selected isothiazol-3-one biocides using the "Toxic Ratio" concept and the "T-SAR" approach.* Submitted to **Environmental Science and Technology**.

Further publications:

1. **Jansen, S**., Arning, J., Dulcks, T., and Beyersmann, D. (2004a). *S-Nitroso compounds interfere with zinc probing by Zinquin*. **Analytical Biochemistry** 332(1), 145-152.
2. **Jansen, S**., Arning, J., Kemken, D., Dulcks, T., and Beyersmann, D. (2004b). *Phospholipase C activator 2,4,6-trimethyl-N-(meta-3-trifluoromethyl-phenyl)-benzene-sulfonamide decays under ultraviolet light and shows strong self-fluorescence*. **Analytical Biochemistry** 330(2), 353-355.
3. **Jansen, S**., Arning, J., and Beyersmann, D. (2003). *Effects of the Ca ionophore A23187 on zinc-induced apoptosis in C6 glioma cells*. **Biological Trace Element Research** 96(1-3), 133-142.

Talks

1. *"Flexible Toxizitätsstudien als integraler Bestandteil eines nachhaltigen Chemikaliendesigns"*. Held on the 26th Osnabrücker Umweltgespräch conference, Deutsche Bundesstiftung Umwelt, December 2006, Osnabrück, Germany.

Curriculum Vitae

03.03.1978	Born in Münster, Germany
1984-1997	Primary, secondary and higher schools in Bremen, Germany. "Abitur" certificate in June, 1997
1997-1998	Alternative service in a kindergarten in Bremen.
1998-2004	Chemistry and biochemistry studies at the University of Bremen. Diploma thesis (submission December, 2003): *"The contribution of the enzyme phospholipase C to the zinc mediated signal transduction pathways in mammalian cells"*. Supervisor: Prof. Dr.rer.nat Detmar Beyersmann
2004-2007	Scholarship holder of the PhD scholarship programme of the German Federal Environmental Foundation (DBU, Deutsche Bundesstiftung Umwelt).
2004-2007	PhD student in the group of Prof. Dr.rer.nat. Bernd Jastorff at the Centre for Environmental Research and Technology (UFT) at the University of Bremen. Submission of the PhD thesis in December 2007.