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Project summary: a critical synopsis of mechanisms of action of low-dose xenobiotics in mammalian organisms as a basis for assessing aggregated effects of chemical mixtures and identifying "new" toxicological end points

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Abstract

Background: European chemicals legislation (registration, evaluation, authorisation and restriction of chemical substances (REACH)) requires a broad assessment of chemicals with respect to, *inter alia*, their health-relevant properties. Due to the extreme number of substances to be assessed and the limited current toxicological knowledge on their respective properties, REACH implicitly requires a paradigm change: away from knowledge generated mainly from costly animal experiments towards the use of mechanistic findings. Moreover, effect mechanisms at the biochemical or cellular level are essential when conclusions shall be drawn about "new" endpoints and mixtures of xenobiotics. This study (funded by the German Federal Environment Agency) describes examples of biochemical processes in the mammalian organism and how xenobiotics interfere with them. Interference with physiological processes expected to lead to adverse health effects is characterised as "toxicity pathway". The study describes toxicological endpoints not usually covered in routine animal testing and the respective toxicity pathways.

Results and conclusions: Screening for chemicals which exert effects via common toxicity pathways and subsequently conducting targeted short-term tests may generate new information about the toxicity of chemicals without performing extensive substance-by-substance animal experiments. Information on common toxicity pathways may also provide input for the assessment of mixture effects. The U.S. Environmental Protection Agency is working intensely on this concept. It involves the use of enormous amounts of data on relevant biochemical and cellular processes, which are generated by "high-throughput screening" methods, and then are combined with substance-specific kinetic data, experimental apical test outcomes and modelling. Current limitations in the regulatory use of this integrated approach on risk assessment will be outlined.

Keywords: risk assessment, regulatory toxicology, mixture, toxicity pathway, (Q)SAR, animal testing, mechanism of action, high throughput screening (HTS), effect mechanism, REACH

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Background

This manuscript comprises the theoretical background and conclusions made in a project initiated and published by the German Federal Environment Agency (see also "Acknowledgments"). The literature work was performed in between February of 2009 and March 2010. Further details beyond this summary can be taken from the original report available at the German Federal Environment Agency (FKZ 3708 61 205) [1]. Theoretical basis of this study is that European chemicals legislation requires the systematic study of marketed chemicals for, *inter alia*, properties of relevance to health (REACH: http://echa.europa.eu/legislation/reach_legislation_en.asp). Animal studies that describe adverse effects on health (or their absence) are mainly used for this purpose, although the underlying mechanism of action does not have to be known. However, carrying out relevant *in vivo* studies is difficult to reconcile with animal welfare concerns and may be extremely costly. Moreover, their validity is limited and incomplete in several respects:

- The animal model is not always suited to detect all xenobiotic-related health effects on humans (in qualitative and quantitative terms).
- Currently, comprehensive tests are generally only required if a large tonnage is to be placed on the market. In the case of small amounts, however, the scope of testing is minimal although potential toxic effects from point exposure sources on consumers and workers, whether directly or indirectly via the environment, are of substantial interest.
- Real contaminant exposures are often based on exposure to several substances. It seems impossible, however, to put such exposure scenarios to comprehensive testing and to describe or even quantify their effects.

Against this background, efforts are increasingly being made to investigate substances' mechanisms of action at the biochemical and cellular levels and to take the results into account in their toxicological assessment. The present study:

- Describes toxic effect mechanisms using biochemical processes influenced by xenobiotics as an example, and
- Subsequently, generates hypotheses of effects that have, up to now, not been observed in animal studies and explains how they can be tested
- For both, individual substances and mixtures of substances.
- It also examines the suitability of already existing instruments such as (quantitative) structure-activity relationship ((Q)SAR) to obtain information on xenobiotics' mechanisms of action and
- Reports on approaches in regulatory toxicology that aim at linking effect-related testing of xenobiotics with generating information on their mechanism of action.

Results

Biochemical and cellular events and the impact of xenobiotics

Metabolic functions, such as the supply of cells with energy, the catalysis of metabolic transformation by enzymes, the transport of substances into the extracellular space or through membranes and communication based on a feedback control system via signalling chains, are fundamental processes in an organism. As an introduction, this study gives examples of biochemical and cellular events that are associated with such metabolic functions, transport processes, or signal transduction, and describes, again by way of example, how some xenobiotics may interfere with the functions mentioned. In cases where all of the underlying steps of xenobiotic interference with the organism are known, one would talk of the substance's "mechanism of action", *i.e.* binding to a specific receptor which elicits a certain subsequent known cascade of events. In cases of a less detailed knowledge of the substance's interaction with the organism, one would refer to "mode of action" as this indicates only a certain area of biochemical interaction, *e.g.* it is known that the substance interferes with DNA repair but without the knowledge of the actual mechanism. In this manuscript, we aim to address the mechanism of action of the mentioned xenobiotic examples.

Energy is supplied through cellular respiration in the mitochondria in the form of high-energy adenosine triphosphate (ATP). However, there are xenobiotics that can penetrate the mitochondrial membrane, then trap protons and transport them away, thereby disturbing cellular respiration, *i.e.* uncoupling ATP synthesis from it, thus leading to energy depletion. Exemplary 2,4-dinitrophenol, weak organic acids or other acidic phenols are able to induce this biochemical uncoupling [2].

In the citric acid cycle, which is also important for energy provision, citrate is converted to isocitrate by the enzyme aconitase. Fluorocitrate is able to inhibit this enzyme by forming a complex with the catalytic centre of the enzyme, thus stopping the citric acid cycle; it may be metabolised in the body from fluoroacetate, a substance often used in the past as a rodenticide [3]. This may induce convulsions and indirectly lead to an acidification of the blood [4].

In the human organism, metals that are widespread due to environmental pollution such as lead behave very similarly to the physiologically important metal ions of calcium or zinc. For instance, lead is able to displace zinc ions in the catalytic centre of the enzyme delta-aminolaevulinic acid dehydratase and may thereby indirectly induce anaemia due to iron deficiency [5].

Xenobiotics may also disturb transport processes in the cell and organism. The process of provision of ATP to supply energy to cells requires transport of protons through the mitochondrial membrane. Specific channels function as routes of transport. Organotin compounds, for example used as stabilisers in plastics, can obstruct these channels and thus stop the flow of protons into the mitochondria [6].

Another transport process necessary for essential bodily functions occurs via a glycoprotein, which transports xenobiotics from the cells and makes them accessible to biliary and renal excretion (efflux transporter). These transporters are mainly expressed in tissues and organs that form a barrier between "outside" and "inside". If such a glycoprotein is confronted with considerable amounts of xenobiotics, its transport capacity may be depleted. For instance, artificial and polycyclic musk compounds, which are present in detergents and cosmetics as fragrances, have a high affinity to the P-glycoprotein and may saturate this efflux transporter and thus contribute to an accumulation of substances in the cells [7].

Environmental chemicals may also influence those communication processes in the organism that take place via signal transduction. Extracellular signal molecules reach a receptor, amplify the signals and after further signal transductions a cellular reaction results. Based on the principles of a feedback control system, feedback will then terminate the reaction. One classical example of this is how the environment influences the function of endocrine glands, *i.e.* thyroidal dysfunction *e.g.* due to dietary iodine deficiency. If the thyroid is not supplied with enough iodine, hormone synthesis does not regularly take place due to a lack of its basic component. The hypothalamic-pituitary feedback system is disrupted, which may lead to the development of an iodine deficiency struma [8].

Permanent excitation of nerve cells due to the absence of negative regulation is another example of the impact of xenobiotics on one or several control elements of signal transduction. Neurotransmitters in the brain are stimulated via the opening of specific ion channels to allow sodium ions to flow into the nerve cell. If required, this activity is modulated or inhibited by chlorine ions which likewise flow into nerve cells through specific channels. Lindane (gamma-hexachlorocyclohexane), which formerly was used as a plant protection product, prevents the influx of these chlorine ions by inhibiting the opening of the relevant ion channels. This results in permanent excitation as mentioned above [9].

It must be stressed, however, that for most xenobiotics it is not known at all or not known in detail via which mechanism they act on the organism. In addition, the following must be borne in mind:

- Substances often act, not via one mechanism alone, but on different processes at several sites. Since these processes may be linked with each other, complex interactions may occur.
- As a rule, the finding of a general impact of a substance (qualitative information) does not allow any conclusions to be drawn as to its quantitative effect (Is the amount absorbed sufficient to induce a disturbance of the physiological process?).
- Extrapolations to similar substances are possible only with considerable restrictions because steric changes in isomers for example may already have major qualitative and quantitative consequences for an interaction with biochemical processes.
- Therefore, findings on the mechanism of action initially represent knowledge-based hypotheses that must be verified by specific testing and/or may influence the priority of further investigations. The findings are generally insufficient to dispense with testing completely.

Identification of "new" toxic end points

Animal testing as characterised in the Organization for Economic Cooperation and Development (OECD) guidelines usually just looks at apical end points and may readily detect clear-cut adverse effects. Therefore, the aim of this study was to investigate whether mechanistic findings on a biochemical or cellular level would allow the identification of more complex or less obvious effects of xenobiotics. We describe three examples of this:

1. Involvement of type-2 alkenes in neurodegenerative processes,
2. Interaction of xenobiotics in delayed hypersensitivity reactions and
3. Mechanistic backgrounds for an increased sensitivity to environmental pollutants in older adults.

Type-2 alkenes and neurodegeneration

Certain type-2 alkenes like propenal can be found in the brains of patients with Alzheimer's disease. These compounds may be formed in the brain endogenously through ubiquitously occurring reactive oxygen species (ROS) and may accumulate there over a lifetime. 4-hydroxy-2-nonenal, for example, is formed in this way. Also, there is a hypothesis on the mechanism by which type-2 alkenes might destroy nerve cells. It assumes that type-2 alkenes mimic the binding of nitrogen monoxide (NO) to proteins and thereby produce similar synaptic signal transduction. This has been confirmed by experimental studies. Since this alkene-protein binding is irreversible, NO-dependent neuromodulation processes are inhibited, resulting in neuron decay [10,11] (see also Figure 1). A similar effect on

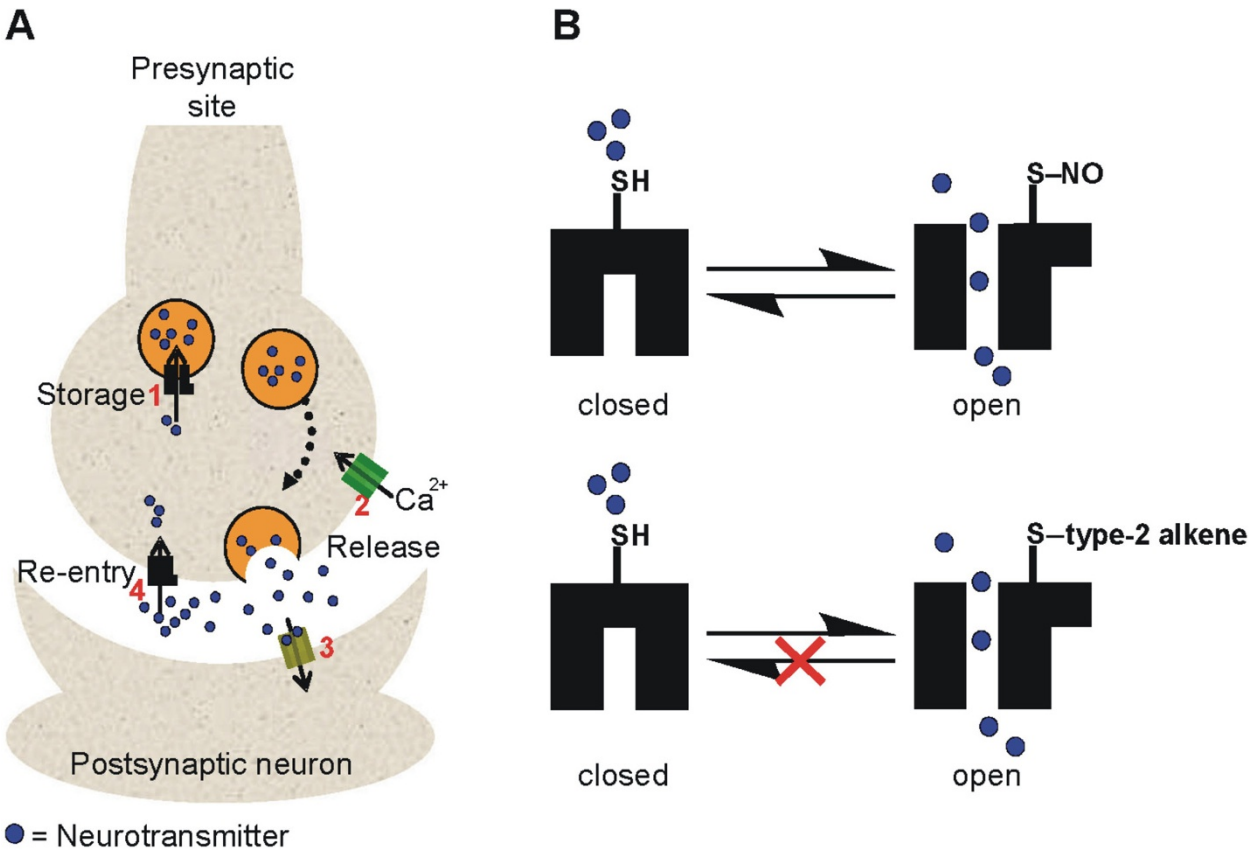

Figure 1 Schematic representation of the molecular mechanism of the neurotoxicity of type-2 alkenes. **(A)** Sulfhydryl-NO-controlled processes in neurotransmitter transport: 1. neurotransmitter storage in vesicles; 2. inflow of calcium for vesicle migration to presynaptic membrane and release of neurotransmitters; 3. uptake of neurotransmitters via post-synaptic membrane; 4. re-entry of excess neurotransmitters via presynaptic membrane. **(B)** Disturbance of pumping function by type-2 alkenes. The sulfhydryl-NO bond is physiologically reversible; to achieve this, pumps and channels are activated, opened and closed as needed (*e.g.* Ca^{2+}-ion channel, above). Binding of type-2 alkenes is irreversible, so that control processes can no longer be modulated.

neurotransmitter activities has been demonstrated for acrylamide, which is structurally related to propenal [12]. It must generally be assumed that other alkenes that are absorbed as xenobiotics may also reach the brain to produce a similar effect. Substances that can enzymatically be broken down into such alkenes, such as certain furan compounds, are also of interest in this context [13].

On the basis of these mechanistic relationships, it is recommended that type-2 alkenes and xenobiotics that metabolise into type-2 alkenes be specifically tested for potential neurodegenerative effects.

Xenobiotics and delayed hypersensitivity reactions

Glucocorticoids act as immune regulators by inhibiting the immunoreaction following binding to the glucocorticoid (GC) receptor. The following focuses on xenobiotics that disturb the function of GC receptors and the release of cytokine Th1 [14]. Glucocorticoids such as dexamethasone, an active ingredient of medicinal products, have an aromatic ring that is necessary to identify the GC receptor, and they have an agonistic reagent that "activates" the receptor. If the agonistic reagent is missing, and the GC receptor is blocked by a substance (antagonistic binding to the GC receptor), a cell-mediated, GC-controlled delayed allergy may result. In the case of structurally similar xenobiotics, it is likely that they disrupt the flow of signals between the GC receptors and the hypothalamus. This causes the inflammatory reaction of the body to persist despite removal of the pathogen. The dyestuff tartrazine is assumed to be able to bind to the GC receptor as tartrazine has a structure very similar to that of non-steroidal anti-inflammatory drugs (NSAIDs). However, if such binding occurred, the receptor would not be activated, since the agonistic reagent necessary for this is missing. Several studies have documented hypersensitivity reactions of the type described here after exposure to tartrazine [15,16]. We have identified some substances that are

similar to tartrazine, including four neonicotinoids which are used as insecticides.

We recommend investigating whether tartazine-like substances also reach sensitive sites, *e.g.* pulmonary epithelial cells, in this form (stability testing) and, if so, whether they sufficiently accumulate there, have a similar interaction with the GC receptor and act as allergens. This might be a "new" end point since the regular chemical testing programme does not include determination of the immunoglobulin D antibody levels to be considered here.

Sensitivity to environmental pollutants in aged people

Protein folding takes place in the lumen of the granular endoplasmic reticulum (ER). If this does not proceed properly, the accumulation of misfolded proteins may result in cytotoxic effects, *e.g.* in the brain [17]. It is assumed that lead, for example, has an impact on the endoplasmic reticulum by interfering with potassium homoeostasis and thus contributes to typical age-related diseases (such as Huntington's disease and Alzheimer's disease) [18,19]. A similar mechanism is postulated for various other substances on the basis of gene expression analyses, *e.g.* for nonylphenol, di-(2-ethylhexyl)phthalate and methoxychlor (analysis of the Comparative Toxicogenomics Database (CTD); http://ctd.mdibl.org/). For an organism which exhibits low efficiency of various specific protective mechanisms due to age (formation of chaperones in the ER, which are responsible for correct folding; degradation of misfolded proteins via the ERAD mechanism; endoplasmic reticulum-associated degradation) it seems to be plausible that such stress caused by the abovementioned xenobiotics in the endoplasmic reticulum would contribute to these typical age-related diseases. Findings on the mechanism of action involved will allow this to be verified in a targeted way. There is also the suspicion of a combined effect in the form of increased damage to the endoplasmic reticulum through concurrent exposure to several of these substances.

Combined effects

As indicated, findings on the mechanism of action of an individual substance also provide indications of combined effects from concurrent exposure to similar substances, whereby both an intensification of effects and competitive behaviour may play a role. We pursued this aspect:

- By analysing the mechanism of the action of substances via the Ah receptor as well as the consequences of an exposure to several chemicals exhibiting the same mechanism of action,
- By discussing the mechanisms whereby different substances are able to induce cardiovascular effects together, either on the basis of their similarity or on the basis of their adverse, but supplementary effects, and
- By evaluating examples from the literature, which (a) dealt with mechanistic considerations of a combined effect or in which (b) mixtures of substances were actually tested and the combined effect was compared with the effect of the individual substances.

Mixtures and Ah receptor-mediated effects

"Toxicity equivalents" of dioxins and dioxin-like substances are a well-known example of the application of biochemical/mechanistic findings in regulation for the assessment of combined effects [20]. In this example, interactions between xenobiotics and the intracellular Ah receptor play a key role (aryl hydrocarbon receptor; a physiological ligand of the receptor is not known to date). The Ah receptor is a transcription factor that is inactively present in the cytoplasm, translocates into the nucleus after activation by xenobiotics and stimulates the transcription of responsive genes there. These genes are associated with cell growth and cell differentiation [21,22]. For various mixtures of tetrachlorodibenzodioxin, pentachlorodi-benzofuran and polychlorinated biphenyl 126, toxicity equivalents correlate very well with the effects. However, a more in-depth analysis also shows that mixtures of substances that interact with the Ah receptor have effects that are inconsistent with a simple linear correlation. For example:

- Metabolites inhibiting the usual Ah receptor-mediated mechanism can be formed via interaction with the Ah receptor (probably by inhibition of an enzymatic degradation of cytochrome or by overexpression of this cytochrome (*e.g.* CYP1A1)),
- A mixture may contain closely related polycyclic aromatic hydrocarbons that act either as receptor agonists (such as benzo(a)pyrene) or as antagonists (such as fluoranthene), which means that consequences from opposite effects that cannot exactly be quantified to date would have to be considered, although the site of action is the same.

These examples show that the observation of an interaction, *e.g.* with the Ah receptor, is not always sufficient in itself to draw conclusions about the mechanism of combined effects with sufficient certainty.

Complex mixtures and cardiovascular diseases

Although cardiovascular diseases are among the most common lifestyle-based conditions, testing of individual substances in bioassays does not explicitly cover this effect. This may mean that too little significance is ascribed to environmental chemicals in that respect. However, in epidemiological studies, a close correlation has been established between exposure to mixtures like dust, diesel soot, or tobacco smoke and cardiovascular diseases [23-25]. Nevertheless, understanding of the effect mechanisms involved is lacking or must be extended.

Hypertension is closely related to cardiovascular diseases. Mechanisms that impair the vasodilatation of blood vessels may play a role in this disease. Redox-reactive hydrocarbons in diesel engine emissions can inhibit the activity of the enzyme that synthesises NO, which may lead to a lowering of the NO level and reduced vasodilatory capacity [26]. CYP450-catalysed epoxidation of fatty acid has a role to play in the endothelial-derived hyperpolarisation factor (EDHF) [27], which is relevant to vasodilatation. Disturbances by reactive oxygene species (ROS) and Ah receptor-active substances interfere with the activity of CYP450 enzymes (*e.g.* lower CYP induction), impairing the EDHF and thus the desired vasodilatation. Polycyclic aromatic hydrocarbons in airborne dust, tobacco smoke, and diesel soot contain Ah receptor-active substances and also cause hypertension in this way [28]; in addition, diesel soot increases ROS production to produce corresponding effects [29].

Reactive oxygen species also cause oxidation of the low-density lipoprotein (LDL) circulating in the blood, which is a factor in the development of atherosclerosis [30-32]. Elevated quantities of oxidised LDL (oxLDL) result in increased expression of a certain receptor (LOX-1) through which oxLDL is taken up into the cell, and this in turn leads to an additional increase of ROS in the cell [30,33]. This ROS production exceeds the detoxification capacity of endogenous antioxidants and induces, *e.g.* necrosis, apoptosis, or cells with damaged DNA [34]. Poly(ADP-ribose) polymerase-1 (PARP-1) cannot cope with excessive DNA damage, resulting, *e.g.* from combined action of the substances mentioned, in base excision repair, and will then induce the above-mentioned cell death instead of repair [35].

The interactions become so complex that it is very difficult to draw more exact quantitative conclusions despite a better understanding of the mechanisms of action (see Figure 2). The oxidising potential of mixtures or their impact on NO production is a helpful parameter to assess their potential contribution to cardiovascular diseases. Substances inhibiting the interaction between PARP-1 and DNA are to be regarded as particularly critical. However, it seems that no quantitative conclusions about the considered combined effects can currently be drawn.

Examples of tested combinations of substances having a common mechanism of action

The widely used pesticides fenarimol, vinclozolin, and acephate have been assessed as individual substances, which showed that all three substances are substrates for enzymes of the cytochrome P450 enzyme family (CYP) and induce CYP in the same way. As a result, CYP-dependent enzymes can also be expressed to an increased or decreased extent. The pesticides mentioned lead to increased hydroxylation of testosterone in the (mouse) liver, as indicated by increased testosterone hydroxylase activity. Tests for CYP induction, NADPH reductase activity and other CYP-dependent enzyme activity changes by certain mixtures of the pesticides showed no clear picture. In any event, the results do not allow the prediction of an antagonistic, synergistic, or additive effect of the substances in the mixture [36]. This demonstrates how questionable it may be to draw conclusions from the testing of individual substances to the interaction of mixtures and how much uncertainty may be involved when extrapolating from one mixture to another.

Ghisari and Bonefeld-Jorgensen [37] investigated the effects of various plasticisers (phthalates and adipates) and structurally related substituted phenols which are used as components of or additives in plastics as well as phenolic components of herbicides. The study focused on the combined oestrogenic effect and on thyroid hormone regulation: Simultaneous exposure below the effect level (no observed adverse effect concentration (NOAEC)) of the individual compounds did not lead to receptor activation. However, simultaneous exposure to the individual effect concentrations (lowest observed adverse effect concentration (LOAEC)) brought a potentiation of effects, which deviated slightly from additivity. The study shows that the conclusion that the oestrogen receptor is always involved in the mechanism of action does not provide sufficient information for defining the type of influence that several components have on the effect. With regard to thyroid hormone regulation, the effect of the mixture at the NOAECs of the individual substances was the same as that of the individual substances. A stimulating effect on proliferation was observed at the LOAECs, but this effect was smaller than that of the most effective individual substances. The effect of the mixture in that regard was subadditive. In the context of the complex influences on triiodothyronine(T3)-induced cell growth, the study showed how difficult it is to formulate a generalisable prediction of the effects of complex mixtures even if mechanistic elements are included in the analysis.

Structure-activity analysis to identify "new" toxic end points

The use of structure-activity relationships (SAR) and quantitative structure-property relationships/quantitative structure-activity relationships (QSPR/QSAR) is one possible approach to avoid animal experiments [38]. In our study, we explored to what extent mechanistic considerations are already integrated in (Q)SAR models today and whether (Q)SAR models may be applied to the "new" end points and example substances characterised above.

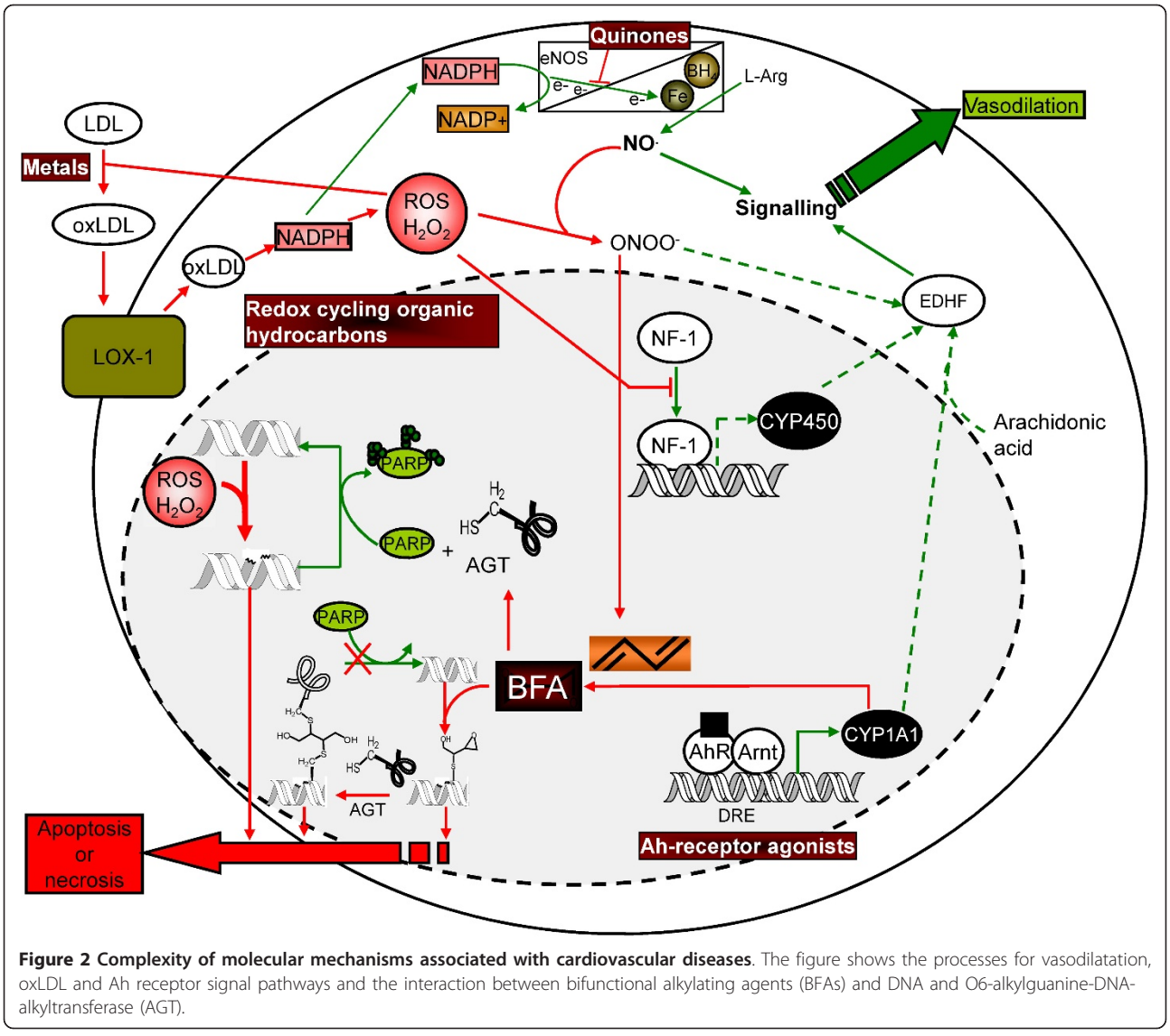

Figure 2 Complexity of molecular mechanisms associated with cardiovascular diseases. The figure shows the processes for vasodilatation, oxLDL and Ah receptor signal pathways and the interaction between bifunctional alkylating agents (BFAs) and DNA and O6-alkylguanine-DNA-alkyltransferase (AGT).

Mechanistic considerations in the analysis of (quantitative) structure-activity relationships

Two different approaches are generally applied to the analysis of (quantitative) structure-activity relationships:

- Rule-based systems and
- Statistical methods.

With rule-based systems, empirical rules established and verified by experts are systematised by means of a computer and applied to predict the presence or absence of specific properties. An essential basis for this is the presence or absence of specific functional groups in a molecule that are known to be responsible for a specific effect. This means that an idea about the mechanism of action usually already exists. The range of experience cannot be extended by means of the expert system, however, since the prediction is based on known rules and therefore requires a defined range of data.

For the statistical approach, it is also desirable to have an idea of the mechanism of action (see validity criteria of the OECD for QSAR), but this is not always the case. It uses different statistical methods to correlate effects and structural elements of a chemical molecule. Deriving a mechanism of action is not possible with this, but would require further expert knowledge. On the other hand, new hypotheses about molecular properties can be established by induction, such as bioavailability or the ability of a substance to inhibit or enhance the function of a specific protein in the organism ("extension of the range of knowledge").

All approaches assume that similar molecules (or at least similar molecular substructures) generally lead to

similar effects and that a minimum amount of experimental data is necessary to obtain reliable results.

Application to findings on "new" end points

For the above-described examples, "type-2 alkenes and neurodegeneration" and "potential glucocorticoid antagonists with allergenic effects", QSAR models were used to investigate whether these models can predict the assumed effect. However, no QSAR models are available for neurodegeneration and delayed hypersensitivity reactions. The same is true in most cases for the more broadly defined end points of neurotoxicity and immunotoxicity, which may be used as approximations. A possible reason for this is the absence of a sufficient amount of experimental data of adequate quality and from the same experimental configuration. This is currently the decisive obstacle to the establishment of models to predict the influence of chemicals on biochemical parameters [39]. Adequate substance-specific experimental tests to verify the postulated effects are currently not available, nor can the relevance of chemicals for the effects considered here be examined by means of QSAR predictions. In the cases discussed here, the mechanistically based hypothesis cannot be verified by QSAR, mainly due to a lack of data. Yet, good QSAR models may also support a mechanistic hypothesis if the available data so warrants (or require such a hypothesis for meaningful data interpretation as a basis for the selection of the experimental data used for modelling). Some modules are being developed for the prediction of immunotoxicity and (delayed) hypersensitivity reactions as well as for "passage of the blood-brain barrier". Therefore, at least for these ("substitute") end points, it is expected that possibilities of prediction will be available in the near future. The use of QSAR in regulatory toxicology would be a major driver for the development of QSAR models (*e.g.* as seen in the pharmaceutical industry during their attempt to optimize active ingredients of medicinal products).

Mechanistic approaches in regulatory toxicology, status, research activities and further approaches

Current relevance in regulatory toxicology

Chemical safety requirements under European chemicals legislation (REACH) are mostly based on effects testing. The majority of the OECD tests available for this purpose are end point-oriented, and the mechanism is left as a black box. Mechanism-based tests exist for only a few end points. The mouse local lymph node assay (LLNA; OECD TG 429) is one example. The use of whole batteries of *in vitro* tests (combinations of different tests) also allows the absence of, *e.g.* genotoxicity to be claimed for a substance with sufficient certainty, without animal experiments. Each of the *in vitro* tests applied for this purpose also provides indications as to the mechanism of genotoxicity.

In other fields, for example in the field of phototoxicity (*e.g.* 3T3 NRU test; OECD TG 432), recognised and standardised *in vitro* alternatives to animal experiments are already being combined with knowledge about mechanisms.

Activities aimed at avoiding animal studies and standardising corresponding methods with a view to the testing of chemicals under relevant EU legislation are collected in a database at the Institute for Health and Consumer Protection of the Joint Research Centre http://ecvam.jrc.it. European legislation requires the search for and application of such alternative, mechanism-based approaches (see REACH). Research is working to increasingly meet this demand. However, in order to satisfy the regulatory requirements, it will be necessary to develop combinations of mechanism-based tests that can be carried out quickly.

Currently, *in vitro* tests are often carried out for screening purposes. Yet, the results from these tests are also being used increasingly for clarifying mechanisms of action or for gaining a better understanding of them. In most cases, *in vitro* tests are not sufficient to replace animal studies. Most of these tests have not yet been validated, and their regulatory relevance is therefore not clear [see reviews 40, 41].

Comparative toxicogenomics database (CTD)

The CTD is a tool to identify genes and their interaction with a specific chemical [42] and the disease that is associated with that interaction. It is based on the journal articles included in the PubMed literature database and uses data from the NLM database OMIM (Online Mendelian Inheritance in Man®) with regard to associations between a gene and disease and from the KEGG (Kyoto Encyclopedia of Genes and Genomes) system for information about signal transduction pathways.

The database is updated every month and currently (as at October 2009) includes about:

- 195,000 chemical-disease associations
- 190,000 chemical-gene interactions
- 670,000 gene-disease associations.

CTD data are prepared with great care and effort, including the standardisation and (hierarchic) organisation of the vocabulary used.

The database offers an abundance of search options and tools, and the search results include many links (up to the underlying PubMed entry). The CTD generally offers the possibility to obtain information about potential diseases mediated by chemicals and is at present unique in this form. However, the information provided is often of an indirect nature, and the underlying data must always be critically reviewed in detail. They can,

nevertheless, be used as guidance for further searches. CTD is only one database with toxicogenomic information, although it is the most important one for toxicological questions.

The concept of the U.S. EPA

In 2007, an expert commission of the U.S. Environmental Protection Agency (U.S. EPA) and of the U.S. National Institute of Environmental Health Services at the National Research Council (NRC) published a fundamental report: "Toxicity testing in the 21st century: a vision and a strategy" [43]. The publication is a central document in current regulatory toxicology. The considerations of the NRC and EPA are based on an analysis of the shortcomings in the current procedure of toxicological risk assessment. The experts specifically consulted for this purpose saw the following limitations in toxicity testing of chemicals as practised to date:

- The uncertainty inherent in using data from animal studies,
- Extrapolation of a high dose (animal study with a small number of animals) to a lower dose relevant to humans,
- Development of ever more end point-related tests, which will continue to be necessary in future, since relevant end points have not adequately been addressed in the present test system making corresponding retesting of substances necessary,
- The ethical issue of animal protection and
- The Aggravation of that problem in view of the call for more extensive testing of additional toxicological end points for further substances (the central issue in REACH), as well as
- The extremely high costs involved,
- The insufficient possibility of a characterisation of mixtures, and finally
- The insufficient assignment to risk groups or life stages.

Against this background, the NRC considers the current test strategy to be a dead end and is proposing a "completely new" concept [also see symposium summary 44] in the form of a bottom-up approach. In this concept, data on physicochemical and biochemical parameters would be determined and used to establish or predict the toxicity of a substance in humans. In rare cases, the prediction would need to be verified via animal studies, which would then be carried out in a targeted manner. Such a concept requires the generation of a large number of substance-specific data that provide information about the interaction of substances with the organism, *e.g.* via *in vitro* systems. State-of-the-art methods (*e.g.* genomics, proteomics and metabonomics) are available for this today.

To predict the properties and toxicity of substances on the basis of already existing data and computer-based models, the EPA has created the electronic information system, Aggregated Computational Toxicology Resource (ACToR) [45], which is associated with the predictive EPA programme ToxCast™ [46]. During the first phase of this programme, a toxicological profile was established for more than 300 chemicals whose toxicological behaviour is known from *in vivo* tests, and these profiles were stored in the database as validation set [47]. Toxicity was also assessed for these substances by means of *in vitro* and *in silico* methods (the CTD discussed above is also integrated in ACToR). Based on the substance activity profiles, an attempt was made to generate a pattern for the prediction of toxicity in *in vivo* tests. In a second phase, predictability will be increased, and a more differentiated selection of chemicals tested. In the third and most important phase, the knowledge that was collected in phases one and two will be used to set priorities for the chemicals to be tested [48,49]. Searches in both ToxCast and ACToR require a harmonised language and conventionalised input of the chemical structures of the substances concerned. The system for this is supplied via the Distributed Structure-Searchable Toxicity Database Network project (DSSTox).

The consistent methodological use of recent knowledge in an integrated approach is something radically new. However, individual elements, *e.g.* determination of toxicity via mechanistic studies, use of genomics or integration of QSAR elements, have been explored for some time now and, in individual cases, have already been applied over the last 10 years.

Discussion and conclusions

The study presented here takes up a highly topical issue of the development of toxicological testing strategies and their integration in regulatory toxicology. Similar discussions in the U.S.A. have started and led to initial provisional results only recently. This issue is being pursued intensely in the U.S.A. by means of the "Toxicity testing in the 21st century: a vision and a strategy" concept. Yet, the fact that the American authorities underline its visionary character also demonstrates that this approach, which is based on mechanistic toxicity pathways, is still at its beginnings. The concept must be further developed before it can be fully integrated into regulatory toxicology.

By focusing on new end points and mixtures, the study deals with one aspect of this paradigm shift in regulatory toxicology that still has a particularly visionary character. On account of the complexity involved, uncertainties due to lacking knowledge, and limited availability of data on "new" end points (such as Alzheimer's disease, specific immunotoxic mechanisms or

ageing), it is not yet possible to establish hypotheses with sufficient reliability and validate them comprehensively. As regards mixtures, the study showed that due to an abundance of competing and interacting mechanistic steps, it is still difficult in that area to identify the toxicity pathway decisive for the resulting total effect and to draw (at least semi-)quantitative conclusions.

In our opinion, it is nevertheless essential to continue the considerations made here and, as regards the effect of chemicals, to draw more attention to those "new" end points that have hardly been considered in the past. Likewise, methods should be developed that provide qualified answers about the adverse effects of complex, heterogeneous mixtures of substances. This could lead to the insight, for example, that the furan compounds considered here should be investigated in more detail in that regard.

Even if it can be expected that it will not be possible to confirm some of the hypotheses put forward on the similarity of the effects of the substances considered, examples of this kind are helpful for broadening effects-based approaches and for more adequately integrating additional conditions that may need to be taken into account.

For mixtures containing a relatively small number of interacting substances, commonly affected end points and/or commonly affected alterations in gene or protein expression could be detected by means of the CTD and alterations postulated in this way could be verified experimentally for the specific mixture. Effects testing could thus be concentrated on particularly relevant end points that can justifiably be assumed to exist.

To promote such a mechanistic approach, all those performing risk assessments for individual substances should take fundamental findings on effect mechanisms (more strongly) into account in the future and document them. Although current risk assessment monographs often contain a passage on "mechanism/mode of action" for the effect regarded as critical, this does not usually include information on how the findings were generated, *e.g.* by "-omics" analyses, and in what form they may be stored in, *e.g.* the CTD. Such considerations may also be of interest for end points that are regarded as non-critical in line with the "new" end points addressed above.

In the case of effect end points for which the available data are still insufficient to allow a final assessment to be made, considerations based on crystallographic methods in conjunction with information on toxicity pathways may be taken into account in their assessment, using a read-across method, even if current (Q)SAR models do not address the end point considered.

Some suitable high-throughput screening methods are already available for end point-related research. They should be used to identify individual priority substances for more specific testing. The base data should be input into existing tools to provide an improved database for other users.

Extensive knowledge of toxicity pathways will present a particular problem for the methodology applied today to assess the risks of and determine limit values for chemical substances and mixtures. The "no observed effect level" and the "lowest observed effect concentration" are core elements of such assessments. In view of biochemical or cellular changes and disturbances far below an adverse effect observable "from outside", it seems obvious that the taxonomy of the adversity concept should be updated.

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Authors' contributions

KH performed a literature review and compilation on relevant biochemical and cellular events and the respective impact of xenobiotics. KH also helped draft and finalise the manuscript. AT contributed to the project by adding chapters on "new" toxic end points and combination effects on xenobiotics. FK was the initiator of this research project, contributed aspects of status and research activities of this effect mechanism-based approach within the regulatory context and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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