Virtual Test Kits for Predicting Harmful Effects Triggered by Drugs and Chemicals Mediated by Specific Proteins

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Summary
Poor pharmacokinetics and toxicity are not only frequent causes of late-stage failures in drug development but also a source for unnecessary animal tests. In drug discovery and for the assessment of the toxic potential of chemicals, in silico techniques are nowadays considered as valuable alternatives to in vivo approaches. Based on a receptor-modelling concept developed at our laboratory (multidimensional QSAR), we have developed and validated virtual test kits for the estrogen, androgen and aryl hydrocarbon receptor (→ endocrine disruption), for cytochrome P450 3A4 (→ metabolic transformations) and most recently for the thyroid receptor. These surrogates have been tested against a total of 430 compounds and are able to predict the binding affinity close to the experimental uncertainty. These results suggest that our approach is suited for the in silico identification of adverse effects triggered by drugs and chemicals. Consequently, we are prepared to offer a free testing to selected academic institutions and non-profit oriented organisations.

Zusammenfassung: *In silico* Tests zur Voraussage rezeptorvermittelter Toxizität von Arzneistoffen und Chemikalien
Ungenügende pharmakokinetische Eigenschaften und Toxizität sind in der pharmazeutischen Entwicklung nicht nur häufige Gründe für sogenannte „late-stage failures", sondern auch eine Quelle für unnötige Tierversuche. In der Entwicklung neuer Arzneistoffe und bei der Abschätzung des toxischen Potenzials von Chemikalien werden computer-gestützte Verfahren heute als wertvolle Alternativen zu in vivo Verfahren eingestuft. Basierend auf einem in unserem Labor entwickelten Rezeptormodellierungskonzept (multidimensionales QSAR) haben wir virtuelle Tests für den Estrogen-, Androgen- und Dioxinrezeptor (→ endokrine Störungen), für Cytochrom P450 (→ Metabolismus) sowie unlängst auch für den Thyroidrezeptor entwickelt und validiert. Diese Surrogate wurden an insgesamt 430 Verbindungen getestet und sind in der Lage, die Bindungsaffinität nahe der experimentellen Ungenauigkeit vorauszusagen. Diese Ergebnisse lassen den Schluss zu, dass unser Ansatz geeignet ist, von Arzneistoffen und Chemikalien ausgehende unerwünschte Nebenwirkungen in silico zu erkennen. In der Folge sind wir bereit, ausgewählten akademischen Institutionen und nicht-profit orientierten Organisationen kostenfreie Tests anzubieten.

Keywords: virtual test kits, virtual experiments, in silico toxicology, multi-dimensional QSAR, reducing animal testing

1 Introduction

Some 40 years ago, Corwin Hansch began quantifying relationships between a compound's physico-chemical properties and its biological activity. His series of papers laid the foundations for a technology referred to as quantitative structure-activity relationships (QSAR). Since then, researchers have employed QSAR to predict the activity of new drugs. A structure-activity relationship has first been established in 1863 by Antoine F. Cros (cf. Borman, 1990) who observed that the toxicity of alcohols for mammals increased as the molecule's water solubility decreased. In 1899, Hans-Horst Meyer (1899) and Charles E. Overton (1901) identified a relationship between the activity of 51 narcotica and their accumulation in the lipophilic phase. Between 1935 and 1938 Louis Hammet correlated electronic properties of organic acids and bases with their pK_a value. In 1964 Spencer M. Free and James W. Wilson formulated their mathematical model allowing to break down the observed biological activity to contributions from individual functional groups and Corwin Hansch and Toshio Fujita (1964) published their seminal work.

In his attempt to formulate a general QSAR model, Hansch went a step further by considering drug transport and distribution as a two-step process (Hansch and Clayton, 1973): after a "random walk" from the site of application to the receptor site, where the drug has to cross several aqueous and lipid barriers, the drug interacts with the protein binding site. The underlying mathematical formulation extended the applicability to *in*

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vivo data. While this and related formulations could quantitatively model the biological activities of congeneric series of neutral molecules, serious problems arose for compounds with significantly different pK_a values which could only be appropriately addressed by including the pK_a into the mathematical formulation (Lien, 1975). Shortly after the advent of Hansch analysis, there were only few parameters (log P, π, hydrophobicity, scales for electronic properties and steric parameters) but later hundreds of parameters describing topology, connectivity, electronic state and others were generated. Already in 1972, John Topliss pointed out that a large number of variables in the models increased the risk of random correlations. Many people neglected these warnings and, as a consequence, literature was deluged with a flood of meaningless models, containing unreasonable combinations of too many parameters (Kubinyi, 2002). In 1973, Ungerer and Hansch defined the good practice in QSAR: "select independent variables, justify the choice of the variables by statistical procedures, apply the principle of parsimony, have a large number of objects as compared to the number of variables, try to find a qualitative model of physicochemical or biochemical significance". Whereas such recommendations constitute a sound ethical guideline, they are not helpful in the derivation of quantitative models if, indeed, many different variables have to be considered. Forward and backward elimination procedures are worthless; even stepwise regression often ends up in a local minimum. The methods of choice for variable selection are evolutionary and genetic algorithms (cf. Kubinyi, 2002 and references cited therein). As with a sufficient number of variables it is possible to "explain everything", it would seem to be of utmost importance to apply the QSAR to a set of test compounds not used to derive the model (see also the "Setubal principles", for example under http://ecb.jrc.it/QSAR).

It is an inherent weakness of classical QSAR that it is restricted to properties and sub-structural features that do not consider the three-dimensionality of real molecules. In 1979, a new approach was proposed to describe molecular properties by fields, calculated in a regular grid (Cramer and Milne, 1979). Vectors representing the clustering of molecular preferences (with respect to a hypothetical binding site) were extracted from these fields by principal component analysis and correlated with biological activities. It was only after 1988 when partial least squares (PLS) analysis was used to correlate the field values with biological activity, the concept – now referred to as Comparative Molecular Field Analysis (CoMFA) – became widely used, particularly to derive quantitative models for protein binding affinities (Cramer et al., 1988; Kubinyi, 1993; Kubinyi et al., 1998a, b). In CoMFA, the first 3D-QSAR concept, the group of congeneric compounds used in a study should act via the same mechanism and, therefore, should fit into a common pharmacophore. In contrast to 2D-QSAR methods, however, they need not to have the same molecular skeleton. The crucial problem of CoMFA studies – their limitation to a single 3D conformation for each ligand – is appropriately addressed by 4D-QSAR (Hopfinger et al., 1997; Ekins et al., 1999; Vedani et al., 2000). Table 1 summarises the philosophy behind different QSAR concepts.

Unfortunately, it is frequently "overlooked" in QSAR studies that the binding affinity of a given compound does not simply depend on its physico-chemical properties but mainly on its interactions with the target protein. Strong binding to a bioregulator is only observed if these interactions overcompensate the price for leaving, say, an aqueous environment. While the latter is often included in QSAR parametrisation, ligand-protein interactions are typically not. With now the 3D structures of over 30,000 proteins available (see http: www.rcsb.org/pdb), it cannot be fathomed why only few QSAR studies include the binding pocket of the protein. Simply analysing the properties of the small molecule may not be sufficient to explain the observed biochemical activity, not to speak of selectivity, e.g. towards receptor subtypes. Why would nature come up with complex proteins if they were not to play a crucial role? This fact should be considered in structure-activity studies by including the protein counterpart. In the following, such studies will be referred to as protein-based QSAR or receptor-modelling studies, respectively. The latter is particularly applied when the 3D structure of the protein is unknown but has been replaced by an atomistic or quasi-atomistic binding-site surrogate.

Accommodation of a ligand molecule in the binding pocket is often facilitated by the adaptation of the protein to the very ligand topology – a phenomenon referred to as induced fit. It may include the rearrangement of few side-chain

Tab. 1. Dimensionality of QSAR approaches

Dimension	Method	Protein
1D-QSAR	Affinity correlates with pK_a, logP, electronic properties, etc.	no
2D-QSAR	Affinity correlates with structural patterns (connectivity, 2D pharmacophore)	no
3D-QSAR	Affinity correlates with the three-dimensional structure of the ligands	possible
4D-QSAR	Substances are represented as an ensemble of conformers, orientations, protonation states, stereoisomers	typical
5D-QSAR	as 4D-QSAR + representation of different induced-fit models	yes
6D-QSAR	as 5D-QSAR + representation of different solvation models	yes

conformations but extend to whole loops, flaps or even extended domains. Although clearly a key phenomenon, induced fit is mostly neglected in QSAR studies. In 1998, the impact of induced fit onto ligand binding in a QSAR context was recognised for the first time and, consequently, incorporated into the *Quasar* software (Vedani et al., 1998). Unfortunately, even with 4D-QSAR, a major unknown persists: manifestation (and magnitude) of the induced fit. To address this problem in an unbiased fashion, Vedani and co-workers extended their concept by a further dimension (5D-QSAR; Vedani and Dobler, 2002; see also below). Since then, this technology was applied to several receptor and enzyme systems (e.g. Vedani et al., 2005a, b; Lill et al., in press; Lill et al., submitted).

Toxic agents, particularly those that exert their actions with a great deal of specificity, sometimes act via receptors to which they bind with high affinity. This phenomenon is referred to as receptor-mediated toxicity. Examples of soluble intracellular receptors, which are important in mediating toxic responses, include the glucocorticoid receptor which is also involved in mediating toxicity associated effects such as apoptosis of lymphocytes as well as neuronal degeneration as a response to stress, the peroxisome proliferator activated receptor, which is associated with hepatocarcinogenesis in rodents, and the aryl hydrocarbon receptor which is involved in a whole range of toxic effects (Gustaffson, 1995). Harmful effects of drugs and chemicals can often be associated with their binding to other than their primary target – macromolecules involved in biosynthesis, signal transduction, transport, storage, and metabolism (Rihova, 1998; Fischer 2000; Lukasiuk and Pitkänen, 2000; Rymer and Good, 2001; Hampson and Grimaldi, 2002; Oliver and Roberts, 2002).

Toxicity testing – mandatory by international regulations for drug development and chemical safety – is still associated with stressful animal tests. While many *in vitro* approaches have been devised for targeting the various aspects of toxicological phenomena, they require a chemical or drug molecule to be physically present (i.e. synthesised) before testing, are time consuming, and the results are often associated with large standard errors. In contrast to *in vitro* assays, computational approaches can be applied to hypothetical substances as their 3D structure can readily be generated *in silico*. The nowadays-available computer power permits to scan larger batches of compounds (e.g. parts of corporate or public databases) in a relatively short time. Toxicity-modelling algorithms are typically based on quantitative structure-activity relationships, neuronal networks, artificial intelligence or rule-based expert systems. In the following, we will demonstrate the feasibility of protein-based QSAR for the quantitative prediction of receptor-mediated adverse effects triggered by drugs and chemicals.

2 Methods

Quasar – a receptor-modelling concept developed at the Biographics Laboratory 3R – is based on 6D-QSAR and explicitly allows for the simulation of induced fit (Vedani and Dobler, 2002; Vedani et al., 2005b). *Quasar* generates a family of quasi-atomistic receptor surrogates that are optimised by means of a genetic algorithm. The hypothetical receptor site is characterised by a three-dimensional surface which surrounds the ligand molecules at van der Waals distance and which is populated with atomistic properties mapped onto it. The topology of this surface mimics the three-dimensional shape of the binding site; the mapped properties represent other information of interest, such as hydrophobicity, electrostatic potential and hydrogen-bonding propensity. The fourth dimension refers to the possibility of representing each ligand molecule as an ensemble of conformations, orientations and protonation states, thereby reducing the bias in identifying the bioactive conformation and orientation (→ 4D-QSAR). Within this ensemble, the contribution of an individual entity to the total energy is determined by a normalised Boltzmann weight. As manifestation and magnitude of induced fit may vary for different molecules binding to a target protein, the fifth dimension in *Quasar* allows for the simultaneous evaluation of up to six different induced-fit protocols (→ 5D-QSAR). The most recent extension of the *Quasar* concept to six dimensions (→ 6D-QSAR) allows for the simultaneous consideration of different solvation models. This can either be achieved explicitly where parts of the surface area are mapped with solvent properties whereby position and size are optimised by the genetic algorithm, or implicitly. Here, the solvation terms (ligand desolvation and solvent stripping) are independently scaled for each different model within the surrogate family, reflecting varying solvent accessibility of the binding pocket. The associated weights are evolved throughout the simulation. Like for the fourth and fifth dimension, a modest "evolutionary pressure" is applied to achieve convergence. Details may be found in the program documentation (see http://www.biograf.ch/PDFS/Quasar.pdf). In the *Quasar* concept, the binding energy is calculated as follows:

$$E_{binding} = E_{ligand-receptor} - E_{ligand\ desolvation} - T\Delta S - E_{ligand\ strain} - E_{induced\ fit}$$

The contributions of the individual entities within an ensemble (conformation/orientation/protonation, induced fit, and solvation) are normalised to unity using a Boltzmann criterion:

$$E_{binding,\ total} = \sum E_{binding,\ individual} \cdot \exp\left(-w_i \cdot E_{binding,\ individual} / E_{binding,\ individual,\ maximal}\right)$$

The concept has been used to quantify the interactions of various large systems, including GPCRs (Vedani et al., 2000; 2005a) and nuclear receptors (Vedani et al., 2005b, 1999; Vedani and Dobler, 2003, 2001; Dobler et al., 2003; Lill et al., in press; Lill et al., submitted), thereby most recently focusing on adverse effects triggered by drugs and chemicals (see also: http://www.biograf.ch/projects.html).

Raptor, an alternative technology developed by our laboratory (Lill et al., 2004) explicitly and anisotropically allows for induced fit by a dual-shell representation of the receptor surrogate, mapped with physico-chemical properties (hydrophobic character and hydro-

gen-bonding propensity) onto it. In *Raptor*, induced fit is not limited to steric aspects but includes the variation of the physico-chemical fields along with it. The underlying scoring function for evaluating ligand-receptor interactions includes directional terms for hydrogen bonding, hydrophobicity and thereby treats solvation effects implicitly. This makes the approach independent from a partial-charge model and, as a consequence, allows to smoothly model ligand molecules binding to the receptor with different net charges. Details may be found in the program documentation (see http://www.biograf.ch/PDFS/Raptor.pdf). In *Raptor*, the binding energy is determined as follows:

$$\Delta G = \Delta G_{\text{hydrogen bonding}} + \Delta G_{\text{hydrophobic}} + T\Delta S + \Delta G_{\text{induced fit}}$$

Albeit to a lesser extent than for 3D-QSAR, the identification of the bioactive conformation is crucial for 6D-QSAR as well – at least to the extent that is must be present in the 4D representation of the ligand data. Depending upon whether or not the 3D structure of the target protein is available at atomic resolution, two fundamentally different concepts are used to identify this bioactive conformation: flexible docking for systems with known 3D structure and pharmacophore hypothesis builder otherwise. For three of the systems presented in this account (estrogen and androgen receptor, cytochrome P450 3A4), the crystal structures are available, for the other (aryl hydrocarbon receptor) it is not.

Flexible docking aims at identifying all potential binding modes (orientations, conformations) of a small molecule within the binding pocket of a protein. The underlying protocol should address two aspects of ligand-protein binding which would seem to be important: 1. the simulation of induced fit, i.e. allowing the protein to adapt its shape to the different orientations and conformations of the small molecule during the search procedure and 2. the consideration of solvent effects (typically water). In our approach (software *Yeti*), the sampling is conducted based on a Monte-Carlo Metropolis protocol which allows to initially (i.e. before energy minimisation) consider apparently less favourable orientations or conformations. Such calculations are quite computing intensive as for a "exhausting search", typically 5,000–10,000 conformations/orientations are generated and 500–1000 are fully minimised. For our studies, we have used the *Yeti* concept (Vedani and Huhta 1990, 1991; see also: http://www.biograf.ch/PDFS/Yeti.pdf). This protocol was applied for the estrogen receptor (Vedani et al., 2005b) and androgen receptor (Lill et al, in press) as well as for cytochrome P450 3A4 (Lill et al., submitted). For the Ah receptor where no experimental structure is available, the alignment was performed based on the molecular skeletons (Vedani et al., 1999; Vedani and Dobler, 2002, 2003).

2.1. The aryl hydrocarbon (Ah) receptor

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds represent serious environmental health hazards, whose effects include tumor promotion, dermal toxicity, immunotoxicity, developmental and reproductive toxicity as well as induction and inhibition of various enzyme activities. TCDD also induces differentiation changes affecting, for example, the

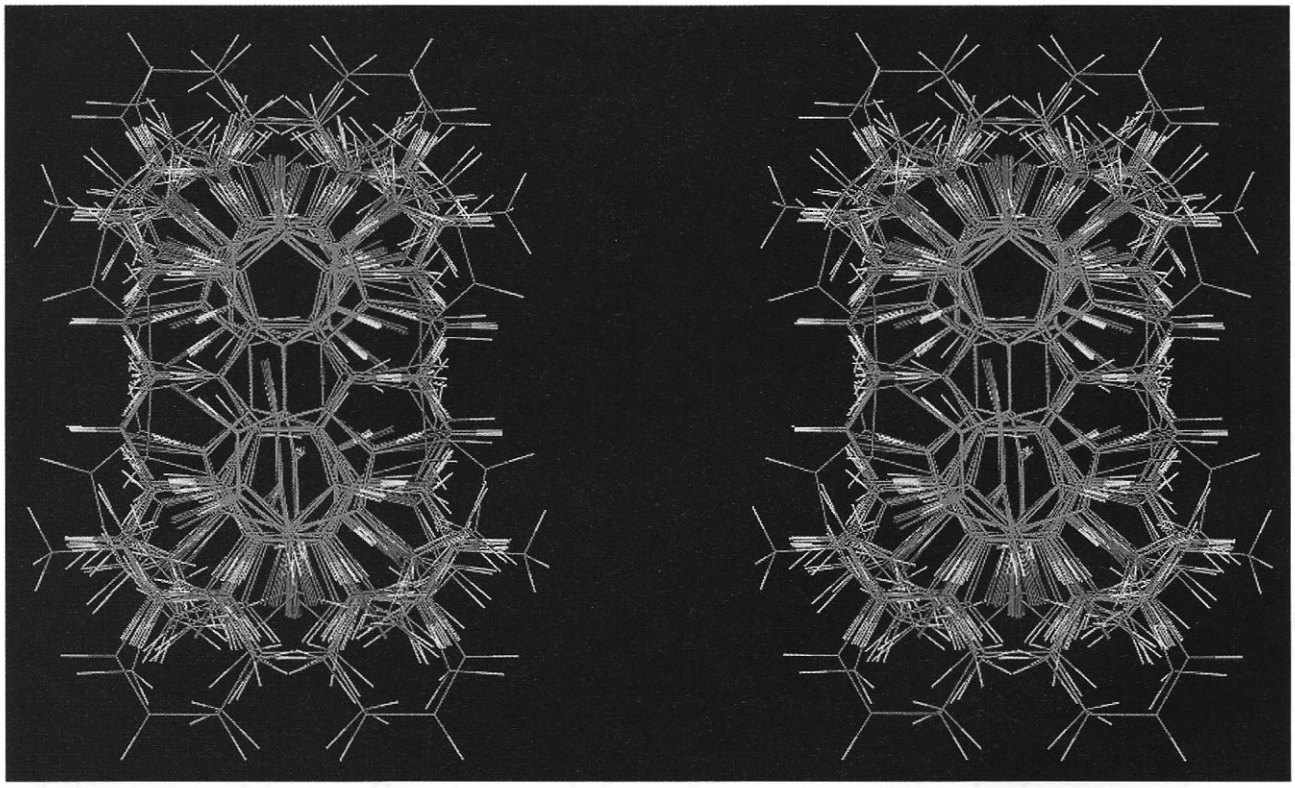

Fig. 1: Stereo view of the 4D representation (461 entities) of the 121 ligands used in the Ah receptor simulation.

human epidermis – manifesting itself as chloracne. There is strong evidence that this effect is mediated by the aryl hydrocarbon receptor (AhR), a regulatory element involved in the mammalian metabolism of xenobiotics. In target cells, TCDD initially binds to the AhR, which accumulates in the nucleus as an "AhR-aryl hydrocarbon nuclear translocator" heterodimeric complex. The nuclear AhR complex acts as a ligand-induced transcription factor, which binds to transacting genomic dioxin/xenobiotics responsive elements located in the 5'-regulatory region upstream from the initiation site. This interaction results in transactivation of gene transcription. Treatment of animals or cells with TCDD and related Ah-receptor agonists can result in decreased enzyme activities and decreased gene expression (see, for example, Safe, 1990; Landers and Bunce, 1991; Rappe, 1993; Swanson and Bradfield, 1993; Whitlock, 1993; Okey et al., 1994; Safe and Krishnan, 1995; Putzrath, 1997).

To establish a QSAR for the Ah receptor, we used a total of 121 dibenzodoxins, dibenzofurans, biphenyls and polyaromatic hydrocarbons (PAHs): 91 represented the training set, 30 the test set (Vedani et al., 1999; Vedani and Dobler, 2003; Dobler et al., 2003). The surrogate family was generated with *Quasar* and included 1000 receptor models, which were evolved over 60,000 crossover cycles (corresponding to 60 generations) by allowing for transcription-error rate of 0.02. The simulation converged at a cross-validated r^2 of 0.816 and yielded a predictive r^2 of 0.763. Both quantities represent values averaged over the 1000 models. A three-dimensional representation of the 4D dataset is depicted in Figure 1, the quasi-atomistic model in Figure 2 and the experimental and calculated IC_{50} values are compared in Figure 3.

The rms deviation for the 91 ligand molecules of the training set corresponds to an uncertainty factor of 1.9 in the binding affinity; the maximal individual deviation is to a factor 8.2 in the IC_{50} value. A total of 30 compounds (not used for model construction) were employed for testing the predictive power of the receptor surrogate yielding a predictive r^2 of 0.773. On the average, the predicted binding affinities of the test ligands deviate by a factor of 2.4 in IC_{50} from the experiment; the maximal observed deviation corresponds to a factor of 9.9 in IC_{50}. This surrogate was then employed to test its ability to handle test compounds – both benign and harmful – that do not belong to any structural class from training or test set. The results suggest that this receptor model is suited to be used as a virtual test kit for identifying toxic effects triggered by small molecules and mediated by the aryl hydrocarbon receptor (Vedani et al., in press).

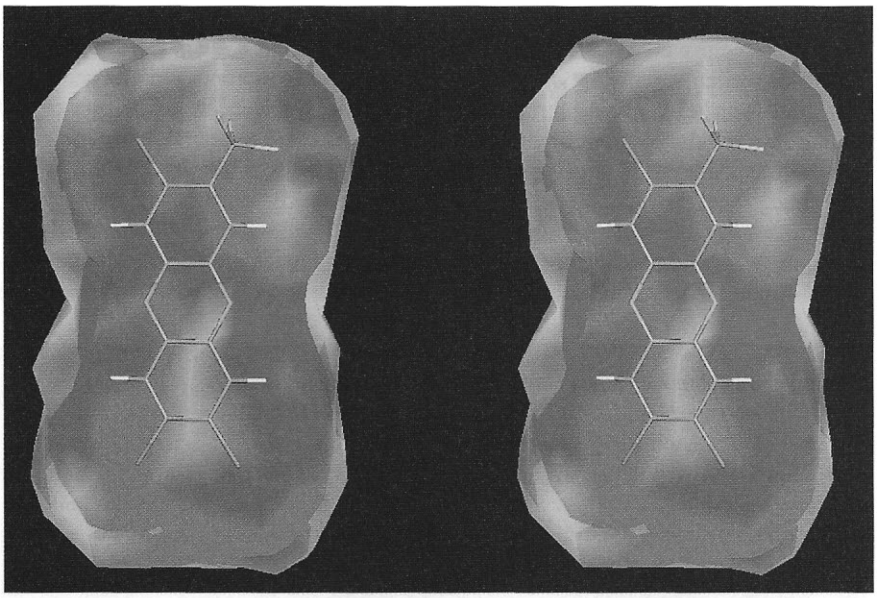

Fig. 2: Stereo view of the Ah receptor surrogate with 2-CF_3-3,6,7-tricholoro-dibenzo-*p*-dioxin bound. For clarity, the front section has been clipped. Areas colored in gray/brown represent hydrophobic properties; areas in green H-bond donor functions (H-bond acceptors and salt bridges are not observed as the ligand data set lacks corresponding groups).

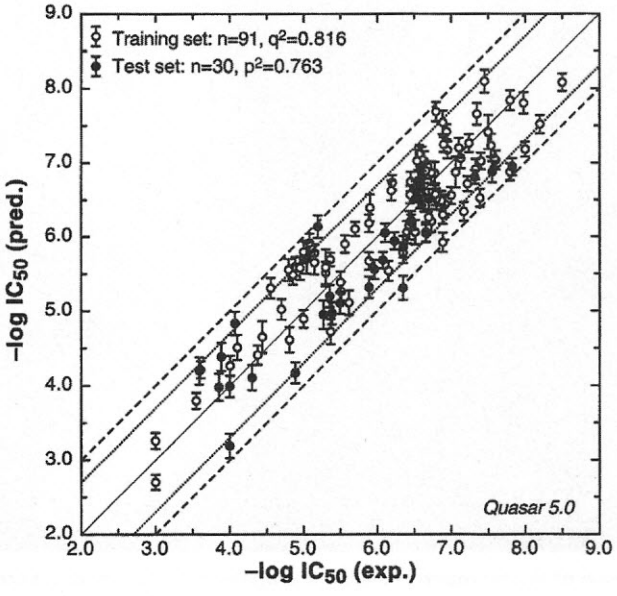

Fig. 3: Comparison of experimental and calculated IC_{50} values for the Ah receptor surrogate.

2.2 The estrogen receptor

Nuclear receptors comprise a family of ligand-dependent transcription factors that transform extra- and intracellular signals into cellular responses by triggering the transcription of target genes. In particular, they mediate the effects of hormones and other endogenous ligands to regulate the expression of specific genes. Among other members, this family includes receptors for the various steroid hormones – e.g. the estrogen, androgen, progesterone, and glucocorticoid receptor. Unbalanced production or cell insensitivity to specific hormones may result in diseases associated with human endocrine dysfunction (Zubay et al., 1995). The presence of hormonally active compounds – endocrine disruptors – in the biosphere has become a worldwide environmental concern. It has been concluded that such compounds elicit a variety of adverse effects in both humans and wildlife including promotion of hormone-dependent cancers, reproductive tract disorders, and a reduction in reproductive fitness. A number of receptor-mediated hormonal responses to toxicity are known, including xenobiotic effects on the thyroid hormone receptor, the epidermal growth factor receptor, the aryl hydrocarbon receptor as well as effects mediated by the androgen and the estrogen receptor, respectively. A variety of compounds in the environment have been shown to display agonistic or antagonistic activity towards the ER, including both natural products and synthetic compounds (Dibb, 1995; McLachlan and Arnold, 1996; Guillette et al., 1995; Colborn, 1995; Feldmann and Krishnan, 1995; Hoare et al., 1994; Korach et al., 1987). The concern over xenobiotics binding to the ER has created a need to both screen and monitor compounds which can modulate endocrine effects. This has been underscored by the US legislation in 1995/6, by mandating that chemicals and formulations must be screened for potential estrogenic activity before they are manufactured or used in certain processes (US Government, 1996a, b). In Switzerland, the necessity for a co-ordinated interdisciplinary approach to the environmental and public health problems caused by endocrine disruption has been recognised and a National Research Programme on Endocrine Disruption (NRP50) has been launched in 2001 (Swiss National Science Foundation).

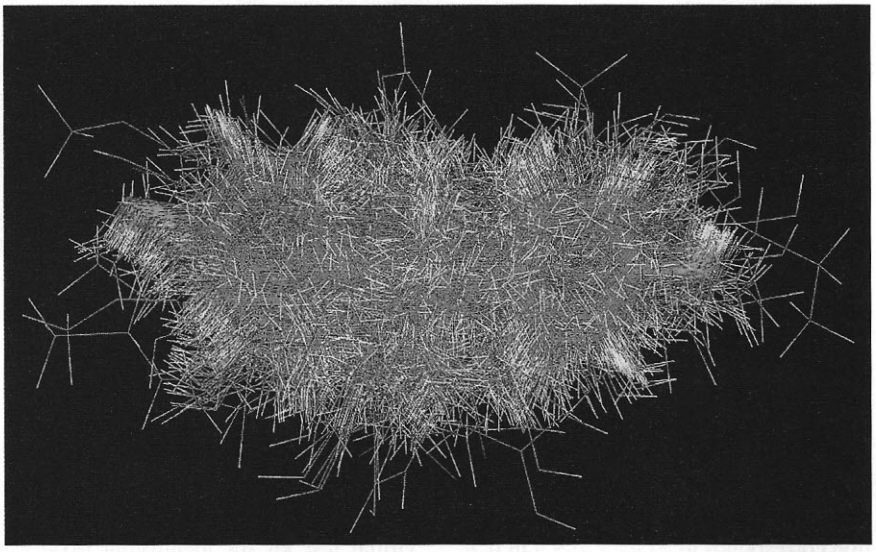

Fig. 4: The 344 entities (4D data set) of the 106 ligands used in the estrogen receptor simulation.

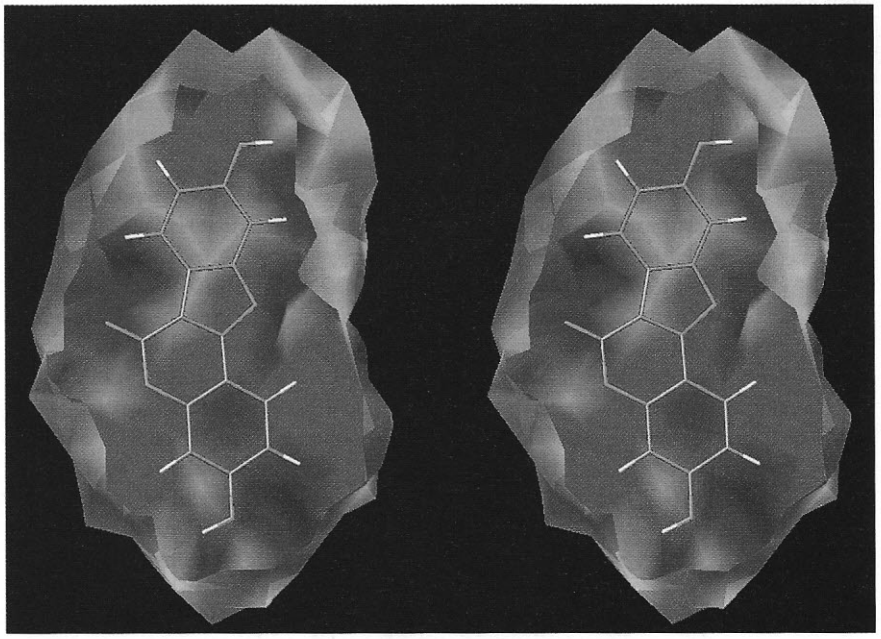

Fig. 5: Stereo view of the estrogen receptor surrogate with Coumestrol bound. For clarity, the front section has been clipped. Areas colored in gray/brown represent hydrophobic properties; areas in green H-bond donor functions (H-bond acceptors and salt bridges are not observed as the ligand data set lacks corresponding groups).

Our 6D-QSAR study was based on the X-ray crystal structure of the human ER α ligand-binding domain with bound diethylstilbestrol (Shiau et al., 1998) which served as template for identifying potential binding modes of the 106 investigated compounds. While for the symmetric diethylstilbestrol molecule the binding mode (the two phenolic hydroxyl groups engage in hydrogen bonds with Glu 353 and His 524, respectively) is unambiguous, different orientations within the binding pocket are feasible for most of the asymmetric compounds used in this study. Here, an only moderate induced fit – involving the rearrangement

of protein side chains – tolerates different ligand orientations (Vedani et al., 2005b). The three-dimensional structures of all 106 compounds were generated and optimised in aqueous solution using MacroModel (Mohamadi et al., 1990); atomic partial charges and solvation energies were calculated using AMSOL (Cramer and Truhlar, 1992). Subsequently, we sampled all feasible arrangements within the binding pocket using a Monte-Carlo search protocol based on a Metropolis selection criterion (software *Yeti*; Vedani and Huhta, 1990). For each compound, a total of 40,000 conformations and orientations within the binding site were randomly generated; 4000 thereof were fully minimised, including an extended binding pocket of 12 Å around the very ligand. For each ligand molecule, up to four energetically favorable arrangements (within 5.0 kcal/mol of the lowest-energy conformation) were retained and composed into a 4-D data set (Fig. 4), totalling in 344 representations for the 106 molecules. The ligands comprise six different substance classes and cover a range in IC_{50} of seven orders of magnitude (2.8 mM - 0.2 nM).

Of the 106 agonist molecules used in our study, 80 defined the training set, and the remaining 26 the test set. In *Quasar*, the surrogate family comprised 200 models that were evolved over 32,000 crossover cycles, corresponding to 160 generations. The simulation reached a cross-validated r^2 of 0.895 and yielded a predictive r^2 of 0.892. The receptor surrogate is depicted in Figure 5; comparison with the binding site at the true biological receptor shows that characteristic properties are well identified by the receptor surrogate (Vedani et al., 2005b). Experimental and calculated IC_{50} values are compared in Figure 6: the rms deviation for the 80 ligand molecules of the training set corresponds to a factor 2.0 in the experimental IC_{50} value; the maximal individual deviation to a factor 8.6 in IC_{50}. On the average, the 26 test compounds deviate by a factor 2.7 in IC_{50} from the experiment; the maximal observed deviation corresponds to a factor 9.5.

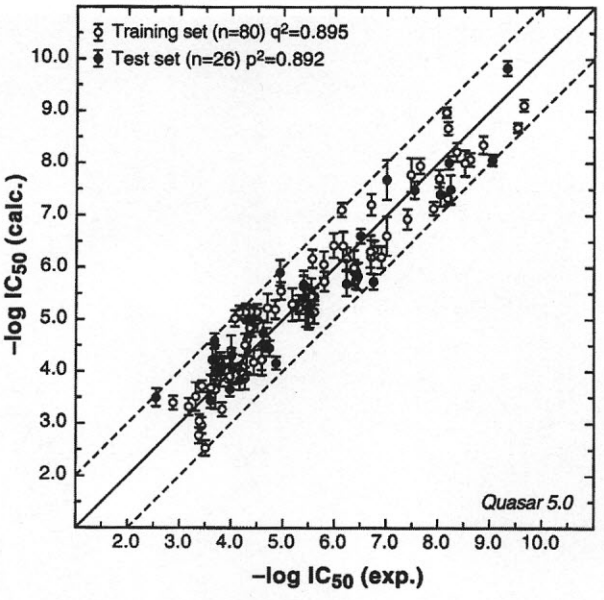

Fig. 6: Comparison of experimental and calculated IC_{50} values for the estrogen receptor surrogate.

2.3. The androgen receptor

Nuclear receptors represent the largest family of ligand-dependent eukaryotic transcription factors transforming extra- and intracellular signals into cellular responses by triggering the transcription of target genes. In particular, they mediate the effects of hormones and other endogenous ligands to regulate the expression of specific genes, thereby regulating development and metabolism. Among other members, this family includes receptors for the various steroid hormones – e.g. the androgen, estrogen, glucocorticoid and progesterone receptor. Unbalanced production or cell insensitivity to specific hormones may result in diseases associated with human endocrine dysfunction. Androgens and the androgen receptor play an essential role in the growth of normal prostate. They are, however, also involved in the development of prostate cancer, representing the most common male malignancy in the United States. Both steroidal and nonsteroidal derivatives have shown clinical benefits as chemotherapeutic agents for prostate cancer. Still, several of these antiandrogens, for example cyprosterone, show overlapping effects with other hormonal systems. Many environmental chemicals, e.g. flavones or kepone, display similar structural properties: a hydrophobic core and one or two terminal polar groups. Consequently, they may bind to a nuclear receptor influencing the balance of the endocrine system. The presence of these so-called endocrine disruptors in the biosphere has become a worldwide environmental concern. It has been concluded that such compounds elicit a variety of adverse effects in both humans and wildlife including promotion of hormone-dependent cancers, reproductive tract disorders, and a reduction in reproductive fitness. A variety of compounds in the environment have been shown to display agonistic or antagonistic activity towards the androgen receptor, including both natural products and synthetic compounds (Kavlock et al., 1996; Colborn et al. 1993; Carlsen et al., 1992). The concern over xenobiotics binding to the androgen receptor has created a need to both screen and monitor compounds expected to modulate endocrine effects. We therefore developed an *in silico* model to quantitatively predict the potential of structurally diverse ligands for binding to the androgen receptor using a multidimensional QSAR technique (software *Raptor*, Lill et al., 2004), specifically allowing for induced-fit. To identify the binding mode at the true biological receptor, a novel stepwise protocol consisting of flexible docking, molecular dynamics (MD)

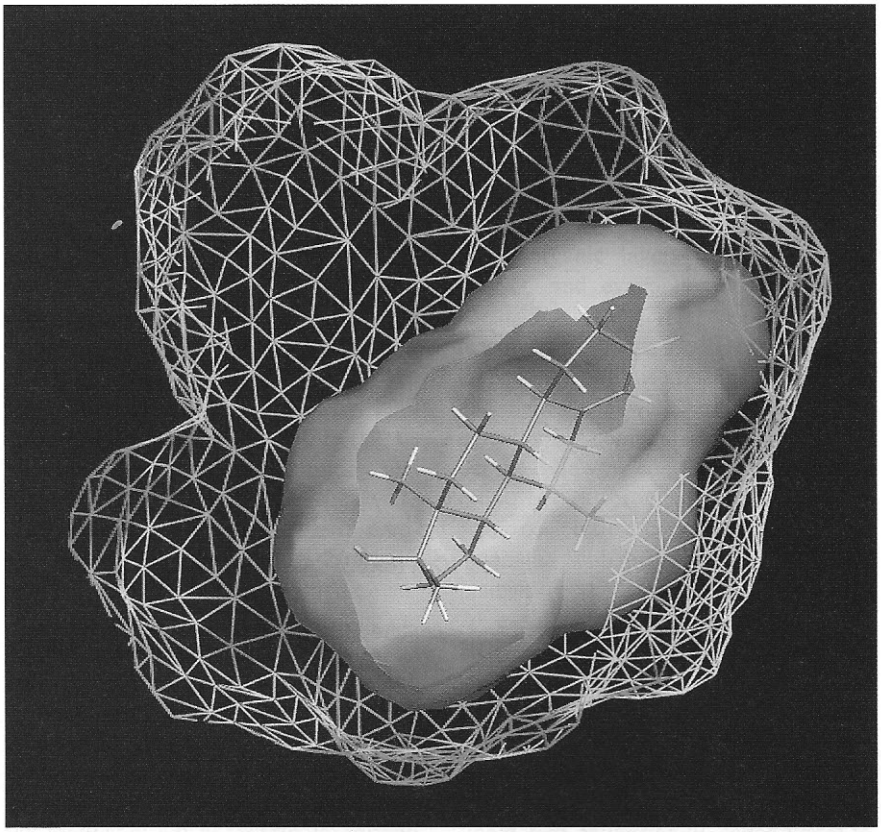

Fig. 7: Dual-shell representation of the androgen receptor surrogate with Mibolerone bound. For clarity, the front section has been clipped. Areas colored in brown represent hydrophobic properties; areas in red correspond to H-bond acceptors, blue to H-bond donors and green to H-bond flip-flops.

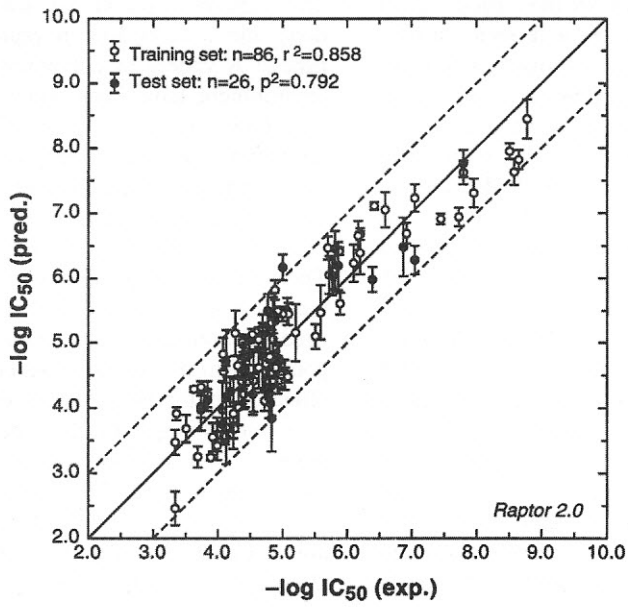

Fig. 8: Comparison of experimental and calculated IC_{50} values for the androgen receptor surrogate. (For clarity, seven threshold compounds have been omitted; cf. Lill et al., in press)

simulations and linear interaction-energy analysis (LIE) was developed (Lill et al., in press).

Our QSAR study was based on 119 ligands; 88 molecules thereof were assigned to the training set and the remaining 31 used as test. Induced fit was accounted for by the dual-shell concept of Raptor (Lill et al., 2004). To allow for topological and physico-chemical variation at the true biological receptor with different ligands bound, the *Raptor* results were averaged over 10 individual models defining a surrogate family.

The model converged at a cross-validated r^2=0.858 for the training compounds and yielded a predictive r^2=0.792 for the test compounds. The model is shown in Figure 7; experimental and calculated IC_{50} values are compared in Figure 8. On the average, the predicted binding affinity of the training ligands deviates by a factor 1.7 from the experiment; those of the test set deviate by a factor 1.6 in IC_{50}. The maximal observed deviation for individual molecules are 7.8 and 13.9, respectively.

2.4. Cytochrome P450 3A4

During multi-drug medication, the individual entities may compete for the same enzyme(s) to be metabolised. This can lead to undesired drug-drug interactions (Tanaka, 1998). In order to envision such interactions, a quantitative prediction of associated pharmacokinetic changes would be beneficiary. A major mechanism is realised by inhibiting Cytochrome P450 3A4 (CYP3A4) mediated biotransformations, as this enzyme is responsible for metabolising 50-60% of orally administered marketed drugs. For this purpose, we developed a computational model to accurately predict the inhibitory potential of a diverse set of ligand molecules using flexible docking and novel multi-dimensional QSAR techniques (Lill et al., submitted).

The three-dimensional structures of 48 compounds were generated and optimised with MacroModel (Mohamadi, 1990). After relaxation, the X-ray crystal structure of the human CYP3A4 enzyme was used as template for the automatic docking of the individual compounds. CYP3A4 can accommodate a wealth of different substrates and

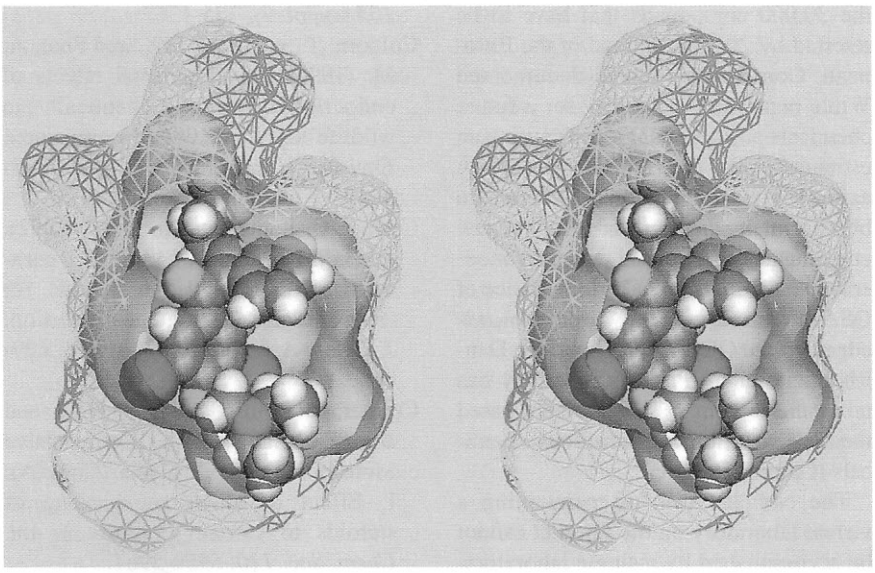

Fig. 9: Dual-shell representation of the cytochrome P340 3A4 surrogate with amiodarone bound. For clarity, the front section has been clipped. Areas colored in brown represent hydrophobic properties; areas in red correspond to H-bond acceptors, blue to H-bond donors and green to H-bond flip-flops.

its binding pocket is rather wide and predominantly hydrophobic in nature. As a consequence, different binding modes and conformations had to be considered for small molecules binding to it. Induced fit may also facilitate ligand binding, thereby hosting compounds varying in molecular volume by as much as a factor of seven. To identify the possible binding modes, we sampled structurally and energetically feasible arrangements within the binding pocket using flexible docking combined with an extended Monte-Carlo search protocol based on a Metropolis selection criterion (software *Yeti*; Vedani and Huhta, 1990) and allowing for an adaptation of the protein side-chains to each individual ligand molecule.

The simulation using the *Raptor* software (Lill et al., 2004) reached a cross-validated r^2 of 0.867 and yielded a predictive r^2 of 0.870. The binding-site model is depicted in Figure 9. Comparison with the binding site at the true biological enzyme shows that both the hydrophobic character and hydrogen-bond properties of the binding pocket are well identified by the model (Lill et al., submitted). Experimental and calculated IC_{50} values are compared in Figure 10. On the average, the predicted binding affinity of the training ligands deviates by a factor 2.4 in IC_{50} from the experiment; the maximal observed deviation is 5.0. For the ligands of the test set, the corresponding values are 2.0 and 5.2, respectively – an appreciable result, as the test set includes diverse molecules not similar in structure to any compound of the training set. The weak- and non-binding molecules were predicted to bind with affinities close to or weaker than the experimental threshold value (Lill et al., submitted).

3 Discussion

3.1. Virtual test kits

The four systems discussed in this account – aryl hydrocarbon receptor, estrogen receptor, androgen receptor, cytochrome P450 3A4 – for which a quantitative structure-activity relationship could be established and validated for a total of 430 compounds by means of multi-dimensional QSAR are part of the envisioned Internet Laboratory for the *in silico* identification of adverse effects triggered by drugs and chemicals (Vedani and Dobler, 2001, 2003; Dobler et al., 2003; Vedani et al., in press). They are already functional as individual test kits and the Biographics Laboratory 3R is prepared to offer a free testing to selected academic institutions and non-profit oriented organisations. The underlying technology (software *Yeti*,

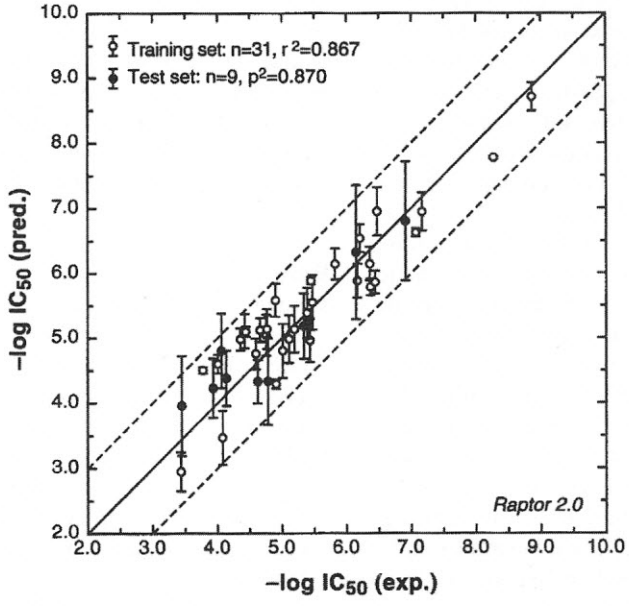

Fig. 10: Comparison of experimental and calculated IC_{50} values for the cytochrome P450 3A4 surrogate. (For clarity, eight threshold compounds have been omitted; cf. Lill et al., submitted)

Symposar, *Quasar*, *Raptor*) runs on Macintosh and PC operating systems. We are presently extending our receptor library by an additional member, the thyroid receptor (Zumstein, 2005). While the compound evaluation (software *Quasar* and *Raptor*) is very fast, the Monte-Carlo search required to identify the binding mode(s) is quite cpu intensive. Presently, up to four compounds/hour may be processed on a laptop computer; on a 32-node Linux cluster as many as 100 compounds/hour (1500 overnight) may be evaluated *in silico*. Any new extensions to the database, the technology or access procedures will be posted at http://www.biograf.ch/projects.html.

3.2 3R relevance

The envisioned Internet laboratory and the already functional virtual test kits can contribute to a significant reduction in animal testing. First, it allows for an early – even before compound synthesis – recognition of potentially harmful substances. By removing those candidate substances from the evaluation pipeline, they will not be forwarded to any *in vivo* toxicity tests. These expectations are supported by the fact that our virtual experiments have so far not produced any false-positive results. Of course, with only a limited number of enzyme/receptor systems known to mediate adverse effects and even fewer accessible in a QSAR context (due to lacking experimental affinity data), false-negative results will always be present. But this is by no means the aim of the approach – instead, it will selectively recognise potentially hazardous compounds associated with major mechanisms (e.g. metabolic degradation, endocrine disruption) and allow to discard them early on. Second, a widely used database of this kind would reduce the number of otherwise doubly-conducted toxicity tests at research laboratories focussed on closely related biomedical targets. The main advantage of the proposed virtual laboratory is that it can be applied to hypothetical substances, is reproducible, fast and cheap.

Another field of application is testing of chemicals for toxicity – for example the 30,000 compounds that have to be retested by 2012 as defined in the European Commission's well-documented White paper on the strategy for a future chemicals policy (2003) – and causing an estimated toll of 10 Million laboratory animals. Here, our system could prove to be a useful *in silico* screening tool as new compounds can be tested with only moderate "human" efforts. The importance of QSARs has more recently been acknowledged by the OECD (2003) and the Danish Environment Protection Agency has taken the lead in use of structure-based methods to prioritise hazardous chemicals (Cronin, 2003).

The complex task of maintaining a virtual laboratory on the Internet cannot be accomplished by a single laboratory. It is therefore our intention to make it freely available as soon as possible – of course, by applying security measures to avoid non-scientific use. The data base will be managed as an "open source mode", implying that all interested, skilled parties may contribute to development and extension. For this purpose, an independent source of continued funding is very important. The Foundation Biographics Laboratory 3R is a non-profit organisation with only limited financial means. We think that financial support by an independent governmental agency would represent an optimal solution. Use of the database would not be associated with any costs as we are prepared to provide to necessary software for free (universities, hospitals, regulatory bodies) or at prime costs for the pharmaceutical industry.

References

Borman, S. (1990). Cros, A. F. A., PhD Thesis, University of Strasbourg, 1863 cited from "New QSAR techniques eyed for environmental assessments". *Chem. & Eng. News 19*, 20-23.

Carlsen, E., Giwercman, A., Keiding, N. and Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *Br. Med. J. 305*, 609-613.

Colborn, T. (1995). Environmental estrogens: health implications for humans and wildlife. *Environ. Health Perspect. 103 (suppl. 7)*, 135-136.

Colborn, T., von Saal, F. S. and Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans (see comments). *Environ. Health Perspect. 101*, 378-384.

Cramer, C. J. and Truhlar, D. G. (1992). AM1-SM2 and PM3-SM3 parametrized SCF solvation models for free energies in aqueous solution. *J. Comp.-Aided Molec. Design 6*, 629-666.

Cramer, R. D. III, Patterson, D. E. and Bunce, J. D. (1988). Comparative Molecular Fields Analysis (CoMFA). I. Effect of shape on binding of steroids to carrier proteins. *J. Am. Chem. Soc. 110*, 5959-5967.

Cramer, R. D. III and Milne, M. (1979). The lattice model: a general paradigm for shape-related structure-activity correlations. *Abstracts of papers of the Am. Chem. Soc. Meeting*, April 1979, COMP 44.

Cronin, M. (2003). A toxic gamble. *Chemistry and Industry 4*, 13-14.

Dibb, S. (1995). Swimming in a sea of estrogens, chemical hormone disrupters. *The Ecologist 25*, 27-31.

Dobler, M., Lill, M. A. and Vedani, A. (2003). From crystal structures and their analysis to the in silico prediction of toxic phenomena. *Helv. Chim. Acta 86*, 1554-1568.

Ekins, S., Bravi, G., Binkley, S. et al. (1999). Three- and four-dimensional quantitative structure-activity relationship analyses of cytochrome P450 3A4 inhibitors. *J. Pharmacol. Exp. Ther. 290*, 429-438.

Feldman, D. and Krishnan, A. (1995). Estrogens in unexpected places: Possible implications for researchers and consumers. *Environ. Health Perspect. 103 (suppl. 7)*, 129-133.

Fischer, B. (2000). Receptor-mediated effects of chlorinated hydrocarbons. *Andrologia 32*, 279-283.

Free, S. M. and Wilson, J. W. (1964). A mathematical contribution to structure-activity studies. *J. Med. Chem. 7*, 395-399.

Guillette, L. J., Crain D. A., Rooney, A. A. and Pickford, D. B. (1995). Organization versus activation: the role of en-

docrine disrupting contaminants EDCs during embryonic development in wildlife. *Environ. Health Perspect. 103 (suppl. 7)*, 157-164.

Gustaffson, J. A. (1995). Receptor-mediated toxicity. *Toxicol. Lett. 135*, 465-470.

Hammet, L. P. (1935). Reaction rates and indicator acidities. *Chem. Rev. 17*, 67-79.

Hampson, A. J. and Grimaldi, M. (2002). 12-hydroxyeicosatetrenoate (12-HETE) attenuates AMPA receptor-mediated neurotoxicity: Evidence for a G-protein coupled HETE receptor. *J. Neurosci. 22*, 257-264.

Hansch, C. and Fujita, T. (1964). ρ-σ-π Analysis. A method for the correlation of biological activity and chemical structure. *J. Am. Chem. Soc. 86*, 1616-1626.

Hansch, C. and Clayton, J. M. (1973). Lipophilic character and biological activity of drugs II: The parabolic case. *J. Pharm. Sci. 62*, 1-21.

Hoare, S. A., Jobling, S., Parker, M. G. et al. (1994). Environmental persistent alkylphenolic compounds are estrogenic. *Endocrinology 135*, 175-182.

Hopfinger, A. J., Wang, S., Tokarski, J. S. et al. (1997). Construction of 3D-QSAR models using 4D-QSAR analysis formalism. *J. Am. Chem. Soc. 119*, 10509-10524.

Kavlock, R. J., Daston, G. P., DeRosa, C. et al. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ. Health Perspect. 104 (Suppl. 4)*, 715-740.

Korach, K. S., Levy, L. A. and Sarver, P. J. (1987). Estrogen receptor stereochemistry: receptor binding and hormonal responses. *J. Steroid Biochem. 27*, 281-290.

Kubinyi, H. (2002). From narcosis to hyperspace: The history of QSAR. *Quant. Struct.-Act. Relat. 21*, 348-356.

Kubinyi, H., Folkers, G. and Martin, Y. C. (1998a). 3D-QSAR in drug design. Volume 2. Ligand-protein complexes and molecular similarity, Kluwer/ESCOM, Dordrecht 1998; also published as *Persp. Drug Disc. Design 9-11*, 1-416.

Kubinyi, H., Folkers, G. and Martin, Y. C. (1998b). 3D-QSAR in drug design. Volume 3. Recent advances, Kluwer/ESCOM, Dordrecht 1998; also published as *Persp. Drug Disc. Design 12-14*, 1-352.

Kubinyi, H. (ed.) (1993). 3D-QSAR in drug design. Theory, methods and applications. Leiden: ESCOM Science Publishers B.V.

Landers, J. P. and Bunce, N. J. (1991). The Ah receptor and the mechanism of dioxin toxicity. *Biochem. J. 276*, 173-287.

Lien, E. J. (1975). Structure-absorption-distribution relationships. In E. J. Ariëns (ed.), *Drug Design* (Volume V, 81-132). New York: Academic Press.

Lill, M. A., Winiger, F., Vedani, A. and Ernst, B. (in press). Impact of induced fit on ligand binding to the androgen receptor: A multidimensional QSAR study to predict endocrine-disrupting effects of environmental chemicals. *J. Med. Chem.*

Lill, M. A., Dobler, M. and Vedani, A. (submitted). Prediction of small-molecule binding to cytochrome P450 3A4: Flexible docking combined with multidimensional QSAR. *Chem. Med. Chem.*

Lill, M. A., Vedani, A. and Dobler, M. (2004). Raptor – combining dual-shell representation, induced-fit simulation and hydrophobicity scoring in receptor modeling: Application towards the simulation of structurally diverse ligand sets. *J. Med. Chem. 47*, 6174-6186.

Lukasink, K. and Pitkanen, A. (2000). GABA(A)-mediated toxicity of hippocampal neurons in vitro. *J. Neurochem. 74*, 2445-2454.

McLachlan, J. A. and Arnold, S. F. (1996). Environmental estrogens. *Am. Sci. 84*, 452-461.

Mohamadi, F., Richards, N. G. J., Guida, W. C. et al. (1990). MacroModel – An integrated software system for modeling organic and bioorganic molecules using molecular mechanics. *J. Comput. Chem. 11*, 440-467.

OECD (2003). http://webdomino1.oecd.org/comnet/env/models.nsf.

Okey, A. B., Riddick, D. S. and Harper, P. A. (1994). The Ah receptor: Mediator of the toxicity of 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. *Toxicol. Lett. 70*, 1-22.

Oliver, J. D. and Roberts, R. A. (2002). Receptor-mediated hepatocarcinogenesis: role of hepatocyte proliferation and apoptosis. *Pharmacol. Toxicol. 91*, 1-7.

Putzrath, R. M. (1997). Estimating relative potency for receptor-mediated toxicity: Re-evaluating the toxicity equivalence factor (TEF) model. *Reg. Tox. Pharmacol. 25*, 68-78.

Rappe, C. (1993). Environmental concentrations and ecotoxicological effects of PCDDs, PCD Fs and related compounds. *Organohalogen Compounds, Dioxin 12*, 163-170.

Rihova, B. (1998). Receptor-mediated targeted drug or toxin delivery. A*dv. Drug Deliv. Rev. 29*, 273-289.

Rymer, D. L. and Good, T. A. (2001). The role of protein activation in the toxicity of amyloidogenic Aβ (1- 40), Aβ (25-35), and bovine calcitonin. *J. Biol. Chem. 276*, 2523-2530.

Safe, S. and Krishnan, K. (1995). Cellular and molecular biology of Aryl hydrocarbon (Ah) receptor-mediated gene expression. In D. H. Degen, J. P Seiler and P. Bentley (eds.), *Archives of Toxicology* (Suppl. 17, 116-124), Berlin: Springer Verlag.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *CRC Crit. Rev. Toxicol. 21*, 51-88.

Shiau, A. K., Barstad, D., Loria, P. M. et al. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen endogenous estrogen 17β-estradiol (E2) and the synthetic nonsteroidal estrogen diethylstilbestrol (DES). *Cell 95*, 927-937.

Swanson, H. I. and Bradfield, C. A. (1993). The Ah-Receptor: Genetics, structure and function. *Pharmacogenetics 3*, 213-230.

Swiss National Science Foundation (2001). http://www.nrp50.ch.

Tanaka, E. (1998). Clinically important pharamcokinetic drug-drug interactions: Role of cytochrome P450 enzymes. *J. Clin. Pharm. Therap. 23*, 403-416.

Topliss, J. G. and Costello, R. J. (1972). Chance correlations in structure-activity studies using multiple regression analysis. *J. Med. Chem. 15*, 1066-1068.

Ungerer, S. H. and Hansch, C. (1973). On model building in structure-activity relationships. A reexamination of adrenergic blocking of β-halo-β-arlyalkylamines. *J. Med. Chem. 16*, 745-749.

US Government (1996a): Safe Drinking Water Act Amendment. Public Law 104-182 (Section 136), http://www.epa.gov.safewater/sdwa/index.html.

US Government (1996b): Food Quality Protection Act. Public Law 104-170 (Section 408), http://www.fda.gov/opacom/laws/foodqual/fqpatoc.htm.

Vedani, A., Dobler, M. and Lill, M. A. (in press). In silico prediction of harmful effects triggered by drugs and chemicals. *Toxicol. Appl. Pharmacol.*

Vedani, A., Dobler, M. and Lill, M. A. (2005b). Combining protein modeling and 6D-QSAR – Simulating the binding of structurally diverse ligands to the estrogen receptor. *J. Med. Chem. 48*, 3700-3703.

Vedani, A., Dollinger, H., Hasselbach, K.-M. et al. (2005a). Novel ligands for the chemokine receptor-3 (CCR3): A receptor modeling study based on 5D-QSAR. *J. Med. Chem. 48*, 1515-1527.

Vedani, A. and Dobler, M. (2003). Internet laboratory for predicting harmful effects triggered by drugs and chemicals – A progress report. *ALTEX 20*, 85-91.

Vedani, A. and Dobler, M. (2002). 5D-QSAR: The key for simulating induced fit? *J. Med. Chem. 45*, 2139-2149.

Vedani, A., Briem, H., Dobler, M. et al. (2000). Multiple conformation and protonation-state representation in 4D-QSAR: The neurokinin-1 receptor system. *J. Med. Chem. 43*, 4416-4427.

Vedani, A., McMasters, D. R. und Dobler, M. (1999). Genetische Algorithmen im 3D-QSAR: Verwendung multipler Wirkstofforientierungen zur verbesserten Voraussage der Toxizität. *ALTEX 16*, 9-14; *ALTEX 16*, 140-143.

Vedani, A. and Dobler, M. (2001). Internet laboratory for predicting harmful effects triggered by drugs and chemicals. *ALTEX 18*, 110-114.

Vedani, A., Dobler, M. and Zbinden, P. (1998). Quasi-atomistic receptor surface models: A bridge between 3-D QSAR and receptor modeling. J. *Am. Chem. Soc. 120*, 4471-4477.

Vedani, A. and Huhta, D. W. (1991). An algorithm for the systematic solvation of proteins based on the directionality of hydrogen bonds. *J. Am. Chem. Soc. 113*, 5860-5862.

Vedani, A. and Huhta, D. W. (1990). A new force field for modeling metalloproteins. *J. Am. Chem. Soc. 112*, 4759-4767.

White paper on the strategy for a future chemicals policy (2003). Cf. http://europa.eu.int/comm/ environment/chemicals/whitepaper.htm.

Whitlock jr., J. P. (1993). Mechanistic aspects of dioxin action. *Chem. Res. Toxicol. 6*, 754-763.

Zubay, G. L., Parson, W. W. and Vance, D. E. (1995). *Principles of Biochemistry*. Dubuque, USA: Wm. C. Brown Communications, Inc.

Zumstein, M. (2005). Establishing a quantitative structure-activity relationship for the α/β selectivity of the thyroid receptor. M.Sc. thesis, University of Basel.

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