From Crystal Structures and Their Analysis to the *in silico* Prediction of Toxic Phenomena[1])

by **Max Dobler**, **Markus A. Lill**, and **Angelo Vedani***

Biographics Laboratory 3R, Friedensgasse 35, CH-4056 Basel
(e-mail: admin@biograf.ch; internet: http://www.biograf.ch)

Dedicated to Professor *Jack D. Dunitz*, mentor and friend, in honor of his 80th birthday

While the development of potential drug molecules based on the known three-dimensional structure of the macromolecular target is doubtless one of the more-potent approaches to rational drug design, the estimation of associated changes in the free energy of ligand binding is all but trivial. Major obstacles include the treatment of long-range electrostatic effects and charge transfer, the calculation of solvation energies, the treatment of entropic effects, and the quantification of induced fit. In the last decade, a number of computational concepts have nonetheless matured into powerful tools for the development of drug-candidate molecules. These concepts have mainly focussed on the binding of the small molecule to a bioregulator. More recently, need has arisen to develop tools for a safe prediction of more-complex phenomena such as metabolism, toxicity, and bioavailability.

We describe the ongoing development of a virtual laboratory on the Internet to allow for a reliable *in silico* estimation of harmful effects triggered by drugs, chemicals, and their metabolites. For this, we used our recently developed underlying technology (5D-QSAR, based on five-dimensional quantitative structure-activity relationships) and compiled a pilot project, including the models of five receptor systems known to mediate adverse effects (the aryl hydrocarbon (*Ah*), 5 HT_{2A}, cannabinoid, $GABA_A$, and estrogen receptor, resp.) which were already validated against 280 compounds (drugs, chemicals, toxins). Within this setup, we could demonstrate that our virtual laboratory is able both to recognize toxic compounds substantially different from those used in the training set as well as to classify harmless compounds as being nontoxic. The results suggest that our approach can be used for the prediction of adverse effects of drug molecules and chemicals and, thus, bears a significant potential to recognize hazardous compounds early in the development process hence improving resource and waste management and reducing animal testing. It is the aim to provide free access to this technology – particularly to universities, hospitals, and regulatory bodies.

Introduction. – In the last two decades, a large number of computer-aided-design (CAD) concepts have been devised and have matured into powerful tools for the development of new drugs or chemicals. While these concepts have reduced the time scale on which new products emerged on the market, they have mainly focussed on a rational and cost-effective lead-candidate optimization. More recently, the need has arisen to further develop such tools to allow for a safe prediction of more complex phenomena such as the acute toxicity or the bioavailability. While most concepts use

[1]) The contributions of the honoree to this concept are manifold. A thorough analysis of small-molecule crystal sructures has lead to the development of a directional force field for optimizing ligand-receptor complexes. The understanding of phenomena such as induced fit or dynamic binding have been pioneered by Professor *Dunitz's* analyses of dynamic properties extracted from 'crystal statics'. His more-recent contributions to the understanding of solvation effects and entropic contributions to ligand binding are still teaching us important lessons on how to model small-molecule–protein complexes.

one-dimensional (1D; *e.g.*, pK_a, log *P*, molecular surface area) or 2D (chemical constitution and connectivity) information, and some are based on 3D data (structure of the drug or chemical target), they seldom consider a major player, *i.e.*, the biological receptor. As biochemical processes at the molecular level are influenced by the mutual adaptation of a drug or chemical and the biological receptor – a process referred to as *induced fit* –, a simulation omitting such a mechanism will hardly be successful in dealing with complex biochemical phenomena.

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.*, synthesized) before testing and are time consuming, and the results are often difficult to reproduce. 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 scanning of large batches of compounds (*e.g.*, parts of corporate or public databases) in a relatively short time. Toxicity-modeling algorithms are typically based on quantitative structure – activity relationships (QSAR), neuronal networks, or artificial intelligence.

QSAR method is an area of computational research, which builds atomistic or virtual models to predict quantities such as the binding affinity, the acute toxicity, or pharmacokinetic parameters of existing or hypothetical molecules. The idea behind QSAR is that structural features can be correlated with biological activity. Structure-activity relationships based on three-dimensional models (3-D QSAR) are very powerful tools in biomedical research as they allow for the simulation of directional forces – H-bonds, metal – ligand contacts, and electrostatic interactions – known to play a key role for both molecular recognition and binding. While at the true bioregulator (enzyme, receptor, DNA, ion channel), only one ligand molecule binds at a time, a QSAR study is typically based on a series of superimposed ligand molecules binding 'simultaneously' to the receptor surrogate. In 3D-QSAR – where each ligand molecule is represented by a single, three-dimensional entity, the identification of the bioactive conformation, orientation, and possibly the protonation state are crucial steps in the procedure. If the ligand alignment (the pharmacophore hypothesis) is based on incorrect assumptions, the resulting receptor surrogate is hardly of any use for predictive purposes. While this problem has long been recognized, only the more recently developed 4D-QSAR technologies would seem to provide decent solutions [1–4]. An unbiased simulation of induced-fit phenomena (5D-QSAR) is a further prerequisite for a realistic simulation of small-molecule (drug or toxin) interactions with a macromolecular receptor at the molecular level [5].

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* ('dioxin receptor'),

which is involved in a whole range of toxic effects [6]. 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 [7–13].

Methods. – The 'heart' of our virtual laboratory (*cf.* below) is a technology referred to as *quasi-atomistic receptor modeling*. It allows to three-dimensionally map an unknown or a hypothetical receptor onto a surface representing its active site, and to quantitatively calculate the affinity of small molecules binding to it. The approach combines receptor modeling based on a genetic algorithm and QSAR techniques. The details of the concept (Quasar software) have been published elsewhere [4][5][14–18]; in this account, we shall focus on its quantitative aspects.

The ligands from both training and test set are represented as an ensemble of conformations, orientations, and protonation schemes, a concept referred to as 4D-QSAR. Within this ensemble, the contribution of an individual entity to the total energy is determined by using a normalized *Boltzmann* factor (see Eqn. 1) where $w = (\Sigma\, E_{\mathrm{bdg,ind}}/E_{\mathrm{bdg,ind,lowest}})^{-1}$ is the normalizing factor.

$$E_{\mathrm{bgd,tot}} = \Sigma\, E_{\mathrm{bdg,ind}} \cdot \exp(-w \cdot E_{\mathrm{bdg,ind}}/E_{\mathrm{bdg,ind,lowest}}) \tag{1}$$

The simultaneous evaluation of the various induced-fit scenarios (5D-QSAR) reduces the bias with the selection of a single representation. Within the surrogate family, each model (typically 200–1000) may select one of the up to six different induced-fit scenarios. In Quasar, those presently include an energy-minimized active-site surface, a linear induced-fit as well as receptor adaptations based on the steric, electrostatic, H-bond, and lipophilicity potential (exerted by the ligands of the training set at the inner surface of the active site), respectively [4][5][15]. Simulations based on 5D-QSAR are superior to *n* 4D-QSAR calculations (by using a single induced-fit scenario) as 5D-QSAR allow for crossover of induced-fit scenarios during a simulation, *i.e.*, different induced-fit scenarios may be dominant at different times during the simulated evolution (*cf. Fig. 1*). To reduce computing time, the algorithm allows to define a parameter *f* that defines the probability of an induced-fit type being actually evaluated depending on its current frequency. When setting $f = 0.0$, all types are always evaluated. When setting $f = 1.0$, an 'extinct type' would never be re-evaluated; by setting $f = 0.9$ (default), such types are still tested at a rate of 10%.

In Quasar, the 'binding energy' of a ligand molecule towards the receptor surrogate is calculated as the mean value towards each member of the receptor family. The latter is determined by *Eqn. 2*.

$$E_{\mathrm{bdg}} \approx E_{\mathrm{force\text{-}field}} + E_{\mathrm{pol}} - T\Delta S_{\mathrm{bdg}} - E_{\mathrm{solv.,lig}} + \Delta E_{\mathrm{int.,lig}} + \mathrm{E}_{\mathrm{ind.fit}} \tag{2}$$

E_{bdg}: resulting binding energy, proportional to ΔG°; $E < 0.0$

$E_{\mathrm{force\text{-}field}}$: force-field energy (*cf. Fig. 2*) of the ligand-receptor interaction [19–21]; $E < 0.0$

E_{pol}: ligand → receptor and receptor → ligand polarization terms [22]; $E < 0.0$

$T\Delta S_{\mathrm{bdg}}$: change in ligand entropy upon receptor binding [23]; $E < 0.0$

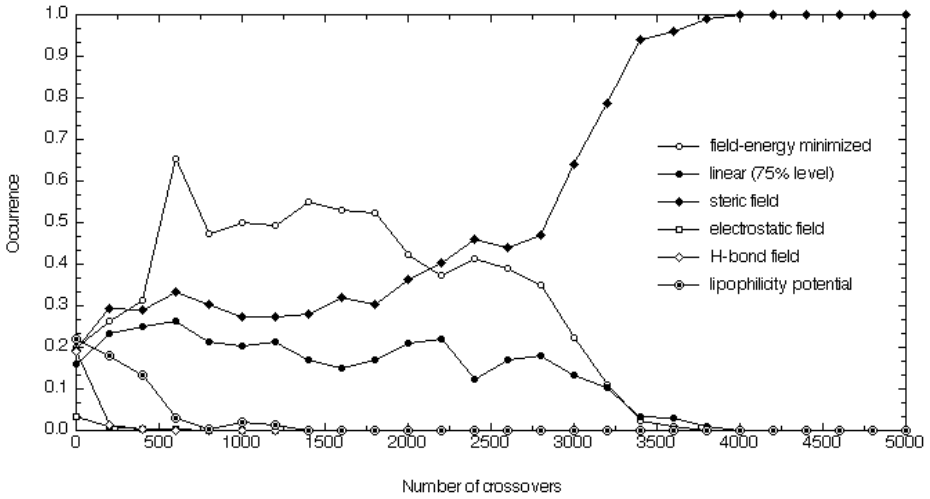

Fig. 1. *Crossover of induced-fit scenarios during the evolution of a receptor surrogate for the enzyme dopamine β-hydroxylase* [15]

$E_{\text{solv.,lig}}$: ligand-desolvation energy [24]; $E < 0.0$ (for all charged and most neutral species)

$\Delta E_{\text{int.,lig}}$: change in ligand internal energy upon receptor binding [25]; $E > 0.0$

$E_{\text{ind.fit}}$: energy uptake for adapting the receptor binding pocket to the ligand topology [4][5][14–16]; $E > 0.0$

The evaluation of the ligand-receptor (surrogate) interactions are based on a directional force field [4][5][14–16][21]. Of particular interest is the expression for H-bonds, interactions known to play a key role for both molecular recognition and selective binding. Its force-field expression (*Fig. 2*) includes a term for the H···Acc separation, a correction term for a nonlinear Don–H···Acc arrangement (Acc = acceptor, Don = donor) and a term for the deviation of the H-bond from the closest lone-pair direction (angle H···Acc–LP; LP = lone pair). The function has been calibrated for 14 different H-bond acceptor types [21] with data retrieved from the *Cambridge Structural Database* (*CSD*) [19][26–29][30]. The polarization terms may significantly contribute to the total energy for ligands including larger polarizable fragments such as aromatic rings, groups (nitro, carboxylate, phosphate), or elements (S, Cl, Br, I).

When calculating interactions between a molecule and a virtual receptor surrogate (a molecular surface populated with atomistic properties, *cf.* [4–5][14–16]), this function cannot be directly applied, as a H-bond property (HBP) has no bond partners and bears no lone-pairs. Therefore, we apply a reduced function to determine the non-electrostatic contribution to the H-bond energy involving a HBP: For the constellation Don–H···HBP, we correct for nonlinearity of the angle suspended at the H-atom (compulsory assuming a perfect directionality at the HBP site). For the arrangement Acc···HBP, we correct for the deviation of the virtual H-bond from the closest lone pair at the acceptor fragment (angle LP–Acc···HBP) and assume perfect linearity of the H-bond. However, this may lead to unrealistic geometries, *e.g.*, perfect linearity

$$E_{\text{total}} = \sum_{\text{bonds}} K_r (r - r_{\text{eq}})^2 + \sum_{\text{angles}} K_\theta (\theta - \theta_{\text{eq}})^2 +$$

$$\sum_{\text{dihedrals}} \frac{V_n}{2} [1 + \cos(n\phi - \gamma)] + \sum_{\text{nb pairs}} \frac{q_i \cdot q_j}{4\pi\varepsilon_o D(r) r_{ij}} + \sum_{\text{nb pairs}} \frac{A}{r_{ij}^{12}} - \frac{B}{r_{ij}^{6}}$$

$$+ \sum_{\text{H-bonds}} \left(\frac{C}{r_{\text{H-Acc}}^{12}} - \frac{D}{r_{\text{H-Acc}}^{10}} \right) \cdot \cos^2(\theta_{\text{Don-H-Acc}}) \cdot \cos^n(\omega_{\text{H-Lig-LP}})$$

$$+ \sum_{\substack{\text{metal-ligand} \\ \text{pairs}}} \frac{q_i^{\text{CT}} \cdot q_j^{\text{CT}}}{4\pi\varepsilon_o D(r) r_{ij}} + \sum_{\substack{\text{metal-ligand} \\ \text{pairs}}} \left(\frac{E}{r_{\text{M-Lig}}^{12}} - \frac{F}{r_{\text{M-Lig}}^{10}} \right)$$

$$+ (E_{\text{MC}} + E_{\text{LFS}}) \cdot \prod_{\substack{\text{indep.} \\ \text{angles}}} \cos^2(\psi_{\text{Lig-M-Lig'}} - \psi_{\text{eq}}) \cdot \frac{1}{n} \sum_{\substack{1^{\text{st}}\text{ shell} \\ \text{ligands}}} \cos^n(\omega_{\text{M-Lig-LP}})$$

Fig. 2. *The directional force-field used in Quasar* [1][10–14][20]

combined with poor directionality or *vice versa*, not in agreement with data retrieved from the *CSD* [30] and the Protein Data Bank [31]. More recently, we, therefore, use the mean value of the two quantities, leading to less-asymmetric arrangements [15].

Free energies of ligand binding, ΔG°, are then predicted by means of a linear regression between ΔG° and E_{bdg} by using the ligand molecules of the training set according to *Eqn. 3*. Slope *a* and intercept *b* are inherent to a given receptor model. As in Quasar, the receptor surrogate is represented by a family of models (typically 200–1000), this quantity is averaged over the *n* models comprising the receptor family. This yields a less-random value for ΔG° and allows each model within the surrogate family to represent aspects of other features at the true biological receptor – induced fit, dynamic behavior, polarizability [32].

$$\Delta G^\circ_{\text{calc}} = |\,a\,| \cdot E_{\text{bdg}} + b \tag{3}$$

The change in entropy upon ligand binding may provide an essential contribution to changes in free energy. By default, the algorithm assigns +0.7 kcal/mol to each freely rotatable bond [23], excluding terminal Me groups. Translational and rotational components to this quantity are not relevant in receptor-modeling studies as only relative ΔG° values are of importance in this context. When analyzing the binding of structurally much different molecules – particularly, such smaller in size – where alternate binding modes can be assumed, the latter terms can no longer be neglected. Molecules that can bind to various positions and orientations are less affected by a change in entropy than those fitting more snugly in the binding pocket. Entropy may become even more dominant when considering the solvent displaced during binding, a phenomenon referred to as *solvent stripping* (*cf.* also below). An interesting aspect of this effect has been discussed by *Dunitz* [33].

The contribution of ligand (de)solvation to the change in free energy of ligand binding can be substantial, particularly when charged species are involved and this energy ranges from 50–60 kcal/mol (compared to 0–10 kcal/mol for neutral mole-

cules). This has two consequences: first, a small error in the computed solvation energy could jeopardize an otherwise robust simulation. Second, in the context of the virtual laboratory, we have to deal with receptor surrogates constructed based on charged ligand species, but we might be forced to test against neutral compounds (and *vice versa*). In such a situation, the compound to be tested will yield too high or much too low affinities. We will, therefore, implement and test an alternative scheme not depending on the actual partial-charge model, *e.g.*, as proposed by *Viswanadhan et al.* [34].

If the molecular volume of a series of ligand molecules used in a multidimensional QSAR study varies considerably, however, the assumptions underlying *Eqn. 2* are no longer valid, as each molecule may displace (strip off) a different number of H_2O molecules bound to the receptor in the free state. Quasar allows us to correct for this effect by scaling the ligand-desolvation energy to the relative molecular volume (calculated based on a 0.5-Å-spaced 3-D grid) of a molecule and the given induced-fit scenario. The algorithm is based on the thought that more H_2O molecules are stripped off when a large receptor-to-ligand adaptation takes place, but only few H_2O molecules are displaced when a negligible or small induced fit occurs, *i.e.*, the binding pocket remains unaltered, allowing for more solvent molecules to remain receptor-bound.

The estimation of internal strain – a small molecule may not bind to a bioregulator in its lowest-energy conformation – is less problematic as we use the same force field for both conformational search and receptor modeling. When data is imported from simulations with different force fields, however, this quantity may have to be recalculated or appropriately scaled. When using a multiple ligand representation (4D-QSAR) for highly flexible molecules, the question arises how energetically 'unfavorable' conformers should be included in the simulation. For practical purposes (*cpu* time), we typically use 4–32 representations per molecule. The 18 systems simulated so far suggest that any conformation more than 10–12 kcal/mol above the global minimum (in aqueous solvent) is hardly accepted as the bioactive conformer; the energetic cost for internal strain would simply be too high.

To reduce the bias associated with the selection of an induced-fit model, Quasar allows for multiple representation of induced-fit scenarios. Presently, up to six different mechanisms (*cf.* above) may be simultaneously evaluated (see *Fig. 3*). In absence of the three-dimensional structure of the true biological receptor, this quantity is difficult to estimate. In our concept, the energy associated with the receptor-to-ligand adaptation is calculated from the r.m.s. shift of the mean (accommodating all ligands of the training set) to an inner surface (snugly fitting to each individual ligand molecule). This shift varies substantially for the six different protocols [5][15] but is typically limited to a r.m.s. shift of 2–3 Å. Larger induced-fit scenarios (as experimentally observed) cannot presently be simulated with our technology.

The Quasar concept has been validated for various receptor systems, representing both pharmacological and toxicological targets [4][5][14–18]. A selection of the results is given in the *Table*.

Results and Discussion. – More recently, we have started to model receptor-mediated toxic phenomena, including the aryl hydrocarbon (*Ah*) receptor [5][17] and estrogen receptor [36], using large data sets of 121 and 106 compounds, respectively.

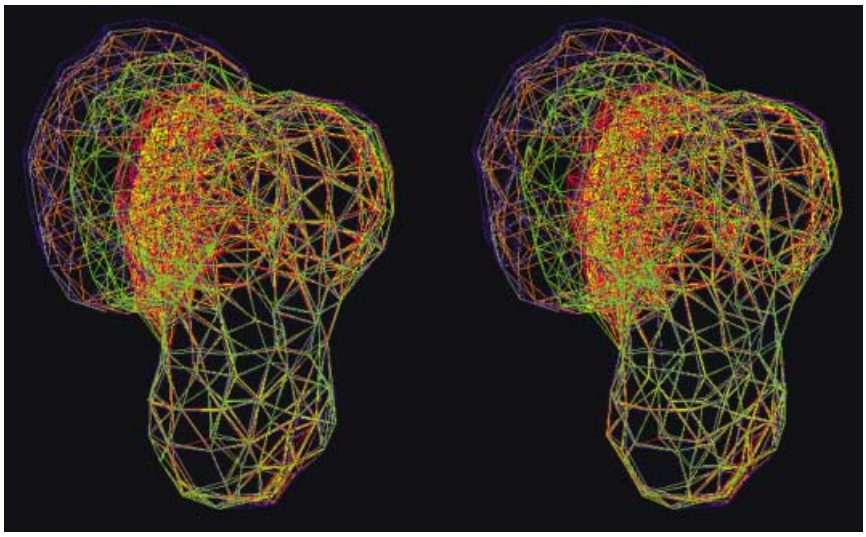

Fig. 3. Fig. 3. *Stereo view of six superimposed induced-fit scenarios for the NK-1 receptor.* Color coding: grey = minimized; yellow = linear, red = steric; orange = electrostatic; blue = H-bond; green = lipophilicity.

Table 1. *Summary of Results Obtained with the 5D-QSAR Software* Quasar

Receptor system	Number of training and test substances	Cross-validated (and predicitive) r^2	R.m.s. deviation of the test set [factor in K]	Max. deviation of the test set [factor in K]
$5\,HT_{2A}$	23 + 7	0.950 (0.860)	2.0	3.0
Aryl hydrocarbon (*Ah*)	91 + 30	0.861 (0.697)	3.2	10.2
Chemokin	81 + 32	0.790 (0.830)	1.6	2.9
Estrogen	84 + 22	0.891 (0.782)	5.2	13.6
Neurokinin-1	50 + 15	0.870 (0.837)	2.3	5.7
Steroid	21 + 10	0.947 (0.912)	1.8	2.8

Fig. 4 shows the results for the simulation of the *Ah* receptor. This model has also been used to predict the toxicity of four new compounds (blue dots) – for those, the mean deviation of the binding affinity from the experiment was calculated to a factor of only 2.2 in K [5].

As the manifestation of a toxic phenomenon is a complex result of a cascade of biochemical events and transformations (*Fig. 5*), it is of utmost importance to demonstrate that a correlation exists between receptor binding and the manifestation of the toxic phenomenon. Unfortunately, this correlation cannot be established for most receptors mediating adverse effects for the simple reason that no quantitative binding data are available. On the other hand, receptor-modeling algorithms tend to fail, for the given data set, when no common underlying mechanism exists. To demonstrate this most-desired property of Quasar, we conducted several so-called *poisoning experiments*, where a different class of molecules is deliberately added to an otherwise consistent set of data. *Fig. 6* shows the result of such a simulation for the *Ah*

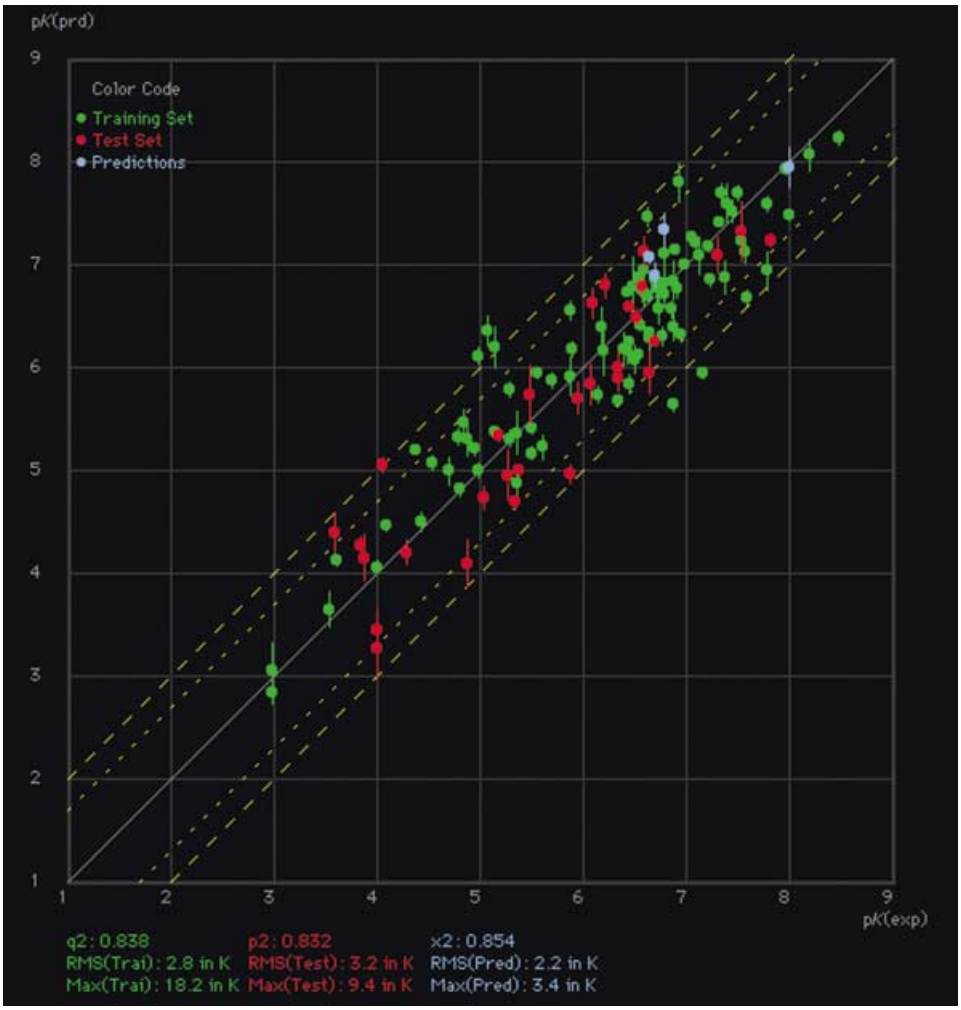

Fig. 4. *Experimental and calculated binding affinities for the aryl hydrocarbon* (Ah) *receptor*. Color coding: green = training set (91), red = test set (30), blue = predictions (4).

receptor system where 16 sulfonamide drugs (all nontoxic) have been added to the 121 toxins (dibenzodioxins, dibenzofurans, biphenyls, and polyaromatic hydrocarbons) comprising the *Ah* data set. While the correct simulation reached a cross-validated r^2 of 0.861, the 'poisoned' simulation converged at a very low value of 0.339, hence demonstrating that no solution is found if no common underlying mechanism exists. It is noteworthy that the 'poisoned data' (random affinities were assigned for those) in the training set represents only 10% of the whole set. That the affinity of these compounds cannot be reproduced is obvious; that the algorithm does not find a solution for the $91 + 30$ true toxins demonstrates that the genetic algorithm is sensitive to the consistency of the ligand data.

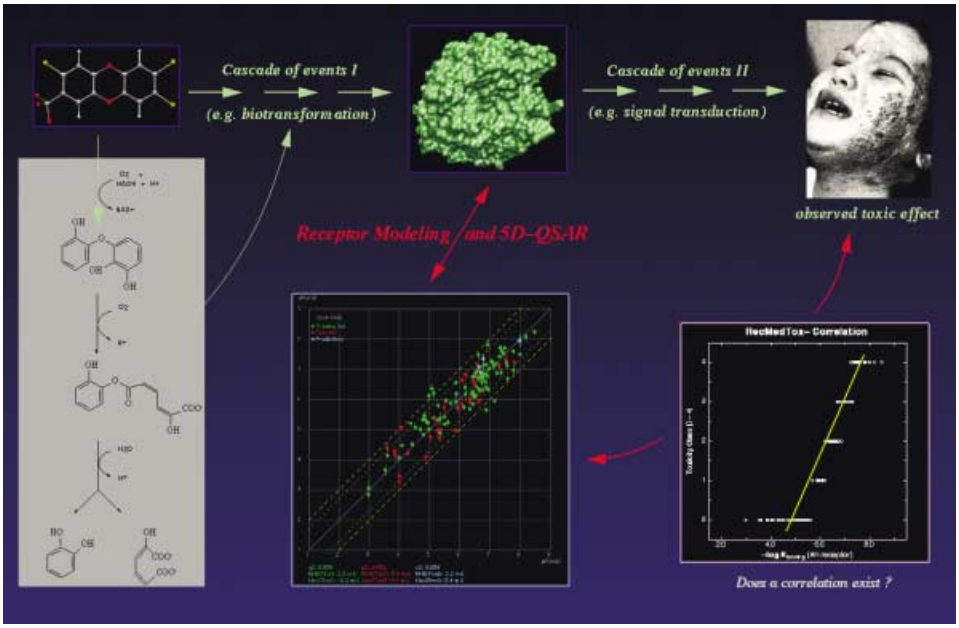

Fig. 5. *Receptor-mediated toxicity: receptor binding and manifestation of adverse effects*

These results stimulated us to establish a virtual laboratory to allow for an *in silico* estimation of harmful effects triggered by drugs, chemicals, and their metabolites, and to make it accessible through the Internet. The philosophy behind our concept is that any existing or hypothetical compound can quickly be tested against a large batch of 3D receptor models (deposited in the database). Should a high affinity be predicted towards any receptor model, the substance is likely to cause adverse effects and should therefore be withdrawn from the evaluation pipeline (drug candidates) or handled with special care (existing chemicals) but definitely not conveyed on to *in vivo* toxicity tests [17][18].

Presently, our database includes validated models for five biological targets mediating adverse effects: the aryl hydrocarbon (*Ah*), the $5\,HT_{2A}$, the cannabinoid, the $GABA_A$, and the estrogen receptor, respectively. The flow chart of the proposed virtual laboratory is shown in *Fig. 7*. Using these data (5 receptor models, 280 compounds) within a pilot setup, we have addressed the following questions:

1) Are nontoxic substances safely identified? To demonstrate that no false-positives are likely to be obtained, we used harmless drug molecules similar in their topology (three-dimensional shape) with toxins known to bind to the *Ah* receptor. The selected 16 drug molecules fit snugly into the binding pocket of the receptor surrogate but did not show any significant binding affinity ($K < 0.1$ mM) – as a matter of fact, only *Furosemide*™ (= 5-(aminosulfonyl)-4-chloro-2-[(furan-2-ylmethyl)amino]benzoic acid; $K = 10$ mM) 'binds' at all, while all other 15 compounds have a positive free energy of ligand binding ($\Delta G^\circ > 0.0$), *i.e.*, they could not trigger any effects *via* the *Ah* receptor even if they were to be massively overdosed (*Fig. 8*).

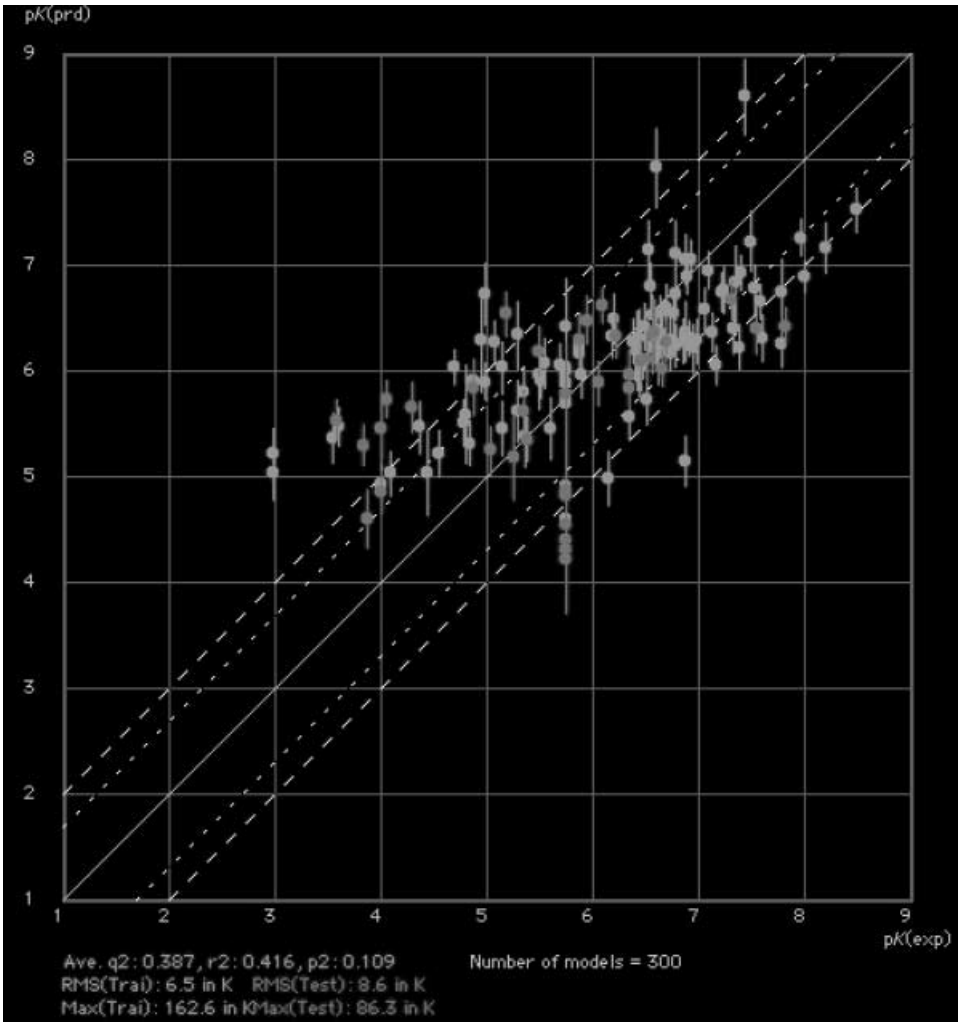

Fig. 6. *Comparison of experimental and predicted binding affinities for the* Ah *receptor system*. The data set has been poisoned 'poisoned' with 10% of nontoxic compounds.

2) Can the algorithm distinguish between toxic and harmless compounds within a foreign data set, *i.e.*, substances that are structurally different from those used to train the system? Again, we have selected the *Ah* receptor but used compounds from different chemical classes: 1,2,3,4-tetrahydroharman-3-carboxylic acid (=1,2,3,4-tetrahydro-1-methyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid = HTCA), harmol (=1-methyl-9*H*-pyrido[3,4-*b*]indol-7-ol), harmalol (=4,9-dihydro-1-methyl-3*H*-pyrido[3,4-*b*]indol), harmine (=7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole), harman (=1-methyl-9*H*-pyrido[3,4-*b*]indole), norharman (=9*H*-pyrido[3,4-*b*]indole), guanabenz (=2-[(2,6-dichlorophenyl)methylene]hydrazinecarboximidamide), and idazoxan

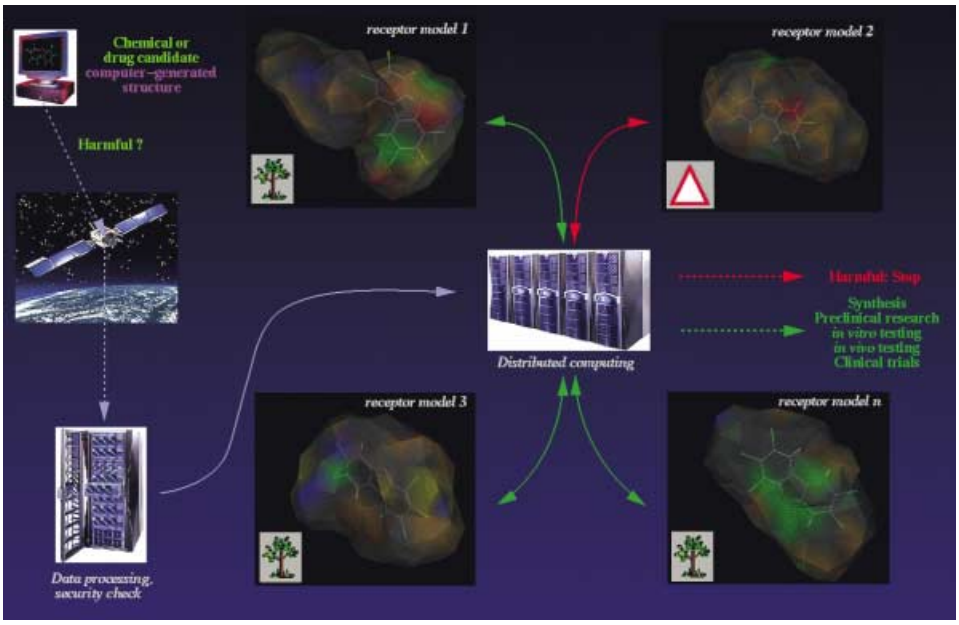

Fig. 7. *Flow-chart of the virtual laboratory for the* in silico *screening for adverse effects*

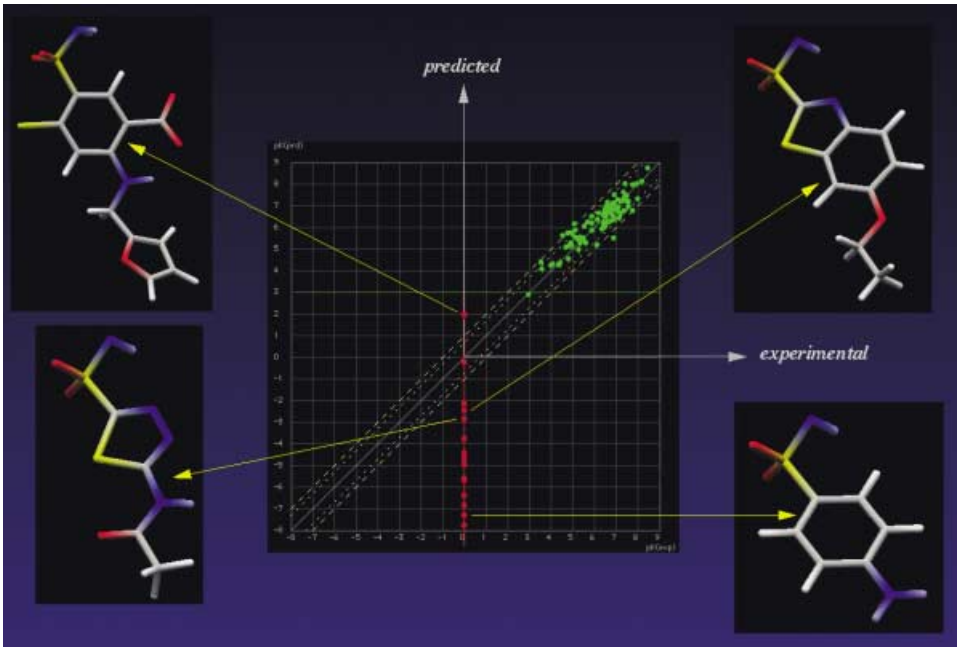

Fig. 8. *Prediction of nontoxic drugs hypothetically binding to the* Ah *receptor* [18]. For the structure of *Furosemide*, see top left.

(=2-(2,3-dihydro-1,4-benzodioxin-2-yl)-4,5-dihydro-1*H*-imidazole) for which semi-quantitative binding data are available [37]. Of these, only HTCA shows a substantial toxic effect mediated by the *Ah* receptor system while all other compounds are nontoxic at low-level doses – some of these compounds bind to monoamine oxidase and display hallucinogenic activity. The result of our simulation is shown in *Fig. 9*. The binding affinity of HTCA is calculated to 112 nM (exper. 60 nM), suggesting a rather high toxicity at low dosage (for comparison: TCDD (=2,3,7,8-tetrachlorodibenzo-[*b*,*e*][1,4]dioxin) binds with an affinity of 10 nM to the *Ah* receptor). The calculated affinity for all other compounds lies in the range of 0.1 – 10 mM, a level at which no adverse effects are expected to be mediated by the *Ah* receptor, which is in agreement with the experimental binding data [37].

Those nine compounds were also tested against the other four receptor systems presently stored in our database: the $5HT_{2A}$, the cannabinoid, the $GABA_A$, and the estrogen receptor, respectively. From their topology, most of them can bind to one or more of these surrogates. However, in the virtual experiment, no binding affinity ($K <$ 0.1 mM) was observed, except for HTCA which has a calculated affinity of 28 μM towards the estrogen receptor and 5.7 nM (!) against the cannabinoid receptor as well as guanabenz, which binds with an affinity of 1.8 μM to the $GABA_A$ receptor.

Within our pilot system, we could demonstrate that this test setup is able to predict both the known toxicity of compounds different from those in the training set and the benign character of currently available drugs. This suggests that our approach can be used for the prediction of adverse effects of molecules prior to their synthesis. The

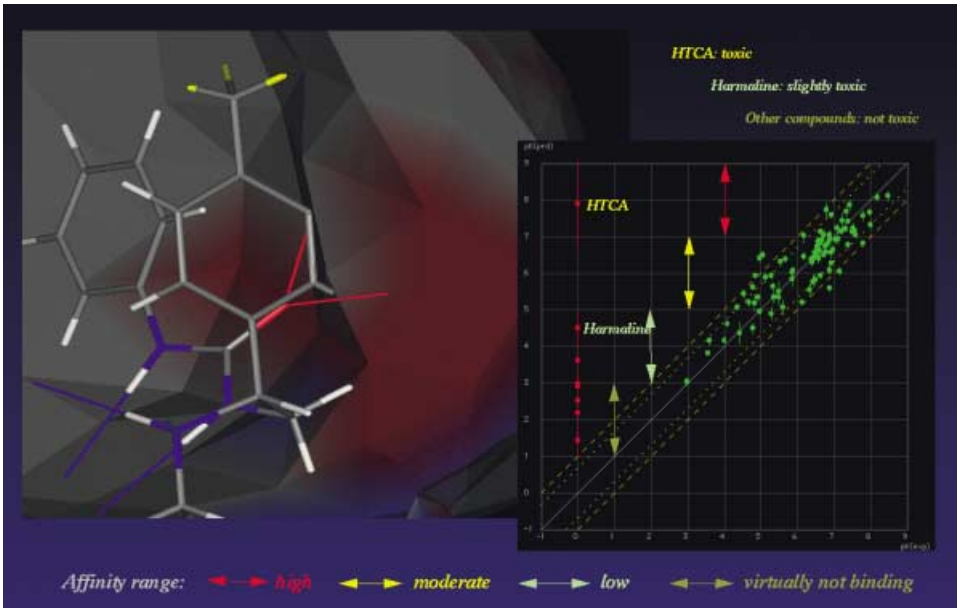

Fig. 9. *Prediction of the binding affinity of nine monoamine oxidase inhibitors* (MAOIs) *also known to bind to the* Ah *receptor* [18][37]

power of the concept lies with a low rate of false-positive predictions, *i.e.*, a compound predicted to trigger adverse effects is most likely to be harmful in reality as well. On the other hand, it is obvious that, no matter how many receptor models are stored in the database, such a virtual laboratory will never be able to identify all toxic substances – this is by no means our objective – as there are many other, more-complex pathways leading to the manifestation of toxic phenomena. As a large body of receptors mediating toxic phenomena exists, the number of false-negative hits can be lowered by increasing the number of validated receptor models stored in the database.

The basic technologies – software Quasar [4][5][15] and Toxar [38], respectively – are available, and the internet protocol for an external access is presently being developed. We therefore plan to make the virtual laboratory available to selected institutions as early as 2005 and to the scientific community as soon as the security measures (*e.g.*, against misusing the virtual laboratory for non-scientific purposes) are considered to be sufficient. We think that our concept has the potential for a significant contribution to laboratory-animal welfare (*in vivo* toxicity tests).

Outlook. – As a next step, we plan to generate and validate receptor surrogates for the following systems: NMDA (*N*-methyl-D-aspartate) receptor involved in *Alzheimer* and *Parkinson* disease pathways; AMPA (2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid) receptor mediating exitotoxicity; histamine H1 (bronchiolar or gastrointestinal smooth-muscle constriction, bronchial hyperreactivity) or histamine H2 receptor (CNS neurotransmission; delirium, confusion, agitation, and seizures); mACh (muscarinic acetylcholine) receptor (urinary retention, blurred vision; *Parkinson*, *Alzheimer*); androgen receptor (side effects during sexual differentiation). *Scramble tests* (*cf.* [4–5][15]) and cross-validation with all data sets and all surrogates in the database will further demonstrate – or disqualify – the validity of each individual model. For the cross-validation, we are using our in-house database including over 400 substances for which not only their 3D structure is available but also their 4D conformational ensemble compiled by means of conformational-search protocols.

Adverse effects may be triggered not only by the interaction of a drug or chemical with a mediating receptor system but also by inhibition of processes associated with both phase-I and phase-II reactions during biotransformation, *e.g.*, the cytochrome P450 system. During such reactions, chemicals may also be metabolized and sometimes lead to toxic (*e.g.*, carcinogenic) products. Therefore, we plan to add a series of surrogates generated based on active inhibitors of these isoenzymes. As several homology models are available, we will use receptor-mediated alignment protocols [39] for model development. The Fe-containing heme portion will be modeled by means of a directional metalloprotein force field [21].

The proposed Internet laboratory could contribute to a significant reduction in animal testing. First, it allows for an early – 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. This would seem to be a realistic scenario as the most important feature of our virtual experiments is not having produced any false-positive results so far. Second, a widely used database of this kind would reduce the number of otherwise doubly-conducted (toxicity) tests at research laboratories focussed on identical or closely related biomedical targets. The

main advantage of the proposed virtual laboratory – for example, when compared with *in vitro* assays – is that it can be applied to hypothetical substances.

Another field of application includes the toxicity testing of chemicals – for example, the 30000 compounds that will 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' [40] – and causing an estimated toll of 10 million laboratory animals. Here, our system could prove to be a useful *in silico* screening tool as any compound can be tested with only moderate 'human' effort. The importance of QSAR has more recently been acknowledged by the OECD [41], and the *Danish Environmental Protection Agency* has taken the lead in use of structure-based methods to prioritize hazardous chemicals [42].

Information on this project is continuously updated under http://www.biograf.ch/projects.html; information about the Quasar software can be found at http://www.biograf.ch/software.html. Corresponding articles published in *ALTEX* may be downloaded from http://www.biograf.ch/publications.html (all in pdf format).

We are most grateful to Professor *Jack D. Dunitz* for 30 years of asking the right questions and, even more importantly, suggesting well-founded answers. This research was made possible through a grant by the *Foundation Research 3R*, Münsingen, Switzerland (http://www.forschung3r.ch) and continued support through the *Foundation for Animal-Free Research*, Zürich, Switzerland (http://www.ffvff.ch) and the *Margaret and Francis-Fleitmann Foundation*, Lucerne, Switzerland.

REFERENCES

[1] A. J. Hopfinger, S. Wang, J. S. Tokarski, B. Q. Jin, M. Albuquerque, P. J. Madhav, C. Duraiswami, *J. Am. Chem. Soc.* **1997**, *119*, 10509.
[2] S. S. So, M. Karplus, *J. Med. Chem.* **1997**, *40*, 4347.
[3] P. W. Sprague, R. Hoffmann, in 'Computer-Assisted Lead Finding and Optimization', Eds. H. van de Waterbeemd, B. Testa, and G. Folkers, VCH, Weinheim, Germany, 1997, p. 223–240.
[4] A. Vedani, H. Briem, M. Dobler, K. Dollinger, D. R. McMasters, *J. Med. Chem.* **2000**, *43*, 4416.
[5] A. Vedani, M. Dobler, *J. Med. Chem.* **2002**, *45*, 2139.
[6] J. A. Gustaffson, *Toxicol. Lett.* **1995**, *135*, 465.
[7] D. L. Rymer, T. A. Good, *J. Biol. Chem.* **2001**, *276*, 2523.
[8] E. V. Hestermann, J. J. Stegemann, M. E. Hahn, *Toxicol. Appl. Pharmacol.* **2000**, *168*, 160.
[9] B. Fischer, *Andrologia* **2000**, *32*, 279.
[10] K. Lukasink, A. Pitkanen, *J. Neurochem.* **2000**, *74*, 2445.
[11] B. Rihova, *Adv. Drug Deliv. Rev.* **1998**, *29*, 273.
[12] A. J. Hampson, M. Grimaldi, *J. Neurosci.* **2002**, *22*, 257.
[13] J. D. Oliver, R. A. Roberts, *Pharmacol. Toxicol.* **2002**, *91*, 1.
[14] A. Vedani, M. Dobler, 'Progress in Drug Research', Ed. E. Jucker, Birkhäuser, Basel – Boston – Berlin, 2000, p. 105.
[15] A. Vedani, M. Dobler, *Quant. Struct.-Act. Relat.* **2002**, *21*, 382.
[16] A. Vedani, D. R. McMasters, M. Dobler, *Quant. Struct.-Act. Relat.* **2000**, *19*, 149.
[17] A. Vedani, D. R. McMasters, M. Dobler, *ALTEX* **1999**, *16*, 140.
[18] A. Vedani, M. Dobler, *ALTEX* **2001**, *18*, 110.
[19] A. Vedani, J. D. Dunitz, *J. Am. Chem. Soc.* **1985**, *107*, 7653.
[20] A. Vedani, M. Dobler, J. D. Dunitz, *J. Comput. Chem.* **1986**, *7*, 701.
[21] A. Vedani, D. W. Huhta, *J. Am. Chem. Soc.* **1990**, *112*, 4759.
[22] A. E. Howard, U. Chandra Singh, M. Billeter, P. A. Kollman, *J. Am. Chem. Soc.* **1988**, *110*, 6984.
[23] M. S. Searle, D. H. Williams, *J. Am. Chem. Soc.* **1992**, *114*, 10690.
[24] W. C. Still, A. Tempczyk, R. C. Hawley, T. Hendrickson, *J. Am. Chem. Soc.* **1990**, *112*, 6127.
[25] A. Vedani, M. Dobler, P. Zbinden, *J. Am. Chem. Soc.* **1998**, *120*, 4471.
[26] P. Murray-Rust, J. P. Glusker, *J. Am. Chem. Soc.* **1984**, *106*, 1018.

[27] R. Taylor, O. Kennard, *Acc. Chem. Res.* **1984**, *17*, 320.
[28] R. S. Alexander, Z. F. Kanyo, L. E. Chirlian, D. W. Christanson, *J. Am. Chem. Soc.* **1990**, *112*, 933.
[29] E. N. Baker, R. E. Hubbard, *Prog. Biophys. Molec. Biol.* **1984**, *44*, 97.
[30] http://www.ccdc.cam.ac.uk
[31] http://www.rcsb.org/pdb
[32] M. Hahn, *J. Med. Chem.* **1995**, *38*, 2080.
[33] J. D. Dunitz, *Science* (*Washington, D.C.*) **1994**, *264*, 670.
[34] V. N. Viswanadhan, A. K. Ghose, U. C. Singh, J. J. Wendoloski, *J. Chem. Inf. Comput. Sci.* **1999**, *39*, 405.
[35] D. Streich, M. Neuburger-Zehnder, A. Vedani, *Quant. Struct.-Act. Relat.* **2000**, *19*, 565.
[36] F. Emery, M. Sc. Thesis, University of Basel, 2002.
[37] S. D. Seidel, V. Li, G. M. Winter, W. J. Rogers, E. I. Martinez, M. W. Denison, *Toxicol. Sci.* **2000**, *55*, 107.
[38] M. A. Lill, M. Dobler, A. Vedani, *J. Med. Chem.*, to be submitted.
[39] P. Zbinden, M. Dobler, G. Folkers, A. Vedani, *Quant. Struct.-Act. Relat.* **1998**, *17*, 122.
[40] http://europa.eu.int/comm/environment/chemicals/whitepaper.htm
[41] http://webdomino1.oecd.org/comnet/env/models.nsf
[42] M. Cronin, *Chem. Ind. (London)* **2003**, *4*, 13.

Received March 17, 2003