Internet Laboratory for Predicting Harmful Effects Triggered by Drugs and Chemicals – A Progress Report

Angelo Vedani, Max Dobler and Markus A. Lill
Biographics Laboratory 3R, CH-Basel

Summary
The main objective of our institution is to establish a virtual laboratory on the Internet to allow for a reliable in silico estimation of harmful effects triggered by drugs, chemicals and their metabolites. In the past two years, we have compiled a pilot system including the 3D models of five receptors known to mediate adverse effects (the Ah, $5HT_{2A}$, cannabinoid, $GABA_A$, and estrogen receptor, respectively) and tested them against 280 compounds (drugs, chemicals, toxins). Within this set-up we could demonstrate that our concept is able to both recognise toxic compounds substantially different from those used in the training set as well as to classify harmless compounds clearly as being non-toxic at low-level doses. This suggests that our approach can be used for the prediction of adverse effects of drug molecules and chemicals. It is the aim to provide free access to this 3D data base, particularly to universities, hospitals and regulatory bodies as it bears a significant potential to recognise hazardous compounds early in the development process and withdraw them from the evaluation pipeline. Hence, for substances recognised as hazardous in silico, subsequent toxicity tests involving animal models become obsolete.

Zusammenfassung: Internet-Labor zur Voraussage schädlicher Wirkungen von Arzneistoffen und Chemikalien – Ein Zwischenbericht
Ziel unserer Arbeit ist der Aufbau eines virtuellen Labors im Internet zur zuverlässigen Voraussage von unerwünschten Nebenwirkungen von Arzneistoffen und Chemikalien sowie deren Metaboliten in silico. In den vergangenen zwei Jahren haben wir ein Pilotsystem aufgebaut, das aus fünf Rezeptoren besteht, welche an der Vermittlung schädlicher Effekte beteiligt sind (der Ah, $5HT_{2A}$, Cannabinoid, $GABA_A$, und Estrogen Rezeptor) und haben diese an insgesamt 280 Substanzen (Arzneistoffe, Chemikalien, Toxine) geprüft. Innerhalb dieser Anordnung konnten wir zeigen, dass unser Konzept in der Lage ist, sowohl toxische Substanzen aus anderen Substanzklassen zu erkennen sowie die Unbedenklichkeit von in niedrigen Dosen nicht-giftigen Arzneistoffen vorauszusagen. Dies lässt den Schluss zu, dass unser Ansatz geeignet ist, unerwünschte Nebenwirkungen von Arzneistoffen und Chemikalien vorauszusagen. Freier Zugang zu dieser 3D-Datenbank soll insbesondere Universitäten, Kliniken und Registrierungsbehörden gewährleistet werden, weil sie unserer Ansicht nach ein beträchtliches Potenzial besitzt, bedenkliche Substanzen in einem frühen Entwicklungsstadium zu erkennen und weitere Prüfungsschritte zu unterlassen. Für Substanzen, die in silico als gefährlich erkannt werden, erübrigen sich nachfolgende Toxizitätstests in vivo.

Keywords: Internet laboratory, virtual experiments, prediction of harmful effects, drugs and chemicals, multi-dimensional QSAR, reduction of animal testing

1 Introduction

In the last two decades, a large number of computer-aided design (CAD) concepts have been devised and 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 development process. More recently, need has aroused to further develop such tools to allow for a safe prediction of more complex phenomena such as the acute toxicity and the oral bioavailability. While most concepts use 1D and 2D information, some are based on the three-dimensional (3D) structure of the drug or chemical target, they do seldom consider a major player: the biological receptor. As 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

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be successful in coping with complex biological 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. synthesised) before testing, 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 to scan large batches of compounds (e.g. parts of corporate or public databases) in a relatively short time. Toxicity-modeling algorithms are mainly based on quantitative structure-activity relationships (QSAR), neuronal networks, or artificial intelligence concepts.

Quantitative structure-activity relationships (QSAR) 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 (3D–QSAR) are powerful tools in biomedical research as they allow for the simulation of directional forces – hydrogen bonds, metal-ligand contacts and the interaction between electric dipoles – known to play a key role for both molecular recognition and binding. While at the true bioregulator (enzyme, receptor, DNA, ion channels) only one ligand molecule binds at the time, a QSAR study is typically based on a series of ligand molecules binding "simultaneously" to the receptor surrogate. In 3D-QSAR – where each substance/compound is represented by a single, three-dimensional entity – the identification of the bioactive conformation, orientation and, possibly, the protonation state is a crucial step in the procedure. If the ligand alignment (i.e. 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 recognised, only the more recently developed 4D-QSAR technologies would seem to provide decent solutions. An unbiased simulation of induced-fit phenomena (5D-QSAR) would seem to be a further prerequisite for a realistic simulation of small-molecule (drug or toxin) interactions with a macromolecular receptor at the molecular level (Vedani and Dobler, 2002).

In pharmaceutical research biologically active compounds (drugs) are specifically designed to selectively bind to specific receptors. On the other hand, toxic agents, particularly those that exert their actions with a great deal of specificity, sometimes act also via specific 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 can act as a model for other receptors but 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 (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; Hestermann et al., 2000; Lukasink and Pitkanen, 2000; Rymer and Good, 2001; Hampson and Grimaldi, 2002; Oliver and Roberts, 2002).

2 Pilot simulation – Results and Discussion

The hub of our virtual laboratory is a technology referred to as *Quasi-atomistic receptor modeling* (software *Quasar*). It allows to map an unknown or a hypothetical receptor in three dimensions and to quantitatively calculate the affinity of small molecules binding to it (Vedani et al., 2000; Vedani and Dobler, 2002). The approach combines receptor modeling and QSAR techniques based on a genetic algorithm. The higher dimensionality (compared to other approaches in the field) allows for a much less biased identification of the bioactive conformation (4D: Vedani et al., 2000) and the induced-fit scenario (5D: Vedani and Dobler, 2002). The *Quasar* concept has been validated for various receptor systems, representing both pharmacological and toxicological targets. A selection of the results is given in Table 1.

Tab. 1: Summary of results obtained with the 5D–QSAR concept Quasar

Receptor system	Number of training and test substances	Cross–validated and predictive r^2	rms deviation of the test set [factor in K]	max. deviation of the test set [factor in K]
5HT2A	23 + 7	0.950 / 0.860	2.0	3.0
Aryl hydrocarbon	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

More recently, we have started to model receptor-mediated toxic phenomena, including the Aryl hydrocarbon (Vedani et al., 1999) and Estrogen receptor (Emery, 2002; Vedani and Dobler, 2003) using large data sets of 121 and 106 compounds, respectively. Figure 1 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 from the experiment was calculated to only a factor 2.2 in K.

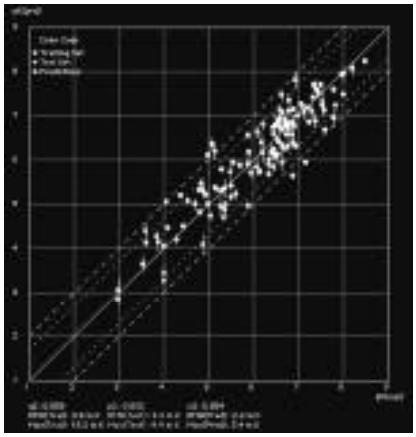

Fig. 1: Experimental and calculated binding affinities for the Aryl hydrocarbon (Ah) receptor
Alle Abbildungen dieses Artikels sind in Farbe auf www.altex.ch zu finden.

As the manifestation of a toxic phenomenon is complex result of a cascade of biochemical events and transformations (Fig. 2), it is of utmost importance to demonstrate that a correlation exists between binding to a specific receptor and the manifestation of the toxic phenomenon. Unfortunately, this correlation cannot be proved for most receptors mediating adverse effects for the simple reason that no quantitative binding data are available. On the other hand, genetic algorithms tend to fail (for the given data set) if no common underlying mechanism exists. To demonstrate this most desired property of genetic algorithms, we have conducted several so-called "poisoning experiments" where a different class of molecules is deliberately added to an otherwise consistent set of data. Figure 3 shows the result of such a simulation for the *Ah* receptor system where 16 sulfonamide drugs (all harmless) have been added to the 121 toxins (dibenzodioxins, dibenzofurans, biphenyls, 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

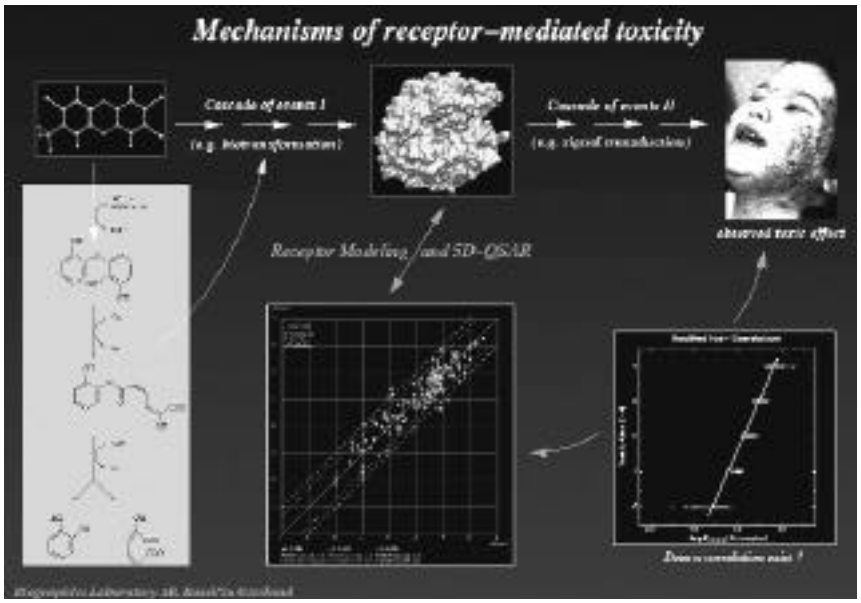

Fig. 2: Receptor-mediated toxicity: receptor binding and manifestation of adverse effects

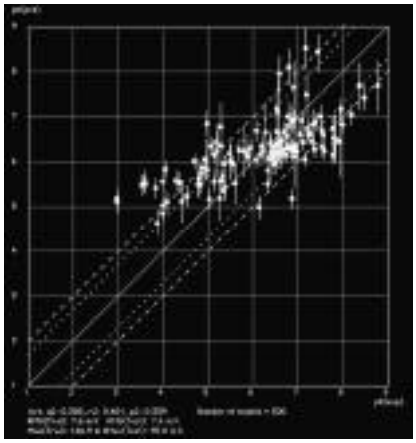

Fig. 3: Comparison of experimental and predicted binding affinities for the *Ah* receptor system
The data set has been "poisoned" with 10% "harmless" compounds

the "poisoned data" (random affinities were assigned for those compounds) 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.

We are presently establishing 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.

Presently, our database includes validated models for five biological targets mediating adverse effects: the Aryl hydrocarbon, the $5HT_{2A}$, the cannabinoid, the $GABA_A$, and the estrogen receptor, respectively. The flow chart of the proposed virtual laboratory is shown in Figure 4. Using these data (5 receptor models, 280 compounds) within a pilot

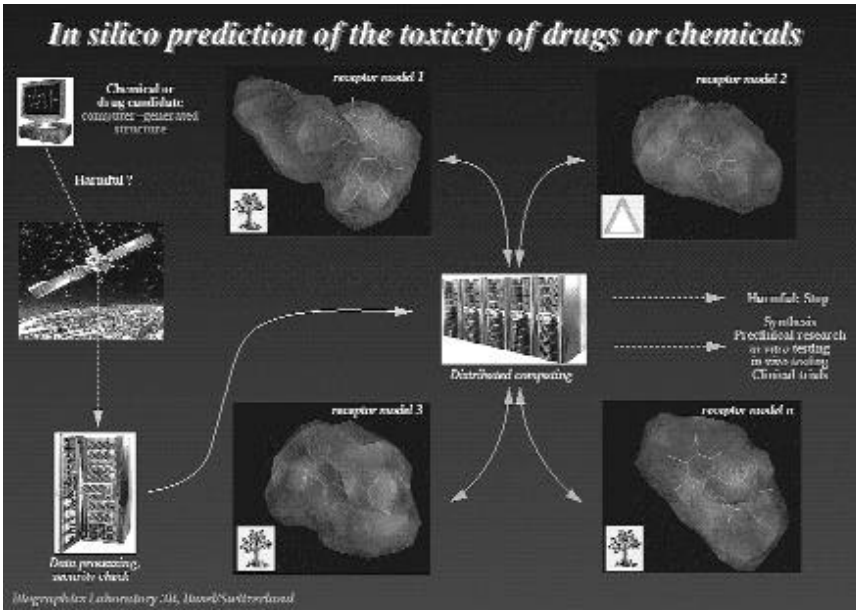

Fig. 4: Flow-chart for the virtual laboratory for the *in silico* screening for adverse effects

set-up, we have addressed the following questions:

1. Are harmless (at low-level doses) substances safely identified? To demonstrate that no false-positive predictions 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 (Ki < 0.1 mM) – as a matter of fact, only Furosemide™ (Ki = 10 mM) "binds" at all, while all other 15 compounds have a positive free energy of ligand binding (ΔG°), i.e. they could not trigger any effects via the *Ah* receptor even if they were to be massively overdosed (Fig. 5).

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: Harman-1,2,3,4-tetrahydro-3-carboxylic acid (*HTCA*), *Harmol*, *Harmalol*, *Harmine*, *Harman*, *Norharman*, *Guanabenz* and *Idazoxan* for which semi-quantitative binding data are available (Seidel et al., 2000). Of these, only *HTCA* shows a substantial toxic effect mediated by the *Ah* receptor system while all others are harmless at low-level doses – some of these compounds bind to Monoamine Oxidase and display a hallucinogenic activity. The result of our simulation is shown in Figure 6. The binding affinity of *HTCA* is calculated to 112 nM (exp: 60 nM), suggesting a rather high toxicity (for comparison: TCDD 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.

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 ore more of these surrogates. However in the virtual experiment, no binding affinity (Ki < 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 bind with an affinity of 1.8 µM to the $GABA_A$ receptor.

Within our limited pilot system (5 receptors, 280 compounds), we could demonstrate that this test set-up 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 power of the concept lies with a low rate of false-

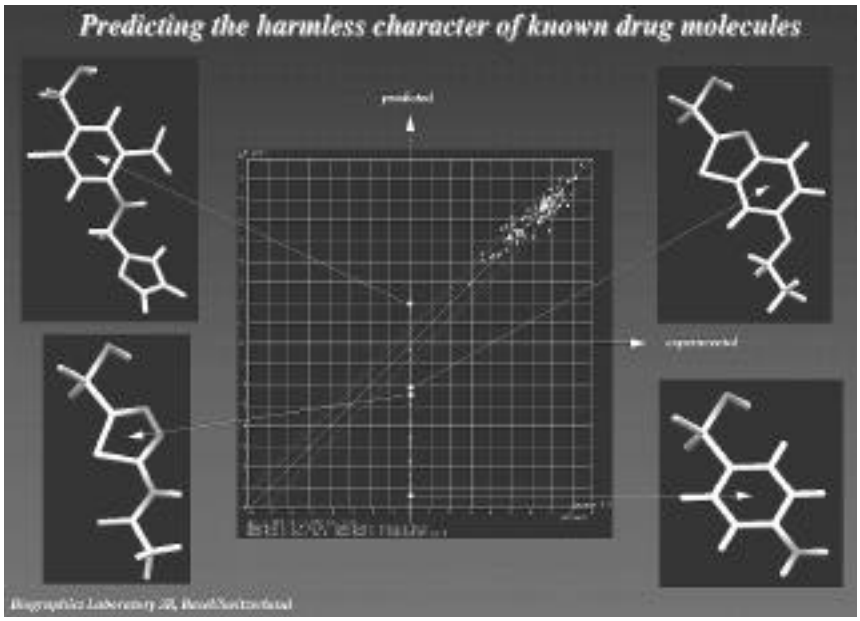

Fig. 5: Prediction of "harmless" substances at low doses hypothetically binding to the *Ah* receptor

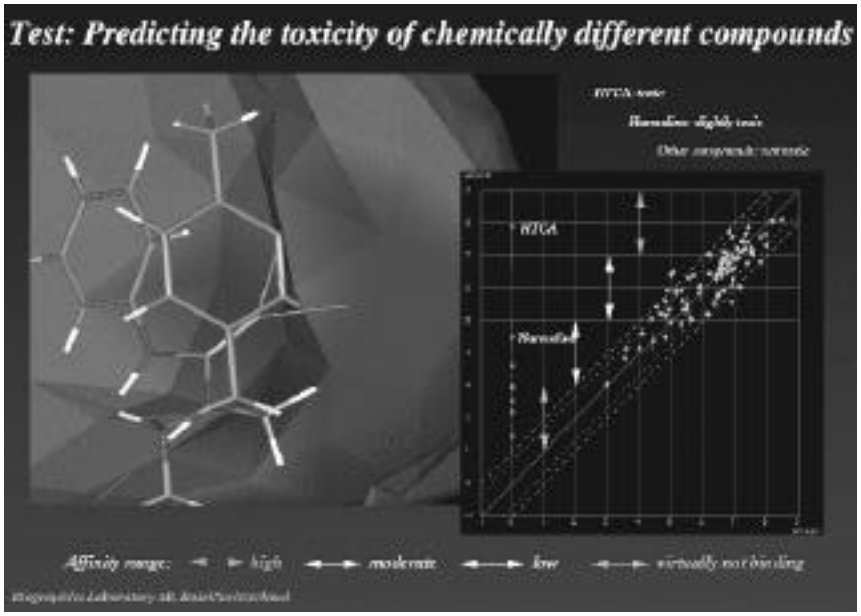

Fig. 6: Prediction of the binding affinity of nine monoamine oxidase inhibitors (MAOIs) also known to bind to the *Ah* receptor

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* (Vedani et al., 2002) and *Toxar* (Lill et al., in preparation), respectively – are available and the Internet protocol for an external access is presently being developed. We therefore think that the virtual laboratory could be made available to selected sites 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). In summary, key aspects of the proposed virtual laboratory include:

1. Potential adverse effects of a compound can be accessed well before its synthesis, i.e. during the first phase of development. Should it test positive for any of the included surrogates, the compound may not be cleared for further studies, in particular *in vivo* toxicity tests.

2. This virtual test is fast: the estimated computing time in *Quasar* is less than one minute per surrogate – i.e. for a database with 1,000 entries this would add up to a total time of 15 hours (on a high-end Macintosh, PC or Unix server). When using distributed computing, an overnight task may handle as much as 5,000–10,000 compounds at a university or corporate laboratory.

3. This surrogate test battery will be available at no costs to non-profit organisations (e.g. universities, hospitals and regulatory bodies) and at moderate costs to others.

4. The content of the database is constantly be augmented and cross-validated; any new experimentally tested compound will be added to the existing data set, thus improving the range of validity as well as its accountability.

5. 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.

6. Most important, there is a 100% data security as the sensitive compound data used to generate and validate the model is not deposited with the database and it cannot be backward regenerated: the dimensionality of the property space (typically, n=10,000-25,000) would seem to be absolutely permissive for such an undertaking.

7. The *Biographics Laboratory* is prepared to assist any party in both the set-up process (structure generation and optimisation, conformational search) as well as during model generation.

8. A mirror of this database can easily be installed on sites outside our laboratory (pharmaceutical industry, academia, regulatory bodies). A receptor-model database with 1,000 entries plus all pertinent software (*Quasar*, *Toxar*, access protocols) is expected to require less than 3.0 GB of disk space; i.e. it could even be installed on a laptop computer.

3 Developments planned for the near future

3.1 Database extension by new receptor surrogates

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 pathway; AMPA (2-Amino-3-(3-hydroxy-5-Methyl-isoxazol-4-yl) Propanoic Acid receptor mediating exitotoxicity; Histamine H1 receptor (bronchiolar or gastrointestinal smooth muscle constriction, bronchial hyperactivity) or Histamine H2 (CNS neurotransmission; delirium, confusion, agitation and seizures); mACh (muscarinic AcetylCholine) receptor (urinary retention, blurred vision; Parkinson, Alzheimer); Androgen receptor (side effects during sexual differentiation). Details of model generation and validation are published (Vedani and Dobler, 2001; Vedani et al., 2002). *Scrambletests* 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 conformational ensemble (4D) compiled using conformational search techniques.

3.2 Extension by models for cyctochrome P450 isoenzymes

Adverse effects may not only be triggered by the interaction of a drug or chemical with a mediating receptor system but also by inhibition of processes associated with phase-I reactions during biotransformation, e.g. the cytochrome P450 system. During such reactions, chemicals may also be metabolised and sometimes lead to toxic (e.g. carcinogenic) products. We therefore 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 (Zbinden et al., 1998) for model development. The Fe-containing heme portion will be modeled using the metalloprotein force field developed by our laboratory (Vedani and Huhta, 1990).

3.3 Improvement of the auto-docking algorithm – Alternative solvation model

The current auto-docking algorithm works fast and reliable for molecules of the approximate size of the binding pocket (Lill et al., in preparation). Larger molecules can be discarded if the associated induced-fit exceeds an *rms* of 1-2 Å. Significantly smaller molecules can in principle bind in a larger number of modes which have all to be explored and, more difficult, be discounted. Presently, we are using a Boltzmann factor for weighting their contribution to the final ensemble but since small differences in large (energy) numbers are involved, the identified solutions may not correspond to biophysical reality. We therefore plan to modify the algorithm by including not only steric and lipophilic criteria but using simulated annealing combined with correlation-coupled refinement (cf. Zbinden et al., 1998), particle swarms instead of a pure genetic algorithm as well as the implementation of a soft directional function, e.g. for hydrogen bonding. Another presently unresolved conflict is associated with the contribution of ligand (de)solvation during the binding process. In *Quasar*, the binding affinity is determined as follows:

$$E_{bdg} = E_{lig\text{-}rec} - T\Delta S_{bdg} - E_{solv.,lig} - E_{int.,lig} - E_{env.adapt.,lig}$$

where $E_{lig\text{-}rec}$ is the interaction energy between ligand and receptor, $T\Delta S_{bdg}$ the entropy change of the ligand binding, $E_{solv.,lig}$ the ligand desolvation energy, $E_{int.,lig}$ the change in internal energy of the ligand binding and $E_{env.adapt.,lig}$ the energy change of the receptor due to adapting to the ligand.

When modeling charged species, the dominant component is the ligand desolvation energy ($E_{solv.,lig}$), typically ranging from 160-240 kJ/mol resulting in a binding affinity (E_{bdg}) in the range of 32-48 kJ/mol. This has two consequences: Firstly, small errors in the computed solvation energy could jeopardise an otherwise robust simulation. Secondly, in the context of the proposed virtual laboratory we have to deal with receptor surrogates constructed based on charged ligand species but might be forced to test neutral compounds against (and *vice versa*). In such a situation, the compound to be tested will yield much too high or much too low affinities just based on this artefact. We will therefore implement and test an alternative scheme – not depending on the actual partial charge model – put forward by Viswanadhan et al. (1999) for this very purpose.

3.4 Internet protocols and security set-up

Easy access and, most important, security are issues of concern before the virtual laboratory and the database can be made freely accessible. The former includes a graphically-driven HTML protocol and the possibility to refer model building to our laboratory. Security shall exclude any access from doubtful institutions over the Internet.

4 3R relevance and future prospects

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. The proposed laboratory has to be validated with classical *in vitro* and *in vivo* toxicology tests. Once validated, it can also direct the design of specific *in vitro* and *in vivo* toxicology tests. The final aim would be to integrate classical *in vitro* and *in vivo* with *in silico* toxicology tests, where computer-based tests will be the initial step during toxicity testing. Another field of application includes toxicity testing of chemicals – for example the 30,000 compounds that have to be retested by 2012 as defined in the Europeans 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 (Fig. 7).

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 in 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 project", implying that all interested, skilled parties may contribute to development

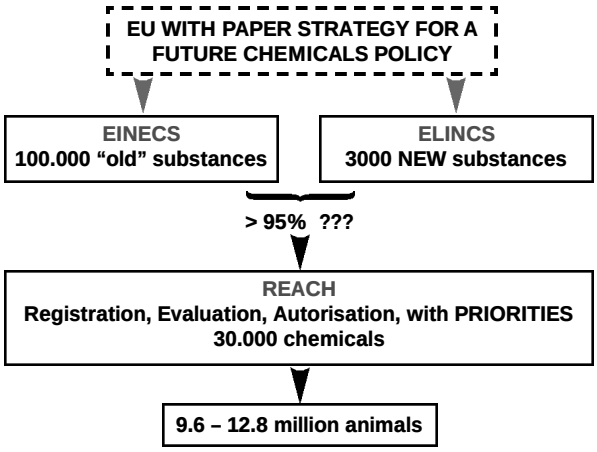

Fig. 7: Consequences of the EU white paper strategy for future chemicals policy (taken from the 2nd ECOPA workshop)

and extension. For this purpose, an independent source of continued funding is very important. The Foundation *Biographics Laboratory 3R* is a non-profit organisation; however, our financial means are limited. We think that financial support by an independent governmental agency would represent an optimal solution. Contribution to 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 moderate cost for the pharmaceutical industry.

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Correspondence to

PD Dr. Angelo Vedani
Biografik-Labor 3R
Friedensgasse 35
CH-4055 Basel
Tel: +41-61-261 4256
Fax: +41-61-261 4258
E-Mail: angelo@biograf.ch
www.biograf.ch