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VirtualToxLab – in silico Prediction of the Endocrine-Disrupting Potential of Drugs and Chemicals

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Abstract: In the last decade, we have developed and validated an *in silico* concept based on multidimensional QSAR (mQSAR) for the prediction of the toxic potential of drugs and environmental chemicals. Presently, the *VirtualToxLab* includes eleven so-called virtual test kits for estrogen (α/β), androgen, thyroid (α/β), glucocorticoid, aryl hydrocarbon, mineralocorticoid and peroxisome proliferator-activated receptor γ as well as for the enzymes cytochrome P450 3A4 and 2A13. The surrogates have been tested against a total of 824 compounds and are able to predict the binding affinity close to the experimental uncertainty with only six of the 194 test compounds giving calculated results more than a factor of 10 off the experimental binding affinity and the maximal individual deviation not exceeding a factor of 15. These results suggest that our approach is suited for the *in silico* identification of endocrine-disrupting effects triggered by drugs and environmental chemicals. Most recently, the technology has been made available through the Internet for academic laboratories, hospitals and environmental organizations.

Keywords: *in silico* prediction of the endocrine-disrupting potential · Molecular modeling · Multidimensional QSAR · VirtualToxLab

Introduction

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.[1] 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.[2–8]

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 toxic effects (*e.g.* endocrine disruption), they require a chemical or drug molecule to be physically present (*i.e.* synthesized) before testing, are time consuming, and the results are often associated with large standard errors, particularly in the low-to-mid activity range (mM–μM).

In contrast to *in vivo* and *in vitro* assays, computational approaches can be applied to hypothetical substances as their three-dimensional (3D) structure can readily be generated *in silico*. Nowadays computer power permits larger batches of compounds (*e.g.* parts of corporate or public databases) to be scanned for their toxic potential in moderate time spans. Toxicity-modeling algorithms are typically based on quantitative structure–activity relationships, neuronal networks, artificial intelligence or rule-based expert systems. The development of the *VirtualToxLab* technology has been previously been described.[9–10] In this account, we focus on the validation status and its implementation on the Internet.

Methods

The *VirtualToxLab* technology is based on a mixed-model approach: The binding mode of a drug or chemical of interest towards a target protein (enzyme, receptor) is identified using flexible docking to the 3D structure of the bioregulatory macromolecule. Its binding affinity – and the associated toxic (or endocrine-disrupting) potential – is then quantified using multidimensional QSAR (mQSAR). Most underlying technologies were developed at our laboratory and are published in detail.[11–15]

Quasar — a receptor-modeling concept developed at the *Biographics Laboratory 3R* — is based on 6D-QSAR and explicitly allows for the simulation of induced fit.[12,15] *Quasar* generates a family of quasi-atomistic receptor surrogates that are optimized

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by means of a genetic algorithm. The hypothetical receptor site is characterized 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 normalized 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 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 optimized 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. Like for the fourth and fifth dimension, a modest 'evolutionary pressure' is applied to achieve convergence. In the *Quasar* concept, the binding energy is calculated as follows:

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

where

$$E_{ligand–receptor} = E_{electrostatic} + E_{van\ der\ Waals} + E_{hydrogen\ bonding} + E_{polarization}$$

The contributions of the individual entities within a 4D ensemble (conformer, orientiomer, protomer, and/or tautomer) are normalized to unity using a Boltzmann criterion:

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

Raptor, an alternative technology developed by our laboratory[13] explicitly and anisotropically allows for induced fit by a dual-shell representation of the receptor surrogate, mapped with physicochemical properties (hydrophobic character and hydrogen-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. In *Raptor*, the binding energy is determined as follows:

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

where

$$E_{ligand–receptor} = f\,(E_{hydrogen\ bonding\ (shell\ 1)} + E_{hydrophobic\ (shell\ 1)}) + (1.0 - f) \cdot (E_{hydrogen\ bonding\ (shell\ 2)} + E_{hydrophobic\ (shell\ 2)})$$

and f is the interpolation weight between the two shells.[13]

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 ten of the proteins included in the *VirtualToxLab* (estrogen α/β, androgen, thyroid α/β, glucocorticoid, mineralocorticoid, peroxisome proliferator-activated receptor γ; cytochrome P450 3A4 and 2A13), the crystal structures are available; for the 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 account for two aspects of ligand–protein binding: i) the simulation of induced fit — allowing the protein to adapt its shape to the different orientations and conformations of the small molecule during the search procedure — and ii) the consideration of solvent effects (typically water). In our approach (software *Yeti*[11,15]), the sampling is conducted based on a Monte-Carlo/Metropolis protocol, which allows to initially considering apparently less favorable poses or conformations. Such calculations are quite computationally expensive as for an 'exhaustive search', typically 5,000–10,000 conformations/orientations are generated and 500–1,000 are fully minimized (Fig. 1). The directional force field – which is incorporated in all of our concepts (*Yeti, Quasar, Raptor, Symposar*) – would seem to be of utmost importance for quantifying hydrogen bonds and metal-ligand interactions (Fig. 2) appropriately.

For the aryl hydrocarbon receptor (AhR), for which no experimental structure is available, the alignment was performed based on the molecular skeletons.[12,15] Within the *VirtualToxLab*, ligands binding to the AhR are docked using the *Symposar* technology.[14] *Symposar* allows both 3D

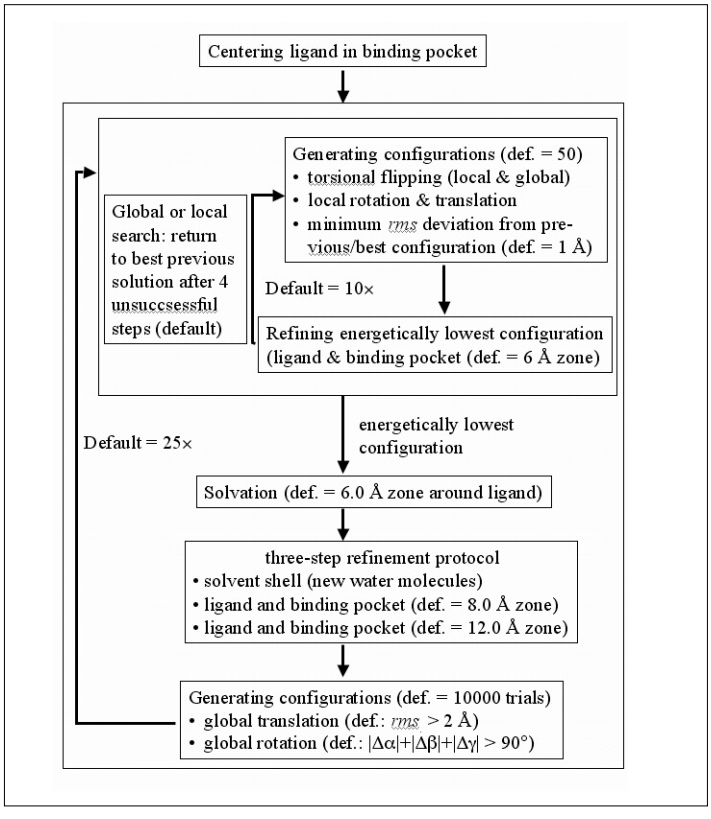


Fig. 1. Flexible docking – flowchart of module *AutoDock* as implemented in *Yeti*

$$E_{total} = \sum_{bonds} K_r (r - r_{eq})^2 + \sum_{angles} K_\theta (\theta - \theta_{eq})^2 +$$
$$\sum_{dihedrals} \frac{V_n}{2} [1 + \cos(n\phi - \gamma)] + \sum_{nb\ pairs} \frac{q_i \cdot q_j}{4\pi\varepsilon_o D(r) r_{ij}} + \sum_{nb\ pairs} \frac{A}{r_{ij}^{12}} - \frac{B}{r_{ij}^{6}}$$
$$+ \sum_{H-bonds} \left(\frac{C}{r_{H-Acc}^{12}} - \frac{D}{r_{H-Acc}^{10}} \right) \cdot \cos^2(\theta_{Don-H-Acc}) \cdot \cos^n(\omega_{H-Lig-LP})$$
$$+ \sum_{metal-ligand\ pairs} \frac{q_i^{CT} \cdot q_j^{CT}}{4\pi\varepsilon_o D(r) r_{ij}} + \sum_{metal-ligand\ pairs} \left(\frac{E}{r_{M-Lig}^{12}} - \frac{F}{r_{M-Lig}^{10}} \right)$$
$$+ (E_{MC} + E_{LFS}) \cdot \prod_{indep.\ angles} \cos^2(\psi_{Lig-M-Lig'} - \psi_{eq}) \cdot \frac{1}{n} \sum_{1^{st}\ shell\ ligands} \cos^n(\omega_{M-Lig-LP})$$

Fig. 2. Directional force field (as implemented in *Yeti*, *Quasar*, *Raptor*, and *Symposar*)

data sets (a single orientation and conformation per ligand molecule) and 4D data sets to be generated. It stores the grid produced and optimized by the ligands of the training set which can then be applied to any new test and prediction set. After the flexible mapping, the conformation of each molecule is minimized within the various grid fields.

The *VirtualToxLab*

Details of model validation are published (*cf.* [10] and references cited therein or [16]). Except for the aryl hydrocarbon receptor, where no experimental structure of the protein is available, all systems are based on docking studies using the human protein. As we used biological data measured in a single laboratory for most systems, the structural diversity is not yet sufficient to consider all models validated (PPARγ, TRαβ, ERβ, GR, MR). For some systems, the activity range would not seem to be sufficiently large (PPARγ, ERβ). The current validation status is summarized in Table 1 and Fig. 3; the appearance of *Quasar* and *Raptor* models is depicted in Fig. 4.

Internet Access Portal

To facilitate access to our technology, we have developed an Internet Access Protocol (IAP), immediately available for academic laboratories (Fig. 5). While the technology itself is freely available, we have to request a modest fee for acquiring and maintaining the underlying hardware and third-party software as well as for customer support. While the *VirtualToxLab* runs fully automated the 3D structure of the compound of interest must be generated and provided by the end user. Several freely available programs exist to accomplish this task; it is nonetheless vital to check the correctness of the structure (constitution, connectivity, stereochemistry).

In a first step (*cf.* Fig. 6), the structure is checked for correctness and the tautomeric and protonation state – at physiological pH – is identified (software Epik[22]). Then, the compound is subjected to a conformational-search protocol (to identify the global minimum conformation) in aqueous solution using the AMBER force field[23] as implemented in the *MacroModel* software.[24,25] Next, MNDO/ESP[26] or CM1[27] atomic partial charges are computed. In the main step, the compound is automatically docked to the target protein(s), thereby allowing the induced fit and sampling of all energetically feasible binding modes (*Yeti/AutoDock*[11,15]→ 4D data set). Thereafter, physicochemical properties (solvation energy, internal strain in the bound mode, entropic contribution to ligand binding) are determined. Finally, the compound's toxic potential is estimated by calculating its binding affinity towards the macromolecular target (*Quasar*[12,15] and *Raptor*[13]). Based on the information available on the data set used to evaluate the model (training and test compounds), the potential toxicological class (from 0 = benign to 4 = extremely toxic) is assigned. The results are then made available to the user *via* the IAP (Fig. 7) both as absolute numbers (binding affinity towards the target protein) and the estimated toxic potential (color coded). At this point, all user data (3D structure of the compound of interest) and all files associated with the procedures described above (compound coordinates, models, physicochemical properties, protocol files) are irreversibly discarded (for security issues, *cf.* below). The user log file may be exported (to a text file) at any time.

Table 1. *VirtualToxLab*: Summary of the currently available test kits (enzyme/receptor models)

System	compounds training+test=total; *compound classes*	q^2	rms training	max. training	p^2	rms test	max. test	fn/ fp[a]	Ref.
Aryl hydrocarbon	105+35=140; *eight*	0.824	1.8	10.2	0.769	2.3	13.5	0/2	[10]
Androgen	88+26=114; *eight*	0.858	1.7	7.8	0.792	1.6	13.9	1/1	[17]
Estrogen α	80+26=106; *six*	0.895	2.0	8.6	0.892	2.9	9.5	0/0	[15]
Estrogen α[b]	80+23=103; *five*	0.787	0.9	2.5	0.682	1.1	3.8	0/0	–
Estrogen β[b]		0.785	1.1	4.8	0.827	0.8	2.4	0/0	[10]
Glucocorticoid	82+28=110; *four*	0.745	1.2	5.9	0.650	2.2	5.5	0/0	[18]
Mineralocorticoid	40+12=52; *three*	0.798	1.6	5.6	0.701	2.5	9.5	0/0	[16]
PPARγ	75+20=95; *two*	0.832	1.4	6.2	0.723	1.4	3.9	0/0	[19]
Thyroid α	64+18= 82; *four*	0.919	1.8	4.3	0.814	2.5	10.0	0/1	[20]
Thyroid β		0.909	2.0	7.7	0.796	2.7	8.8	1/0	[20]
CYP3A4	38+10=48; *eighteen*	0.825	2.7	7.0	0.659	3.8	7.1	0/0	[21]
CYP2A13	18+6=24; *six*	0.900	0.6	1.5	0.830	0.3	0.7	0/0	[16]

q^2 = cross-validated r^2, p^2 = predictive r^2; the rms and maximal deviation from the experimental binding affinity is given as factor in K_i or IC_{50}.
[a]fn = false-negative, fp = false-positive compounds: a factor 10.0 or more off the experimental value
[b]different compounds (diphenolic azoles) than for the 80+26 model above.

Test Compounds

As an example, we tested six compounds: bisphenol-A, a polymer additive; 3,4,5,3',4',5'-hexabromodiphenylether, a flame-retarding chemical; erythrosine, a food dye; methylparaben, a preservative and fungicide; paracetamol, an NSAID; and methyltrienolone, a very potent ste-

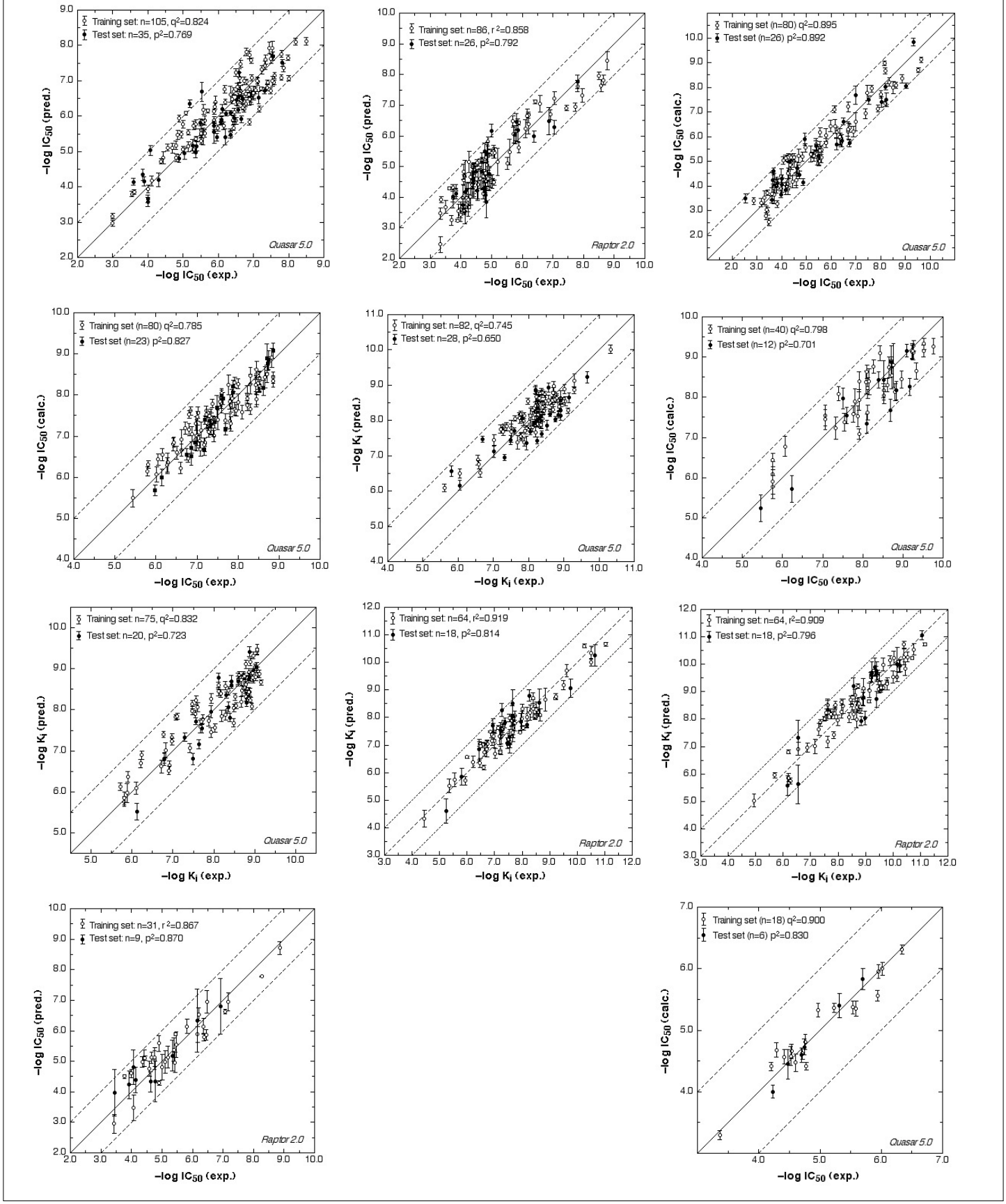


Fig. 3. Comparison of experimental and calculated binding affinities of the models underlying the *VirtualToxLab*. From left to right and top to bottom: AhR, AR, ERα; ERβ, GR, MR; PPARγ, TRα, TRβ; 3A4 (simulation without the eight threshold ligands; *cf.* ref. [21]), 2A13. The ligands of the training set are shown as open circles, those of the test set as filled circles. The error bars correspond to the variation of the 200–500 models comprising the model families. Dashed lines are drawn at ± 1.0 log unit from the experimental value.

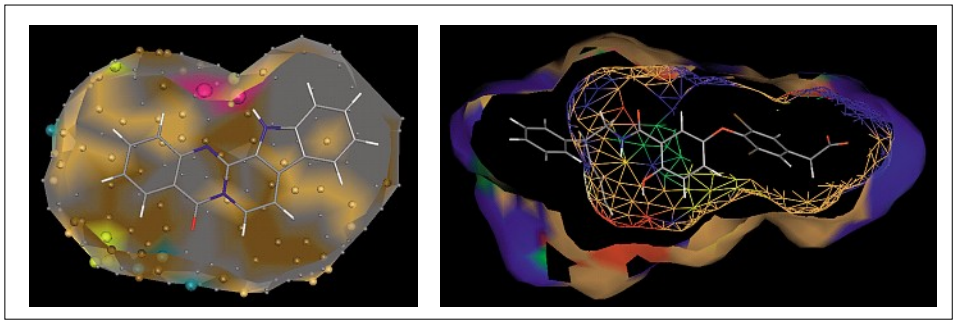


Fig. 4. *Quasar* model (6D-QSAR, left) and *Raptor* surrogate (dual-shell 5D-QSAR, right). The bound ligand is shown as stick representation (atom coloring: gray = carbon, white = hydrogen, red = oxygen, blue = nitrogen). The quasi-atomistic properties of the receptor are mapped onto the surface(s): blue = positively charged salt bridge, red = negatively charged salt bridge; brownish colors = hydrophobic properties, pink = hydrogen-bond flip flop. The *Quasar* model represents the Aryl hydrocarbon receptor;[10] the *Raptor* model depicts the thyroid receptor β.[17]

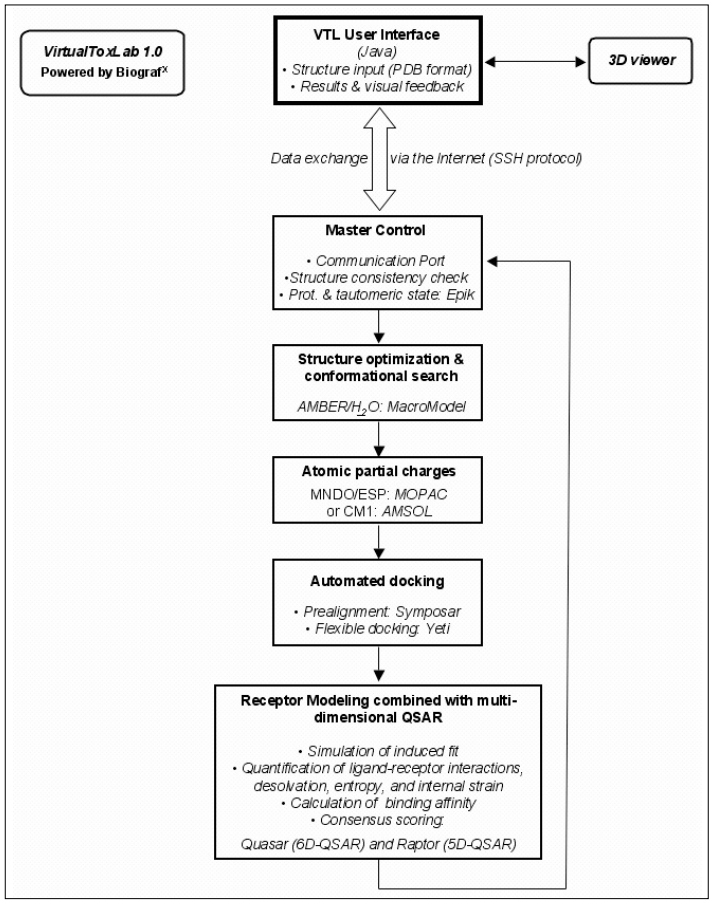


Fig. 5. Flow-chart of the *VirtualToxLab*

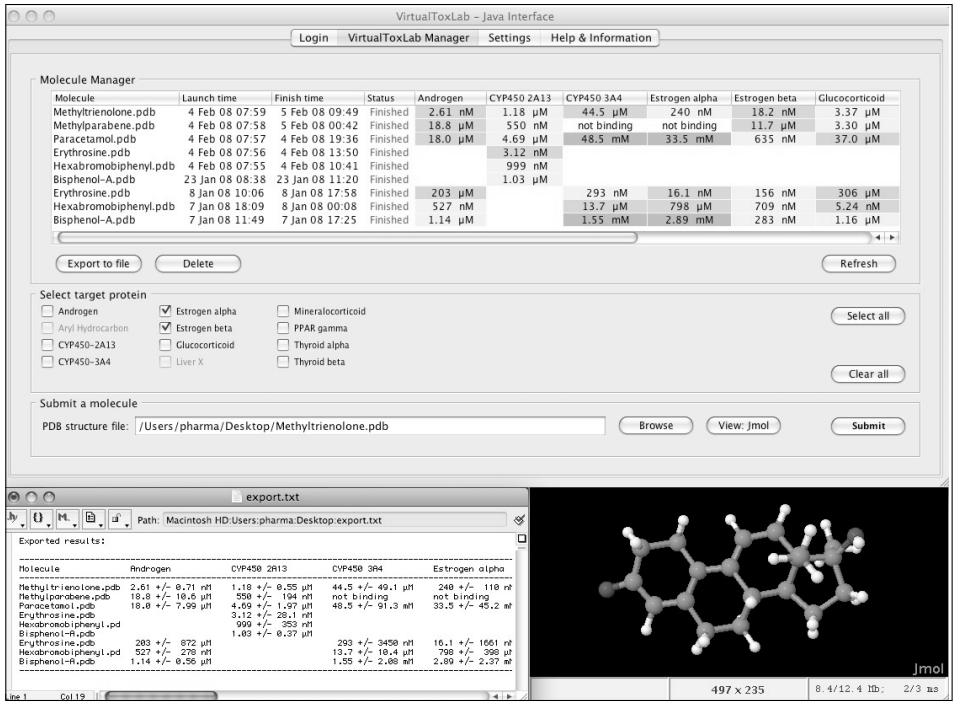


Fig. 6. Appearance of the Internet Access Portal to the *VirtualToxLab*

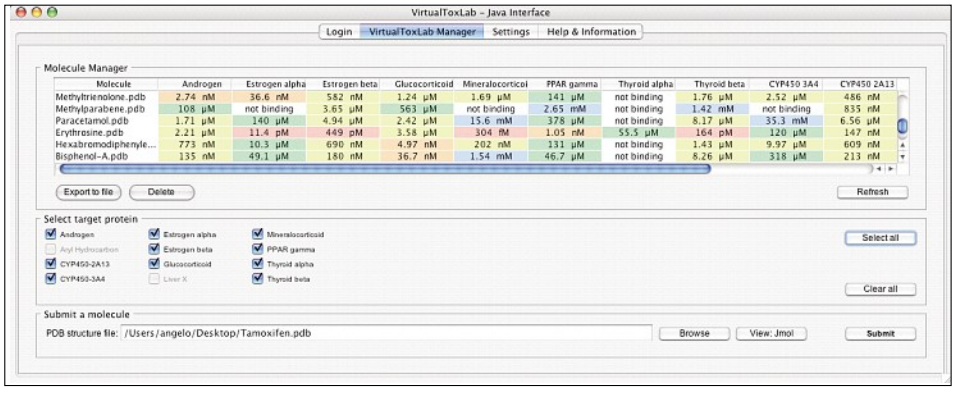
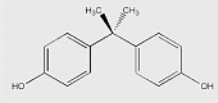
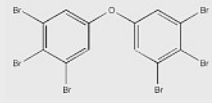
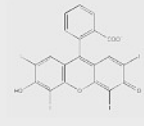
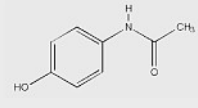
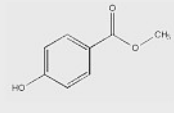
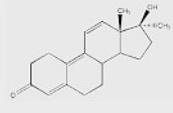


Fig. 7. Main dialog box of the IAP to the *VirtualToxLab*

Table 2. *VirtualToxLab*: Toxic potential profile of six selected compounds

	 Bisphenol A	 Hexabromo-diphenylether	 Erythrosine
Aryl hydrocarbon receptor	(–)	+++	++++
Androgen receptor	+++	+++	+++
Estrogen receptor α	++	++	not determinable
Estrogen receptor β	+++	+++	not determinable
Glucocorticoid receptor	++++	++++	+++
Mineralocorticoid receptor	(+)	+++	not determinable
PPARγ	++	++	(++++)
Thyroid receptor α	–	–	(++)
Thydorid receptor β	(+++)	(+++)	(+++++)
CYP450 3A4	++	+++	(++)
CYP450 2A13	+++	+++	(+++)
	 Paracetamol	 Methylparaben	 Methyltrienolone
Aryl hydrocarbon receptor	(+)	–	+
Androgen receptor	+++	++	++++
Estrogen receptor α	++	–	++++
Estrogen receptor β	+++	+++	+++
Glucocorticoid receptor	+++	++	+++
Mineralocorticoid receptor	(+)	–	+++
PPARγ	++	+	++
Thyroid receptor α	–	–	–
Thydorid receptor β	(+++)	(+)	+++
CYP450 3A4	+	–	+++
CYP450 2A13	+++	+++	+++

Toxic potential *in silico*			
	–	none	K_i or IC_{50} > 100mM
	+	very low	100mM > K_i or IC_{50} > 1mM
	++	low	1 mM > K_i or IC_{50} > 10 µM
	+++	medium	10 µM > K_i or IC_{50} > 100 nM
	++++	high	100 nM > K_i or IC_{50} > 1nM
	+++++	very high	K_i or IC_{50} < 1nM

Values in brackets indicate a high standard deviation and should be interpreted with caution; "not determinable" indicates very high esd's.

roid. The three-dimensional structures of all compounds were generated using *Bio*[28] and processed with the standard protocol of the *VirtualToxLab*. The resulting toxic potentials are given in Table 2.

Outlook

Up to date, the *VirtualToxLab* concept has only produced a few false-positive results. At the current level, however, false-negative predictions are still obtained, as a compound of interest cannot be tested against all potential receptors it may bind to *in vivo* (some macromolecular targets will remain unknown, for others no experimental structure exists or too few affinity data are available to establish a QSAR). We therefore plan to extend the current concept by implementing a set of virtual filters, which can recognize benign compounds. Among others, criteria include the molecular weight, drug-like properties (→ Lipinski's rule of five), and the presence/absence of characteristic structural motifs. We are also considering a combination of our *VirtualToxLab* with *LigandScout,* a fast screening technology based on protein-ligand structures.[29] Any new models and results are continuously updated on our website[30] from where the *VirtualToxLab* documentation can be downloaded.

Effective April 1, 2008, the technology has been made available through the Internet for academic laboratories, hospitals and environmental organizations. Interested parties should request the licensing agreement. After signing the document, they will receive a personal account/password and the URL to access the *VirtualToxLab* on-line.

In the *VirtualToxLab,* the data are transferred using the SSH (Secure Shell) protocol. After completion of the task, all data – compound coordinates, models, physicochemical parameters – are irreversibly discarded. The entries in the user log file (containing the calculated binding affinities) may be deleted by the user at any time.

Disclaimer: It is understood the *VirtualToxLab* may only generate reliable results for compound classes used to train the model of the respective target protein (which are given in the pertinent publications[9–21]). The standard deviation of a given result is also a safe quality indicator.

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