Marie Curie Initial Training Network

Environmental ChemOinformatics (ECO)

Final Report

September 2010 - August 2013

General Mechanisms in Biotransformation of Chemicals

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Starting Date: 1st September 2010

General Mechanisms in Biotransformation of Chemicals

DESCRIPTION OF THE PROJECT

REACH legislation[1] encourages the use of models for the risk assessment of chemicals. In bioaccumulation models, biotransformation is one of the processes which decrease the concentration of metabolizable compounds in an organism, together with elimination through other physicochemical processes. Rates of elimination via with air, water, and food can be predicted quite accurately from properties of chemical substances and biological species.[2-3] On the contrary, biotransformation rates (k_m, s^{-1}) are difficult to obtain. Recently, many efforts have been made to develop *in silico* methods to derive k_m values of chemicals, for example with Quantitative Structure-Activity Relationships (QSARs) for fish[4] or by comparing accumulation and elimination of stable and labile compounds for various biological species groups.[5] Methods have also been developed to scale *in vitro* estimates to *in vivo* k_m values in fish.[6]

Though biotransformation rates apply to a specific combination of a chemical and a species, some general patterns are noted. For instance, accumulation of metabolizable compounds appears to be a factor of about 50 lower than that of persistent equivalents.[7] This suggests that the underlying mechanisms may be (somewhat) more universal than usually thought. So far, such general patterns have hardly been investigated. Information on the overall principles determining biotransformation may be helpful in predicting metabolic rates.

In this 3-year project, the focus was on **general mechanisms in biotransformation of chemicals**, which have been studied using two approaches:

1. Evaluation of the **differences between the physicochemical properties of xenobiotics and their metabolites**. Parent compounds are usually transformed by enzymes into more polar metabolites to be excreted more rapidly. So far, this change in lipophilicity has been scarcely investigated; yet, it could shed light on general patterns of metabolism.
2. Investigation of the **enzymatic action of metabolism**, which involves two processes. Firstly, the chemical needs to reach the enzyme and bind with it; secondly, a catalytic reaction must take place. The binding of the chemical and its successive catalysis are described by two enzymatic parameters: the Michaelis constant (K_m), and the maximum rate of the reaction (V_{max}), respectively.[8] The K_m value is the substrate concentration at half V_{max} and is independent of the enzyme concentration.[9] Measured K_m and V_{max} data are lacking for many chemicals and species, thus models are helpful to explain these metabolic processes, and extend this knowledge to a broader range of chemicals. For reactions that exhibit Michaelis-Menten kinetics and on condition of non-saturating substrate concentration, the ratio between V_{max} and K_m provides an estimation of the intrinsic clearance (CL_{int}).[10-11] This parameter, which is a measure of enzyme activity towards a compound, can be extrapolated to equivalent whole-body *in vivo* metabolic rate (k_m),[6] which can be used to refine bioaccumulation models.

DESCRIPTION OF ACCOMPLISHMENTS

The aim of the project was twofold:

1. Estimation of the difference in lipophilicity, expressed by the octanol-water partition coefficient (K_{ow}), between parent compounds and their metabolites for a number of organic pollutants in mammals.
2. Prediction of K_m and V_{max} in mammals based on the characteristics of the chemicals.

These two topics will be described separately.

1. Estimation of the difference in lipophilicity between parent compounds and metabolites

The octanol-water partition coefficient (K_{ow}) is often used in risk assessment to predict intake, accumulation, and excretion rates of chemicals.[2] Elimination rate constants for persistent chemicals generally decrease with the K_{ow} (Figure 1).[12-13] Biotransformation usually reduces the lipophilicity of the compound, facilitating its excretion via aqueous fluids,[14] but this process is neglected in bioaccumulation models, which may lead to overestimation of the elimination rates of metabolizable compounds. If the parent compound is immediately and totally metabolized, it can be assumed that the elimination of the metabolite is similar to that of a persistent compound that is as lipophilic as the metabolite. As an example, Figure 1 shows the increase of the elimination rate constant by a factor of 10, from about 0.08 to 0.80, as a result of the reduction of the K_{ow} by two orders of magnitude, i.e., from 10^5 to 10^3. The objective of the present study was to estimate the difference in lipophilicity, expressed by the K_{ow}, between parent compounds and their metabolites for organic pollutants. This approach can be considered as a first indication of increased elimination to be used in exposure and risk assessment if empirical data and refined models are lacking. This study has been published as a paper (see the list of publications at the end of the report).

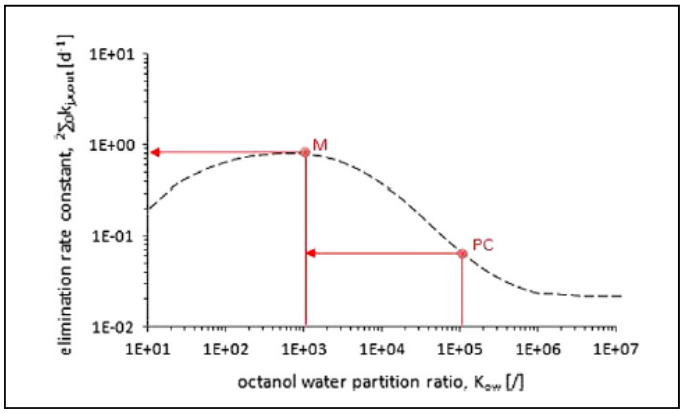

Figure 1 – Effect of a K_{ow} reduction from parent compound (PC) to metabolite (M) on the elimination rate constant. Background graph taken from [2]. The dashed line refers to elimination rate constants representing total physical-chemical elimination of persistent compounds, i.e. without biotransformation, in 10^{-1} kg mammals.

Information on the metabolic pathways of a set of environmental pollutants (parent compounds) was taken from the scientific literature and from two publicly available databases: Hazardous Substances Data Bank (HSDB, http://toxnet.nlm.nih.gov/) and Toxin and Toxin Target Database (T3DB, http://www.t3db.org/). Only those pollutants that have one main metabolic pathway in mammals were considered. The parent compounds were grouped according to their first "metabolite", i.e., to the reaction they undergo. We considered the following biotransformation reactions: alcohol oxidation (by ADH), aldehyde oxidation (by ALDH), and the more common types of P450 reactions, [15-16] i.e., hydroxylation, dihydroxylation, epoxidation, and heteroatom

(N, S) oxygenation. The K_{ow} values of parent compounds and metabolites were taken from the ChemSpider database (http://www.chemspider.com/). ChemSpider reports the experimental Log K_{ow} values (when available in the database), as well as the predicted values calculated by the ACD/LogP program. The Log-transformed octanol-water partition coefficients of the metabolites, Log $K_{ow\,(metabolite)}$ were related to the parent compounds, Log $K_{ow\,(parent)}$, according to Log $K_{ow\,(metabolite)}$ = a·Log $K_{ow\,(parent)}$ + b. A first set of regressions was built using Log K_{ow} values calculated by the ACD/LogP program and a second one using experimental Log K_{ow} values, when available for at least 5 parent compounds and their relative metabolites.

The Log K_{ow} decreased by a factor that varies between 0 and -2, depending on the metabolic pathway considered. For reactions mediated by CYP, the decrease in K_{ow} was one order of magnitude for hydroxylated and epoxidated compounds and two orders of magnitude for dihydroxylated and sulphoxidated xenobiotics. On the other hand, no significant change in lipophilicity was observed for compounds N-hydroxylated by CYP and for alcohols and aldehydes metabolized by ADH and ALDH. These trends could be anticipated by the calculus method of Log ACD/LogP.[17] Yet, they were validated using experimental Log K_{ow} values, when available. These relationships estimate the extent to which the elimination of pollutants is increased by biotransformation. Thus, the quantification of the K_{ow} reduction can be considered as a first necessary step in an alternative approach to anticipate biotransformation rates, which are difficult to estimate with existing methods.

2. Prediction of enzymatic constants

The scientific literature was searched in order to gather information on turnover of biotic compounds and on biotransformation of xenobiotics. Only few QSARs relating metabolic parameters and chemical structure were found, mainly focused on drugs oxidized by the microsomal cytochrome P450 (CYP), e.g.[18-19]. Compound lipophilicity seems to play a major role in the binding of xenobiotics for CYP (see reviews[8, 20]). Indeed, it has been shown that substrate binding affinities obtained from K_m data exhibit linear correlations with lipophilicity, expressed in terms of the Hansch relationship: Log ($1/K_m$) = a·Log K_{ow} + b. It is also important to emphasize that other factors might be involved in the enzyme binding, such as ionic interactions and hydrogen bonding properties.[21-22] On the other hand, the catalytic process (represented by V_{max}) results less controlled by substrate lipophilicity and more influenced by electronic properties of the substrates, such as electrophilicity.[8] However, these models focussed on single CYP isoenzymes and small datasets, mainly drugs. We extended the analysis to a broader set of chemicals and enzymes, in order to find generic patterns of metabolism across enzymes. We collected K_m and V_{max} values for four enzyme groups in mammals: alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), flavin-containing monooxygenase (FMO), and cytochrome P450 (CYP). Liver ADH catalyses the reversible transformation of alcohols to the corresponding aldehydes or ketones. ALDH enzymes oxidise a wide range of aldehydes to their corresponding carboxylic acids.[23] FMO oxygenates a wide range of xenobiotics, such as pesticides and drugs, containing a nucleophilic heteroatom (usually sulphur and nitrogen).[24] The oxygen abstraction takes place before binding via a nucleophilic attack by the substrate. The P450 enzymes usually catalyse mono-oxygenase reactions, which involve the insertion of an oxygen atom into a

substrate.[25] The chemicals investigated were xenobiotics, such as alcohols, aldehydes, pesticides, and drugs. We developed Quantitative Structure-Activity Relationships (QSARs) and the influencing descriptors in the QSARs were explained in relation to the catalytic cycles of the enzymes.

First, we investigated the relationship between Log ($1/K_m$) and lipophilicity (Log K_{ow}), and the outcomes have been published in an article (see the list of publications at the end of the report). Successively, we developed QSARs for both Log ($1/K_m$) and Log V_{max} with two approaches: (i) using descriptors chosen on the basis of mechanistic considerations; (ii) using theoretical descriptors selected with a genetic algorithm (GA), in collaboration with the Helmholtz Zentrum Munich. The outcomes of these two different approaches have been collected in two papers: one is under review and the second one is being finalized. These three works will be elucidated in the following three paragraphs.

2.1 Prediction of K_m using lipophilicity as descriptor

Experimental K_m values (*in vitro*) were collected from scientific literature and from a database (http://www.brenda-enzymes.info/) for compounds metabolized by four enzyme groups: alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), flavin-containing monooxygenase (FMO), and CYP enzymes. Only *in vitro* assays of purified liver enzymes in mammals were considered. The octanol-water partitioning coefficients (K_{ow}) were compiled from the KOWWIN™ software v. 1.67. Experimental K_{ow} values, when available, were preferred over estimated ones. Furthermore, each compound was assigned to relevant chemical classes using the ECOSAR™ program v 1.0 present in EPI Suite™. For each enzyme family, data were grouped per species (i.e., human, horse, rat, mouse, pig, and rabbit), and isoenzymes[1]. Regressions were developed for each combination of a species and isoenzyme (specific regressions). In addition, all species and isoenzymes were merged into one regression per enzyme family (general regression). The datasets of the specific and the general regressions consisted of the average values of $1/K_m$. For each dataset, linear regression analysis was performed using the Ordinary Least Squares (OLS) method: $\text{Log}(1/K_m) = a \cdot \text{Log}\, K_{ow} + b$.

For all regressions, $1/K_m$ increased with compound K_{ow}, which can be understood from the tendency to biotransform lipophilic compounds into more polar, thus more easily excretable metabolites. Lipophilicity was relevant to the binding of most of the substrate classes of ADH, ALDH, and CYP. The resulting slopes had 95% Confidence Intervals covering the value of 0.6-0.7, typically noted in protein-water distribution (Log K_{pw}) and Log K_{ow} regressions.[26-27] A reduced slope (0.2-0.3) was found for FMO: this may be due to a different reaction mechanism involving a nucleophilic attack.[24] The general patterns of metabolism were mechanistically interpreted in terms of partitioning theory.

2.2 QSARs for K_m and V_{max} using descriptors chosen on the basis of mechanistic considerations

Experimental V_{max} values (*in vitro*) were taken from the same papers for which K_m values were collected (ADH, ALDH, FMO, and CYP enzymes in liver of mammals). All species and isoenzymes were merged into one

[1] The isoenzymes are any of the several forms of an enzyme, all of which catalyze the same reaction but are characterized by varying properties (e.g. electrophoresis, chromatography, kinetics criteria, chemical structure, etc).

regression per enzyme family, for a total of four datasets each for Log ($1/K_m$) and Log V_{max}. We compiled a list of physico-chemical descriptors based on mechanistic considerations. These descriptors had been previously used in the QSARs for P450[25, 28-30] and can be interpreted mechanistically. They reflect partitioning (log K_{ow}), geometric (area, etc.) and electronic properties (hydrogen bond donor and acceptor, etc.) of the substrates. The descriptors were computed for the compounds in our datasets with Chemaxon (http://www.chemaxon.com) through the OCHEM platform[31] (http://ochem.eu) and with the semi-empirical molecular orbital program MOPAC2009[32] (Hamiltonian AM1) using the software Vega ZZ[33] v2.4.0 (http://vegazz.net). General linear models (GLM) were developed for Log ($1/K_m$) and Log V_{max} with the software R v.2.15.1[34] (http://www.R-project.org), using the R package 'bestglm'.[35] The models were cross-validated with the leave-one-out (LOO) procedure.

The explained variance of the QSARs varied between 20% and 70%, and it was larger for Log ($1/K_m$) if compared to Log V_{max}. Common features were found within the QSARs for Log ($1/K_m$) and Log V_{max}, despite the different reaction types of the four enzymes considered in this study. Log ($1/K_m$) was largely controlled by compound hydrophobicity (logP), as well as area (A) and frontier orbital energy (ΔE_{L-H}), while the rate (V_{max}) was mainly influenced by electronic parameters, such as dipole moment (v), hydrogen bonding properties (HBD and HBA), and energy of the lowest occupied molecular orbital (E_{LUMO}). The difference in the molecular properties controlling Log ($1/K_m$) and Log V_{max} was expected from the nature of the processes underlying these two constants. The inverse of K_m is usually assumed equal to the affinity constant for enzyme binding, which is generally a desolvation process, thus it is controlled mainly by hydrophobicity. The V_{max} represents the catalytic process, which is characterized by the cleavage and formation of covalent bonds (strong interactions), thus it is more influenced by electronic properties of the substrates.[8] The present study may be helpful to understand the underlying principles of the chemical specific activity of four important oxidizing enzymes.

2.3 QSARs for K_m and V_{max} using theoretical descriptors selected with a genetic algorithm (GA)

In this study, QSARs were developed for Log ($1/K_m$) and Log V_{max} of four oxidizing enzyme groups in mammals; few descriptors were selected by Genetic Algorithms from a pool of 1500 to 2000 total descriptors, depending on the dataset. We calculated 0D-3D descriptors using the Online CHEmical Modeling environment platform (OCHEM) (http://ochem.eu).[31] A Genetic Algorithm (GA) was applied to descriptors in order to find the subsets of variables that allow the calculation of the models with the highest predictive power. The models were built with multiple linear regression (MLR) and validated with the leave-one-out method.

The explained variance of the QSARs varied between 40% and 80%, and it was generally larger for K_m if compared to V_{max}. For Log ($1/K_m$), the packing density index (PDI) was the most influential descriptor for ADH and CYP. PDI is a molecular property related to the folding; its positive coefficient indicates that for ADH and CYP less folded chemicals have higher affinity and binding increases with the size of the molecules, in accordance with our previous works (see paragraphs 2.1 and 2.2). For the K_m of FMO, the hydrogen bond acceptor was the most important descriptor with a negative correlation coefficient. The substrates of FMO are nucleophiles, i.e. electron donors, thus the lower is the acceptor site number, the higher is the possibility of the

chemical to interact with these enzymes. For the K_m of ALDH, the most important descriptor was the E-state index SeaC2C3aa, referred to the aromatic bond between two C atoms present in aromatic aldehydes. Log V_{max} was particularly influenced by the structural fragments present in the compounds. The most important descriptors for ADH and ALDH were the number of aliphatic aldehydes and the number of halogens (Cl, Br, I, F) on an aromatic ring, respectively. For the V_{max} of CYP and FMO, the most important descriptors were, respectively, the E-state indexes Se1C1C3sd, related to a single bond between two C atoms (RC(=O)-C), and Se2C3O1s, which represents the carbonyl group (R'(R)C=O). The importance of local structural characteristics in determining the extent of metabolism has been shown also for *in vivo* biotransformation half-lives in fish, which have been predicted by a QSAR using exclusively fragments as descriptors.[36] The present study may be helpful to predict the metabolic constants in case measurements are lacking and to understand the chemical specific activity of four important oxidizing enzymes.

CONCLUDING REMARKS

Investigations on general mechanisms of biotransformation are still ongoing, due to the prolongation of my contract at Radboud University until December 2013. The focus is on biotransformation potential measured in mammals in different metabolizing test systems for liver. K_m and V_{max} values were searched for xenobiotics metabolized *in vitro* and measured in isolated hepatocytes, liver microsomes, or S9 liver fractions.[37-38] The data have been collected in a database; we decided to proceed only with data for microsomes, as they are the most abundant (>90% of the total). This dataset will be used to develop QSARs to predict K_m and V_{max} in liver microsomes. The results will be compared to the outcomes obtained in the QSARs for the single enzymes. In addition, we will calculate the ratio between V_{max} and K_m experimental values, i.e., the intrinsic hepatic clearance *in vitro* ($CL_{INT,vitro}$), which can be extrapolated to *in vivo* biotransformation rates (k_m) using scaling factors. We collected in literature the allometric relationships to obtain the scaling factors (i.e., liver weight (LW), hepatic blood flow (Q_H), etc.) using only species characteristics (mainly weight) and chemical properties (mainly K_{ow}). This would allow to predict *in vivo* k_m values from *in vitro* data knowing only species weight and the chemical properties. The effectiveness of the method can be tested by incorporating the k_m values (min^{-1}) obtained with IVIVE in a bioaccumulation model (e.g., the OMEGA model[2]) and comparing the results with experimental bioconcentration values The aim is to see if the bioconcentration models can be refined when biotransformation is considered (as already done for few compounds in fish[37-39]), and for which species and/or chemicals this inclusion leads to a better assessment of bioconcentration.

Additional Information

PUBLICATIONS

- Pirovano A, Huijbregts MAJ, Ragas AMJ, Veltman K, Hendriks AJ, 2013. Mechanistically based QSARs to describe metabolic constants in mammals, *Submitted*.
- Pirovano A, Huijbregts MAJ, Ragas AMJ, Hendriks AJ, 2012. Compound lipophilicity as a descriptor to predict binding affinity ($1/K_m$) in mammals. *Environmental Science & Technology*, 46, pp. 5168–5174, DOI: http://dx.doi.org/10.1021/es204506g.
- Pirovano A, Borile N, Hendriks AJ, 2012. A comparison of octanol–water partitioning between organic chemicals and their metabolites in mammals. *Chemosphere*, 88, pp. 1036-1041, DOI information: http://dx.doi.org/10.1016/j.chemosphere.2012.03.033.

SECONDMENT

- August 2012, Helmholtz Zentrum München, Munich (Germany).

COURSES AND CONFERENCES ATTENDED

1. Generic skills training courses organized by Radboud University

- Sept-Nov 2012, Advanced Conversation (1.5 ECTS)
  Content: Practical assignments aimed at improving English conversation skills and pronunciation.
- Feb - May 2012, Education in a Nutshell (1.5 ECTS)
  Content: Basic principles of didactics and tools for teaching courses.
- Nov 2011 - Feb 2012, Academic Writing (3 ECTS)
  Content: Practical assignments aimed at improving English writing skills.

2. Language courses

- Jan 2010 - Jun 2011, Nederlands als 2de taal (NT2) voor beginners, niveau A0->A1.
- Oct 2011 - Mar 2012, Nederlands als 2de taal (NT2) voor iets gevorderden, niveau A1-A2.
- Oct 2012 - Apr 2013, Nederlands als 2de taal (NT2) voor gevorderden, niveau A2-B1.

3. ECO schools

- 25 Feb - 01 Mar 2013, 3rd Winter School organized by Linnaeus University, Kalmar (Sweden).
- 11-15 June 2012, 3rd Summer School organized by the University of Milano-Bicocca, Milan (Italy) and held in Verona (Italy) together with the annual meeting of the International Academy of Mathematical Chemistry.
- 27 Feb - 02 Mar 2012, 2nd Winter School organized by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid (Spain)
- 19-30 Sept 2011, 2nd Summer School organized by Universiteit Leiden, Leiden (Netherlands).
- 21-25 Feb 2011, 1st Winter School organized by Hochschule Fresenius, Idstein (Germany).
- 18-22 Oct 2010, 1st Summer School organized by Helmholtz Zentrum München, Munich (Germany).

4. Conferences attended

- CEMEPE/SECOTOX 4th International Conference, Mykonos (Greece), 24-28 June 2013.
  Presentation: Pirovano A, Huijbregts MAJ, Ragas AMJ, Veltman K, Hendriks AJ, "Quantitative Structure-Activity Relationships (QSARs) to predict metabolic constants ($1/K_m$ and V_{max}) in mammals."
- SETAC 3rd Young Environmental Scientists (YES) Meeting, Krakow (Poland), 11-13 Feb 2013.
  Poster: Pirovano A, Huijbregts MAJ, Ragas AMJ, Veltman K, Hendriks AJ, "Quantitative Structure-Activity Relationships (QSARs) for metabolic affinity ($1/K_m$) and maximum velocity (V_{max}) in mammals."
- CADASTER Workshop, Munich (Germany), 7.-9 Oct 2012.
- SETAC 6th World Congress, Berlin (Germany), 20-24 May 2012.
  Poster: Pirovano A, Huijbregts MAJ, O'Connor IA, Ragas AMJ, Hendriks AJ, "Compound lipophilicity as a descriptor to predict metabolic affinity (K_m) in mammals."
- SETAC Europe 21st Annual Meeting, Milan (Italy), 15-19 May 2011.
  Poster: Pirovano A, Huijbregts MAJ, O'Connor IA, Ragas AMJ, Hendriks AJ, "Biotransformation of chemicals: linking octanol-water partitioning to Michaelis constants to search for general mechanisms."

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