European Journal of Pharmaceutical Sciences 39 (2010) 310–321

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Prediction of the *in vitro* intrinsic clearance determined in suspensions of human hepatocytes by using artificial neural networks

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ARTICLE INFO

Article history:
Received 21 July 2009
Received in revised form
15 December 2009
Accepted 20 December 2009
Available online 7 January 2010

Keywords:
Human hepatic clearance
In vitro intrinsic clearance
Human hepatocytes suspension
In silico prediction
Artificial neural network

ABSTRACT

Use of *in vitro* suspensions of human hepatocytes is currently accepted as one of the most promising tools for prediction of metabolic clearance in new drugs. The possibility of creating computational models based on this data may potentiate the early selection process of new drugs. We present an artificial neural network for modelling human hepatocyte intrinsic clearances (CL_{int}) based only on calculated molecular descriptors. *In vitro* CL_{int} data obtained in human hepatocytes suspensions was divided into a train group of 71 drugs for network optimization and a test group of another 18 drugs for early-stop and internal validation resulting in correlations of 0.953 and 0.804 for the train and test group respectively. The model applicability was tested with 112 drugs by comparing the *in silico* predicted CL_{int} with the *in vivo* CL_{int} estimated by the "well-stirred" model based on the *in vivo* hepatic clearance (CL_H). Acceptable correlations were observed with *r* values of 0.508 and 63% of drugs within a 10-fold difference when considering blood binding in acidic drugs only. This model may be a valuable tool for prediction and simulation in the drug development process, allowing the *in silico* estimation of the human *in vivo* hepatic clearance.

1. Introduction

Undesirable pharmacokinetic properties are still one of the major reasons for drug failures and the low success rate of drug discovery programs (Singh, 2006). Due to lack of reliable predictive models in early development phases, new drug entities are subjected to a large number of *in vitro* and animal *in vivo* tests before they are ultimately administered in man. During phase 1 clinical studies, development is frequently discontinued after a large number of data has already been produced. Due to cost, time and ethical reasons, the use of *in silico* methods based on this data to predict fundamental ADME (absorption, distribution, metabolism and elimination) properties is increasing, and already plays a role in the early drug candidate selection (Richon, 2008).

Hepatic clearance (CL_H) is a major pharmacokinetic parameter, as it is related both to the drug elimination and to the drug oral bioavailability. Due to those two facts, and since it influences the dose and dose regime in man, it is a major factor for drug selection in the development phase of new drug candidates. Several methods exist to predict CL_H in humans, namely interspecies extrapolation (Wajima et al., 2002), allometric scaling (Hu and Hayton, 2001), *in vitro–in vivo* correlations (Naritomi et al., 2003), physiologically based *in vitro–in vivo* prediction (Ito and Houston, 2004) and *in silico* methods (Ekins et al., 2000). A recent review (Fagerholm, 2007) recommended that, of these, physiologically based *in vitro–in vivo* prediction using hepatocytes intrinsic clearances (CL_{int}), should be applied and improved. In this type of approach, the CL_{int} value determined in human hepatocytes suspensions is used to predict the CL_H in human after inclusion on a mathematical liver model with physiological parameters. The "well-stirred" model (Rowland et al., 1973) is one of the most used, due to its simplicity and reasonable results (Ito and Houston, 2004). In its original form, the "well-stirred" model emphasizes the relationship between the blood perfusion (Q), metabolic activity (CL_{int}) and blood binding (f_B) in the hepatic clearance of drugs. However, inclusion of f_B for all drugs often results in poor predictions mainly for basic, neutral and zwitterionic drugs and various authors proposed extrapolations without the inclusion of this parameter (Jacobson et al., 2007; Lau et al., 2002; McGinnity et al., 2004; Reddy et al., 2005).

Some *in silico* methods are already being published with variable success (Chang et al., 2008; Ekins, 2003; Ekins and Obach, 2000; Jolivette and Ward, 2005; Lee et al., 2007; Li et al., 2008; Schneider et al., 1999; Turner et al., 2004; Wajima et al., 2002; Zuegge et al., 2001). These could be divided into methods employing only *in silico*-derived parameters or combining *in vitro* and *in vivo* data. The first class is extremely useful, as it would provide clearance prediction before the drug actually exists. Moreover, since *in silico* methods are, by nature, only the first line of drug selection, the type of parameter predicted should also play a role when developing an

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doi:10.1016/j.ejps.2009.12.007

Table 1
In vitro CL_{int} values collected in the literature. Values were determined in human hepatocytes suspensions using the substrate depletion method without the addition of serum in the incubation medium.

Compounds	Data set	log *in vitro* CL_{int} (μl/min/10^6 cells)												*In silico* predicted
		A	B	C	D	E	F		G		H	I	J[a]	
7-Hydroxycoumarin	Train	–	–	–	–	–	–	–	–	–	1.4	–	–	1.37
4-Methylumbelliferone	Val	–	–	–	–	–	–	–	–	–	–	–	1.51	1.14
Acebutolol	Train	–	–	–	–	–	–	0.26	–	–	–	–	–	0.2
Acetaminophen	Train	−0.11	–	–	–	–	–	–	–	–	–	–	–	−0.13
Antipyrine	Train	–	−0.33	–	–	–	–	–	–	–	–	–	–	−0.36
Atenolol	Train	–	–	–	–	–	–	−1.00	–	–	–	–	–	−0.95
Benzydamine	Train	–	–	–	1.31	–	–	–	–	–	–	–	–	1.13
Bepridil	Train	–	–	–	–	–	–	0.30	–	–	–	–	–	0.45
Betaxolol	Train	–	–	–	–	–	–	0.40	–	–	–	–	–	0.37
Bisoprolol	Test	–	–	–	–	–	–	0.20	–	–	–	–	–	0.01
Bosentan	Train	–	–	–	–	–	0.18	–	–	–	–	–	–	0.13
Bromocriptine	Train	–	–	–	–	–	–	1.57	–	–	–	–	–	1.8
Bufuralol	Train	–	–	–	–	–	–	–	–	–	0.85	–	–	1.09
Caffeine	Train	–	0.15	–	–	–	–	0.52	–	–	–	–	–	0.46
Carbamazepine	Train	–	–	–	–	−0.10	–	0.30	–	–	–	–	–	0.18
Carvedilol	Train	–	–	–	–	–	–	1.54	–	–	–	–	–	1.37
Cerivastatin	Val	–	–	–	–	–	–	–	–	–	–	–	0.23	0.7
Cetirizine	Train	–	–	–	–	–	–	−1.00	–	–	–	–	–	−0.95
Chlorpheniramine	Train	–	–	–	–	–	–	0.45	–	–	–	–	–	0.95
Chlorpromazine	Test	–	1.04	–	–	–	–	–	–	–	–	0.71	–	1.36
Chlorprothixene	Train	–	1.15	–	–	–	–	–	–	–	–	–	–	1.43
Cimetidine	Train	–	–	–	–	–	–	0.08	–	–	–	–	–	0.15
Clozapine	Test	–	–	–	–	–	–	0.78	–	–	–	–	–	1.06
Codeine	Train	–	–	–	–	–	–	1.36	–	–	–	–	–	1.46
Desipramine	Train	–	1.04	–	–	–	–	0.48	–	–	–	–	–	0.92
Dextromethorphan	Test	–	–	–	–	–	–	0.88	1.20	1.18	–	–	–	1.03
Diazepam	Train	0.13	0.15	–	–	–	–	−0.52	−0.05	−0.05	0.30	–	–	−0.02
Diclofenac	Test	–	–	–	–	–	–	–	1.67	1.64	0.90	–	–	0.42
Diltiazem	Train	1.37	0.67	–	–	–	–	0.95	1.11	1.11	–	–	–	1.07
Diphenhydramine	Train	–	–	–	–	–	–	0.78	–	–	–	–	–	0.7
Doxepin	Train	–	–	–	–	–	–	1.11	–	–	–	–	–	0.78
Ethinylestradiol	Test	–	–	–	–	–	–	0.85	0.85	0.70	–	–	–	0.29
Famotidine	Train	–	–	–	–	–	–	−1.00	–	–	–	–	–	−1.03
FK079	Train	1.11	–	–	–	–	–	–	–	–	–	–	–	0.98
FK1052	Train	0.41	–	–	–	–	–	–	–	–	–	–	–	0.38
FK480	Train	0.51	–	–	–	–	–	–	–	–	–	–	–	0.64
Fluoxetine	Train	–	–	–	–	–	–	0.00	–	–	–	–	–	−0.11
Furosemide	Test	–	−2.00	–	–	–	–	–	–	–	–	–	–	−1.37
Gemfibrozil	Train	–	–	–	–	–	–	–	1.38	1.30	–	–	–	1.3
Granisetron	Train	–	–	–	–	–	–	0.95	–	0.85	–	–	–	0.41
Ibuprofen	Train	–	0.62	–	–	–	–	–	–	–	–	–	–	0.67
Imipramine	Train	–	0.91	–	–	–	–	–	–	0.85	–	–	–	1.15
Irbesartan	Val	–	–	–	–	–	–	–	–	–	–	–	0.81	−0.28
Isradipine	Train	–	–	–	–	–	–	1.26	–	–	–	–	–	0.79
Ketoprofen	Test	–	–	–	–	–	–	–	0.60	0.00	–	–	–	−0.23
Lidocaine	Train	–	–	–	–	–	–	–	–	–	–	1.13	–	0.88
Lorazepam	Test	–	−0.57	–	–	–	–	0.00	–	–	–	–	–	0.04
Mephenytion	Val	–	–	–	–	–	–	–	–	–	–	–	0.32	−0.56
Methylprednisolone	Train	–	0.99	–	–	–	–	–	–	–	–	–	–	1.14
Metoprolol	Train	–	–	–	–	–	–	0.85	0.85	1.00	–	0.81	–	0.76
Midazolam	Train	–	0.85	–	–	–	1.10	1.15	1.15	1.04	0.90	–	–	0.8
Morphine	Train	–	–	–	–	–	–	1.38	–	–	–	–	–	1.38
Nadolol	Test	–	–	–	–	–	–	−1.00	–	–	–	–	–	−0.88
Naloxone	Test	–	1.45	–	–	–	1.90	2.33	–	–	–	–	–	1.52
Naproxen	Train	–	–	–	–	–	–	–	0.70	0.48	–	–	–	0.98
Nifedipine	Train	–	0.89	–	–	–	–	0.75	–	–	–	–	–	0.93
Nitrendipine	Test	–	–	–	–	–	–	0.87	–	–	–	–	–	1.03
Nortriptyline	Train	–	–	–	–	–	–	–	–	–	–	0.44	–	0.46
Omeprazole	Train	–	–	–	–	–	–	0.23	–	–	–	–	–	0.24
Ondansetron	Train	–	–	–	–	–	–	0.15	–	–	–	–	–	0.7
Oxazepam	Train	–	0.30	–	–	–	–	–	–	–	–	–	–	0.19
Oxyprenolol	Val	–	–	–	–	–	–	–	–	–	–	–	0.34	1.03
Phenacetin	Train	–	–	–	–	–	–	–	–	–	0.88	–	–	1.02
Phenytoin	Train	–	–	–	–	0.47	–	–	–	–	–	–	–	0.57
Pindolol	Test	–	–	–	–	–	–	0.45	–	–	–	–	–	0.58
Pirenzepine	Train	–	–	–	–	–	–	−1.00	–	–	–	–	–	−0.94
Prazosin	Train	–	–	–	–	–	–	0.36	–	–	–	–	–	0.58
Prednisone	Train	–	0.99	–	–	–	–	–	–	–	–	–	–	0.88
Procainamide	Train	–	–	–	–	0.11	–	–	–	–	–	–	–	0.09
Propafenone	Test	–	–	–	–	–	–	–	–	–	–	1.76	–	0.54
Propofol	Train	–	–	–	–	–	–	2.03	2.03	1.96	–	–	–	1.52

Table 1 (*Continued*)

Compounds	Data set	log *in vitro* CL_{int} (μl/min/10^6 cells)											*In silico* predicted
		A	B	C	D	E	F		G		H	I	J[a]	
Propranolol	Test	–	1.00	–	–	–	–	1.00	1.18	1.28	–	1.11	–	0.98
Quinidine	Train	–	–	–	–	0.74	–	–	–	–	–	–	–	0.51
Quinotolast	Train	−2.00	–	–	–	–	–	–	–	–	–	–	–	−1.92
Ranitidine	Train	–	–	–	–	–	–	0.00	–	–	–	–	–	0.02
Ritonavir	Train	–	–	–	–	–	–	0.32	–	–	–	–	–	0.31
Scopolamine	Train	–	–	–	–	–	–	0.85	–	–	–	–	–	1.02
Sildenafil	Train	–	0.72	–	–	–	–	–	–	–	–	–	–	1.03
Sulpiride	Train	–	−2.00	–	–	–	–	–	–	–	–	–	–	−1.83
Temazepam	Test	–	–	–	–	–	–	0.30	–	–	–	–	–	−0.14
Tenoxicam	Train	–	0.41	–	–	–	–	–	–	–	–	–	–	0.18
Terbutaline	Train	–	−2.00	–	–	–	–	–	–	–	–	–	–	−2
Theophylline	Test	–	−0.28	–	–	−0.32	–	–	–	–	–	–	–	−0.56
Tolbutamide	Train	–	0.20	–	–	–	–	–	–	–	–	−0.25	–	−0.07
Triazolam	Train	–	–	–	–	–	–	0.00	–	–	–	0.34	–	0.17
Triprolidine	Train	–	–	–	–	–	–	0.63	–	–	–	–	–	0.42
Troglitazone	Train	1.32	–	–	–	–	–	–	–	–	–	–	–	1.4
Valproic acid	Test	–	–	–	–	−0.31	–	–	–	–	–	–	–	0.67
Verapamil	Train	–	1.20	–	–	–	–	1.26	1.62	1.63	–	–	–	1.54
Vinpocetine	Train	–	–	2.41	–	–	–	–	–	–	–	–	–	1.76
Warfarin	Train	–	0.04	–	–	–	–	–	–	–	–	–	–	0.09
Zidovudine	Train	0.51	–	–	–	–	–	–	–	–	–	–	–	0.41
Zileuton	Train	–	–	–	–	–	–	0.32	–	–	–	–	–	0.45
Zolpidem	Train	–	–	–	–	–	–	–	–	–	–	0.56	–	0.62

Data set A–J were obtained from references (Naritomi et al., 2003; Lau et al., 2002; Szakacs et al., 2001; Fisher et al., 2002; Bachmann et al., 2003; Blanchard et al., 2005; McGinnity et al., 2004; Floby et al., 2004; Reddy et al., 2005; Jacobson et al., 2007) respectively.

[a] 2.5% of BSA in the incubation medium.

in silico method. For example, various methods exist that directly predict human CL_H. Since estimation of the *in vivo* CL_H is frequently made by considering that the CL_H is equal to the *in vivo* non-renal clearance in man, this results in an overestimation of the CL_H value. Another drawback in these types of modelling approaches is that prediction confirmation is only possible when the drug is administered to humans, leaving no room for "fine-tuning" during the development phase.

In this study, a new *in silico* model was developed based on the CL_{int} of 89 drugs determined *in vitro* with suspensions of human hepatocytes by the substrate depletion method in absence of added serum. Only *in silico* derived parameters were used, and an artificial neural network was optimized to relate these molecular descriptors to the *in vitro* data. The model applicability was tested, in a physiologically based *in silico–in vivo* approach, with another 112 drugs from which a reliable estimation of the human *in vivo* CL_H and the *in vivo* CL_{int} determined by "well-stirred" model was possible. With this ANN model it is possible to provide estimates of CL_{int}, which could be early validated in the pipeline process of new drug development and that, by using physiologically based *in silico–in vivo* prediction, could be used to estimate the CL_H itself.

2. Materials and methods

2.1. Data collection

In vitro CL_{int} values (Table 1) were obtained from published studies of drug metabolism in human hepatocytes using the substrate depletion method in absence of added serum. Drugs were randomly divided between a train ($n = 71$) and a test group ($n = 18$) used in the ANN optimization process. An external validation group ($n = 5$) of data was also considered based on drugs not included in the train and test group from the study of Jacobson et al. (2007). In this study, although 2.5% of BSA was included in the incubation medium, no differences in CL_{int} were described by the authors when BSA was not added to the incubation medium. In order to test the ability of the *in silico* model to predict *in vivo* intrinsic clearances, two additional data sets were also obtained (Tables 2 and 3). These consisted of drugs with human intravenous *in vivo* pharmacokinetic data that were available (Goodman et al., 2006). In these cases, data on intravenous total plasma clearance, free fraction in plasma, fraction of drug eliminated by the kidneys, as well as oral bioavailability were collected. Experimentally determined values of the drug blood-to-plasma concentration ratio (R_B) were used whenever available, or calculated according to Paixao et al. (2008). This model provided predictions of R_B with a percentage of correct values within a 1.25-fold error of 86, 84 and 87% in the train, test and validation data set respectively.

2.2. In silico calculation of the molecular descriptors

The following methodology was used for the *in silico* descriptors: SMILES notation of each molecule was obtained using the on-line PubChem Compound database (http://www.ncbi.nlm.nih.gov). Ionization descriptors ($pK_{a\,acid}$; $pK_{a\,base}$), lipophilicity ($\log P$) and water solubility ($\log S$) were obtained using the on-line ALOGPS 2.1 program (Tetko and Bruneau, 2004). For drugs without an acid ionisable group, a value of 15 was attributed to $pK_{a\,acid}$. For drugs without a basic ionisable group, a value of −1 was attributed to $pK_{a\,base}$. The remaining descriptors, related to size, compactness, lipophilicity and others, were obtained from the on-line E-Dragon 1.0 software using CORINA to convert the SMILES notation to the 3D representation of the molecule (Tetko et al., 2005). A total of 233 molecular descriptors were calculated, consisting of 10 molecular properties, 48 constitutional descriptors, 73 topological descriptors, 22 geometrical descriptors, 47 information indices and 33 WHIM descriptors.

2.3. ANN model building

The Artificial Neural Network (ANN) non-linear regression was performed using the backpropagation neural modelling system Qnet for Windows v.2000 build 751 (Vesta Services Inc., USA) and an in-house developed Microsoft Excel® VBA routine for process automation. CL_{int} values were log transformed and randomly

divided between a train ($n = 71$) and a test group ($n = 18$). More than one CL_{int} experimental value was available for some drugs. In these cases, all values were considered but with a weighting factor of $1/n$ being n the number of independent determinations of CL_{int} for the individual drug. Both the input and output variables were normalised, and a sigmoid transfer function was used in all connections. Early stopping based on the degradation of the root mean square error (RMS) of the test group was used to avoid over fitting. Each network was started 20 times with random initial values to avoid convergence to local minima.

Network optimization was performed in a two step process. The first step consisted of the reduction of the molecular descriptors space. This was initially done by removing highly correlated ($r > 0.90$) descriptors, allowing the removal of molecular descriptors with information contained within another descriptor. Then, an ANN with a network architecture of 1 hidden layer with 3 hidden neurons was optimized for each of the molecular descriptors class, and the relevance of each descriptor within each class of molecular descriptors was tested against the percent contribution for the final output of a random input variable, allowing the removal of molecular descriptors that were uncorrelated with the output variable. Finally a pruning procedure was undertaken, using also a network architecture of 1 hidden layer with 3 hidden neurons and including all the remaining molecular descriptors. At the end of each optimization, the percent contribution for the final output was calculated to all descriptors and the descriptor presenting the worst value was removed. This procedure was repeated until a significant degradation of the RMS with a descriptor removal was observed.

The second step consisted of the optimization of the network architecture for the most relevant molecular descriptors. Several networks were made varying the number of hidden layers (1–3) and the number of hidden neurons (1–5).

2.4. Model applicability

Typically, *in vitro* CL_{int} data determined in human hepatocytes suspensions is used to predict the *in vivo* CL_H as this is a major parameter in order to characterize the drug elimination and bioavailability in man. For that purpose, *in vitro* CL_{int} data is introduced in a mathematical liver model, such as the "well-stirred" model, and used to predict the human CL_H value. With the same purpose, we tested the ability of the ANN model to predict both the *in vivo* CL_{int} and the *in vivo* CL_H using the drugs from Tables 2 and 3. Since predictive errors by both the *in vitro* data and the "well-stirred" model were expected, drugs from Table 2 that were present in the training and test data sets of Table 1 were used to test the ability of the *in vitro* data to predict the *in vivo* CL_{int} and the *in vivo* CL_H by using the "well-stirred" model. The 112 drugs from Table 3 were effectively used to test the ANN model performance in the same situation.

The *in vivo* CL_H values from Tables 2 and 3 drugs were determined by using Eq. (1) (Naritomi et al., 2003),

$$CL_H = \frac{CL_{total}^{plasma}}{R_b} \cdot (1 - f_{renal}) \quad (1)$$

This equation assumes that total blood clearance, determined by the ratio between the described total plasma clearance (CL_{total}^{plasma}) to the drug blood-to-plasma concentration ratio (R_B), is the sum of hepatic and renal clearance the last being determined by using the fraction of drug eliminated by the kidneys (F_{renal}).

Table 2
In vivo pharmacokinetic data for the drugs included in the ANN model building and used to evaluate the ability of the *in vitro* hepatocytes suspensions in estimating the *in vivo* CL_{int} and CL_H by the "well-stirred" model. P indicates data determined in plasma; Bl data determined in blood; F is the drug oral bioavailability; F_{renal} is the percentage of parent drug eliminated in urine; R_B is the blood to plasma concentration ratio of the drug; F_p is the percentage of drug bound to plasma proteins; A acid, B basic, N neutral and Z zwitterionic drug and pH 7.4.

	CL	F (%)	F_{renal} (%)	F_p (%)	CL_{total} (ml min^{-1} kg^{-1})	R_B	Drug class	CL_{blood} (ml min^{-1} kg^{-1})	CL_H (ml min^{-1} kg^{-1})
Acetaminophen	P	88	30	20	5	1.04	B	4.8	2.40[e]
Atenolol	P	58	94	5	2.4	1.07[d]	B	2.24	0.13
Carvedilol	P	25	2	95	8.7	0.72	B	12.12	11.88
Cimetidine	P	60	62	19	8.3	0.97[d]	B	8.56	3.25
Clozapine	P	55	1	95	6.1	1.13	B	5.41	5.36
Diazepam	P	100	1	98.7	0.38	0.58[d]	N	0.66	0.65
Diclofenac	P	54	1	99.5	4.2	0.56	A	7.54	7.46
Diltiazem	P	38	4	78	11.8	1.00[d]	B	11.8	11.33
Diphenhydramine	P	72	1.9	78	6.2	0.65[d]	B	9.54	5.60[e]
Furosemide	P	61	66	98.6	2	0.55	A	3.61	1.23
Gemfibrozil	P	95	1	97	1.7	0.55	A	3.08	1.00[e]
Granisetron	P	60	16	65	11	0.86	B	12.81	8.00[e]
Lidocaine	P	0.35	2	70	9.2	0.84[d]	B	10.95	10.73
Lorazepam	P	93	1	91	1.1	1.05	N	1.05	1.04
Methylprednisolone	P	82	4.9	78	6.2	0.78	N	7.94	3.60[e]
Metoprolol	P	38	10	11	15	1.00[d]	B	15	13.5
Midazolam	P	44	1	98	6.6	0.80[d]	N	8.25	8.17
Morphine	P	24	4	35	24	0.95	B	25.22	15.20[e]
Naloxone	P	2	0	30[c]	22	1.22[d]	B	18.03	18.03
Nifedipine	P	50	0	96	7	1.63	N	4.28	4.28
Nortriptyline	P	51	2	92	7.2	1.50[d]	B	4.8	4.7
Ondansetron	P	62	5	73	5.9	0.83[d]	B	7.11	6.75
Prazosin	P	68	4	95	3	0.70[d]	N	4.29	4.11
Propranolol	Bl	26	0.5	87	16	0.89[d]	B	16	14.80[e]
Quinidine	P	80	18	87	4.7	0.88	B	5.31	4.36
Ranitidine	P	52	69	15	10.4	1.03	B	10.11	3.14
Sildenafil	P	38	0	96	6	0.99	N	6.04	6.04
Valproic acid	P	100	1.8	93	0.14	0.64[d]	A	0.22	0.21
Zidovudine	P	63	18	25	26	1.06	N	24.5	7.40[e]
Zolpidem	P	72	1	92	4.5	0.76[d]	N	5.92	5.86

Values were collected in Goodman et al. (2006) except a) Ritschel (2000), b) Rowell et al. (1980), c) Garrett et al. (1985) and d) were *in vitro* value from Paixao et al. (2008). For data in e) CL_H was determined according to Eq. (2). Values in f) were limited to 19.5 ml min^{-1} kg^{-1}.

Table 3
In vivo pharmacokinetic data for the drugs not included in the train process and used to evaluate the ANN model applicability in estimating the *in vivo* CL_{int} and CL_H by the "well-stirred" model. P indicates data determined in plasma; Bl data determined in blood; *F* is the drug oral bioavailability; F_{renal} is the percentage of parent drug eliminated in urine; R_B is the blood to plasma concentration ratio of the drug; F_p is the percentage of drug bound to plasma proteins; A acid, B basic, N neutral and Z zwitterionic drug and pH 7.4.

	CL	*F* (%)	F_{renal} (%)	F_p (%)	CL_{total} (ml min^{-1} kg^{-1})	R_B	Drug class	CL_{blood} (ml min^{-1} kg^{-1})	CL_H (ml min^{-1} kg^{-1})	*In silico* predicted log CL_{int} (μl/min/10^6 cells)
Acyclovir	P	30	75	15	6.19	1.08	N	5.75	1.44	0.53
Alendronate	P	7	45	78	1.11	1.7	Z	0.65	0.36	−2.54
Alfentanil	P	–	1	92	6.7	0.63[d]	N	10.63	10.53	−0.14
Allopurinol	P	53	12	2[a]	9.9	1.09	N	9.11	8.02	−0.49
Alprazolam	P	88	20	71	0.74	0.78[d]	N	0.95	0.76	0.49
Amikacin	P	–	98	4	1.3	0.97	B	1.34	0.03	−2.93
Amiodarona	P	46	0	99.9	1.9	0.73[d]	B	2.6	2.6	1.08
Amitriptyline	B	48	2	95	11.5	0.86[d]	B	11.5	10.40[e]	0.93
Amlodipine	P	74	10	93	5.9	1.2	B	4.91	4.42	−0.09
Amoxicillin	P	93	86	18	2.6	1.04	A	2.51	0.35	−0.06
Aprepitant	P	65	0	95	1.29	0.6	N	2.14	2.14	1.11
Atropine	P	–	57	18	8	0.99	B	8.12	3.49	0.16
Azathioprine	P	60	2	30[a]	57	1.05	N	54.23	8.00[e]	1.07
Bupivacaine	Bl	–	2	95	7.1	0.73[d]	B	7.1	6.96	0.25
Buprenorphine	P	–	0	96	13.3	1	B	13.3	13.3	2.04
Buspirone	P	3.9	0	95	28.3	0.62	B	45.99	19.22[e]	0.35
Butorphanol	P	17	1.9	80	40	1.9	B	21.03	16.60[e]	−1.12
Calcitriol	P	61	10	99.9	0.43	0.55	N	0.78	0.7	−0.02
Candesartan	P	42	52	99.8	0.37	0.55	A	0.67	0.32	0.53
Cefazolin	P	90	80	89	0.95	0.56	A	1.7	0.34	0.44
Cefepime	P	–	80	18	1.8	0.74	A	2.43	0.49	−2.19
Cefixime	P	47	41	67	1.3	0.62	A	2.11	1.25	−2.22
Ceftazidime	P	–	84	21	1.92	0.72	A	2.68	0.43	−1.54
Celecoxib	P	–	3	97	6.6	0.57	N	11.61	11.26	−0.44
Cephalexin	P	90	91	14	4.3	1.02	A	4.21	0.38	0.27
Chlorthalidone	Bl	64	65	75	0.04	0.73[d]	N	0.04	0.01	−0.42
Cinacalcet	P	20	0	95	18	0.64	B	28.28	16.00[e]	0.18
Ciprofloxacin	P	60	50	40	7.6	1.07	Z	7.09	3.55	−0.37
Clindamycin	P	87	13	93.6	4.7	0.76	B	6.15	2.60[e]	1.11
Clonidine	P	95	62	20	3.1	1.04	B	2.97	1.13	1.33
Cyclophosphamide	P	74	6.5	13	1.3	1.06	N	1.22	1.14	−0.1
Dapsone	P	93	15	73	0.6	1.04[d]	N	0.58	0.49	−2.85
Dicloxacillin	P	50	60	95.8	1.6	0.55	A	2.89	1.16	1.66
Didanosine	P	38	36	5	16	1.08	N	14.85	9.5	0.14
Docetaxel	P	–	2.1	94	22.6	0.55	N	40.93	19.50[f]	2.12
Dofetilide	P	96	52	64	5.23	0.72	B	7.3	3.5	−0.11
Doxycycline	P	93	41	88	0.53	1.7	Z	0.31	0.18	−2.43
Entacapone	P	42	0	98	10.3	0.55	A	18.66	11.60[e]	0.87
Ethambutol	P	77	79	18	8.6	0.96	B	8.98	1.89	0.31
Etoposide	P	52	35	96	0.68	0.55	N	1.23	0.8	1.57
Fentanyl	P	–	8	84	13	0.97[d]	B	13.4	12.33	0.59
Finasteride	P	63	1	90	2.3	0.56	N	4.12	4.08	0.91
Flecainide	P	70	43	61	5.6	0.89	B	6.3	3.59	−0.23
Fluconazole	P	90	75	11	0.27	1.06	N	0.26	0.06	−1.06
Flumazenil	P	16	0.2	40	9.9	1.00[d]	N	9.9	9.88	1
Fluorouracil	P	28	10	10	16	1.09[d]	N	14.62	13.16	1.51
Fluphenazine	P	2.7	0	92[b]	10	0.69	B	14.55	14.55	0.66
Foscarnet	P	9	95	15	1.6	1.27	A	1.26	0.06	−2.91
Fulvestrant	P	–	1	99	9.3	1	N	9.3	9.21	0.64
Gabapentin	P	60	66	3	1.6	1.1	Z	1.46	0.49	−0.36
Galantamine	P	95	2	18	5.7	1.04	B	5.48	1.00[e]	−0.68
Ganciclovir	P	4	91	1	3.4	1.08	N	3.16	0.28	−0.98
Gentamicin	P	–	90	10	1.52	1	B	1.52	0.15	−2.93
Glimepiride	P	100	0.5	99.5	0.62	0.55	A	1.12	1.11	0.92
Glyburide	P	90	0	99.8	1.3	0.56	A	2.34	2.34	0.65
Hydrochlorothiazide	P	71	95	58	4.9	1.70[d]	N	2.88	0.14	0.17
Hydromorphone	P	42	6	7.1	14.6	1.07	B	13.62	12.8	1.18
Imatinib	P	98	5	95	3.3	0.64	B	5.17	0.40[e]	1.19
Indomethacin	P	100	15	90	1.4	0.56	A	2.51	2.14	1.36
Irbesartan	P	70	2.2	90	2.12	0.64	Z	3.34	3.26	−0.28
Isosorbide dinitrate	P	22	1	28	46	1	N	46.06	15.60[e]	0.99
Isosorbide-5-mononitrate	P	93	5	0	1.8	1.08	N	1.67	1.59	−1.01
Lamivudine	P	86	50	36	4.95	1.06	N	4.67	2.33	0.41
Lansoprazole	P	81	1	97	6.23	0.56	N	11.17	3.80[e]	−0.03
Letrozole	P	99.9	3.9	60	0.58	0.92	N	0.63	0.61	0.12
Levetiracetam	P	100	66	10	0.96	1.07	N	0.89	0.3	−0.63
Levofloxacin	P	99	70	30	2.52	1.05	Z	2.41	0.72	1.36

Table 3 (*Continued*).

	CL	F (%)	F_{renal} (%)	F_p (%)	CL_{total} (ml min^{-1} kg^{-1})	R_B	Drug class	CL_{blood} (ml min^{-1} kg^{-1})	CL_H (ml min^{-1} kg^{-1})	*In silico* predicted log CL_{int} (μl/min/10^6 cells)
Linezolid	P	100	35	31	2.1	0.73	A	2.86	1.86	0.01
Losartan	P	35.8	12	98.7	8.1	0.55	A	14.68	12.92	0.6
Meloxicam	P	97	1	99.4	0.15	1.22	A	0.12	0.12	−0.3
Melphalan	P	71	12	90	5.2	0.96[d]	Z	5.42	4.77	0.96
Meperidine	P	52	5	58	17	0.87[d]	B	19.54	9.60[e]	0.79
Mercaptopurine	P	12	22	19	11	1.2	N	9.2	7.18	−2.71
Metformin	P	52	99.9	0	7.62	1.04	B	7.33	0.01	−2.91
Methadone	P	92	24	89	1.7	0.75[d]	B	2.27	1.60[e]	0.85
Methotrexate	P	70	81	46	2.1	0.71	A	2.94	0.56	−2.92
Metoclopramide	P	76	20	40	6.2	0.96	B	6.44	4.80[e]	0.96
Metronidazole	P	99	10	11	1.3	1.07	N	1.21	1.09	0.75
Montelukast	P	62	0	99	0.7	0.55	A	1.27	1.27	1.48
Moxifloxacin	P	86	21.9	39.4	2.27	1.05	Z	2.16	1.69	0.76
Nalmefene	P	40	9.6	34	15	1.11	B	13.54	12.24	0.99
Neostigmine	P	–	50	0[a]	16.7	1.07	N	15.6	7.8	−1.14
Nitrofurantoin	P	90	47	62	9.9	0.76[d]	A	13.03	2.00[e]	1.22
Oxycodone	P	42	19	45	12.4	1.03	B	12.04	9.75	1.59
Pancuronium	P	–	67	7	1.8	1.02	N	1.77	0.58	1.56
Phenobarbital	P	100	24	51	0.06	0.86[d]	N	0.07	0.05	−0.55
Pravastatin	P	18	47	45	13.5	0.55[d]	A	24.55	13.01	−1.02
Quetiapine	P	9	1	83	19	0.9	B	21.11	18.20[e]	1.14
Quinine	P	76	16	90	0.9	0.91	B	0.99	0.83	0.49
Repaglinide	P	56	1	97.4	9.3	0.55	A	16.85	8.80[e]	0.8
Riluzole	P	64	1	98	5.5	1.7	N	3.24	3.2	1.74
Risedronate	P	1	87	24	1.5	1.07	A	1.4	0.18	−2.91
Risperidone	P	66	3	89	5.4	0.67[d]	B	8.06	7.82	1.2
Rizatriptan	P	47	28	14	12.3	1.04	B	11.85	8.53	0.24
Rocuronium	P	–	17	25	4.25	1	N	4.26	3.54	1.46
Ropivacaine	P	–	1	92	6.3	0.93	B	6.81	6.74	0.49
Sulfamethoxazole	P	100	14	53	0.31	0.79	A	0.39	0.34	−0.96
Sumatriptan	P	14	22	17.5	22	1.03	B	21.37	16.67	0.29
Tamsulosin	P	100	12.7	99	0.62	0.55[d]	B	1.13	0.98	0.87
Tegaserod	P	11	0	98	18	0.72	B	25.04	17.80[e]	−0.97
Tenofovir	P	25	82	1	2.6	1.05	A	2.47	0.44	−2.75
Terazosin	P	82	12.5	92	1.15	0.84	N	1.37	1.2	0.43
Tetracycline	P	77	58	65	1.67	1.7	Z	0.98	0.41	−2.29
Timolol	P	61	8	10	7.7	0.87	B	8.88	7.80[e]	0.11
Tramadol	P	70	20	20	8	1.03	B	7.75	6.2	0.34
Trazodone	P	81	1	93	2.1	0.81	N	2.61	2.58	−0.24
Trimethoprim	P	63	63	37	1.9	1.03	N	1.84	0.68	0.47
Valsartan	P	23	29	95	0.49	0.55	A	0.89	0.63	0.42
Vecuronium	P	–	20	69	6.4	0.97	N	6.6	5.28	1.37
Vinorelbine	P	27	11	87	21	0.58	B	36.34	14.60[e]	2.11
Zaleplon	P	31	1	60	15.7	0.99	N	15.84	13.80[e]	0.55
Ziprasidone	P	59	1	99.9	11.7	0.81	B	14.52	8.20[e]	1.27

Values were collected in Goodman et al. (2006) except a) Ritschel (2000), b) Rowell et al. (1980), c) Garrett et al. (1985) and d) were *in vitro* value from Paixao et al. (2008). For data in e) CL_H was determined according to Eq. (2). Values in f) were limited to 19.5 ml min^{-1} kg^{-1}.

Some drugs may have other non-renal elimination routes besides the hepatic one. In these cases, it is expected that CL_H determined by Eq. (1) would be overpredicted. To minimise this, *in vivo* CL_H was also determined by Eq. (2) (Iwatsubo et al., 1997),

$$CL_H = Q_H \cdot (1 - F_{oral}) \quad (2)$$

This equation assumes that oral bioavailability (F_{oral}) is only a result of the first pass-effect in the liver, allowing the determination of the liver extraction ratio (E_H) and the CL_H by multiplying E_H with the hepatic blood flow rate (Q_H) with a value of 20 ml min^{-1} kg^{-1}. Although Eq. (2) could also provide overpredicted values for CL_H, in theory these would be the maximum possible values for this parameter. For this reason, when comparing CL_H determined by Eq. (1) to the value obtained with Eq. (2), if the first is bigger the latter prevails.

The *in vivo* CL_{int} values were determined by using the "well-stirred" liver model rearranged in Eq. (3),

$$CL_{int} = \frac{Q_H \cdot CL_H}{f_B \cdot (Q_H - CL_H)} \quad (3)$$

with the previously determined *in vivo* CL_H and the free fraction of drug in blood (f_B) determined by the ratio between the free fraction of drug in plasma (F_{uP}) by the drug blood-to-plasma concentration ratio (R_B). Hepatocellularity was considered to be 107×10^6 cell/g liver (Wilson et al., 2003) and it was also assumed that liver weighed 20 g/kg of body weight in order to scale the *in silico* CL_{int} values from unities of μl/min/10^6 cells to ml min^{-1} kg^{-1}.

2.5. Statistical analysis

Correlation between the predicted and observed values was determined by means of the Pearson correlation coefficient (r) for the train and test groups for the log(CL_{int}) data. In order to assess the precision and bias of the network, root mean squared prediction error (RMSE) and mean error (ME) were also estimated by using the following equations.

$$RMSE = \sqrt{\frac{\sum \left(CL_{int\,pred} - CL_{int\,obs}\right)^2}{N}} \quad (4)$$

Table 4
Summary of the 21 molecular descriptors used in the ANN model.

Molecular descriptor	Description	Molecular descriptor	Description
AMW	Average molecular weight	PJI2	2D Petitjean Shape Index
BAC	Balaban Centric Index	PW3	Randic Shape Index Path/Walk 3
Gm	G Total Symmetry Index weighted by atomic masses	PW5	Randic Shape Index Path/Walk 3
HNar	Narumi Harmonic Topological Index	Ram	Ramification Index
Hy	Hydrophilic factor	RBN	Number of rotable bonds
IC4	Information Content Index (neighborhood symmetry of 4-order)	SIC2	Structural information content (neighborhood symmetry of 2-order)
log *P*	Oil:water partitioning coefficient	SPI	Superpendentic Index
Me	Mean atomic sanderson electronegativity (scaled on carbon atom)	TI1	Second Mohar Index
Ms	Mean electrotopological state	Tot_Fn	Fraction of neutral drug
MSD	Mean Square Distance Index	TPSA (NO)	Topological polar surface area (No)
nCIR	Number of circuits		

Detailed information of the presented molecular descriptors may be obtained in Todeschini and Consonni (2000).

$$\mathrm{ME} = \frac{\sum \left(\mathrm{CL}_{\mathrm{int\,pred}} - \mathrm{CL}_{\mathrm{int\,obs}}\right)}{N} \quad (5)$$

Percentage of correct values within a 2-fold error (difference of log predicted to log observed values outside the interval −0.3 to 0.3) was determined in order to assess the quantitative ability of the network. Additionally, percentage of correct values within a 10-fold error (difference of log predicted to log observed values outside the interval −1 to 1) was also determined in order to assess the qualitative ability of the network.

3. Results

3.1. ANN model building

Optimization of the ANN model was made as described under methods. Regarding the reduction of the molecular descriptors space, the removal of correlated descriptors reduced the number of descriptors to 92. The next two procedures were preformed using an ANN with 3 hidden neurons in 1 hidden layer. This ANN structure was chosen as a compromise between simplicity, in order to avoid over parameterization and memorization, and complexity to allow an adequate learning ability of the network. To escape local minima each network was run 20 times with random initial values and learning was early-stopped based on the degradation of test RMS error to avoid memorization. After 20 runs, the network with the lower average test RMS error was kept, and the relative contribution of each input on the $\log(\mathrm{CL}_{\mathrm{int}})$ prediction was established using the "input node interrogator" option in QNet. In this option, inputs contribution are determined by cycling each input for all training patterns and computing the effect on the network's output response. Of the 92 non-correlated descriptors, 43 were considered non-relevant, since their input contribution was equal or inferior to a random input and were also removed. Finally, by the pruning procedure described under methods, it was considered that 21 molecular descriptors were needed to characterise the output response, as a degradation of the test RMS error was observed when further removals were undertaken. The final model was constructed with the 21 molecular descriptors presented in Table 4. Based on these descriptors, a network architecture optimization was pursued using a brute force approach. In order to maintain a practical computational time, the network architectural space was swept between 1 to 3 hidden layers and 1 to 5 hidden neurons by layer. Additionally, the ratio between the number of patterns to the number of connections was maintained above 1 in order to reduce the ability of the network to memorise the data and avoid overfitting (So and Richards, 1992; Turner et al., 2004).

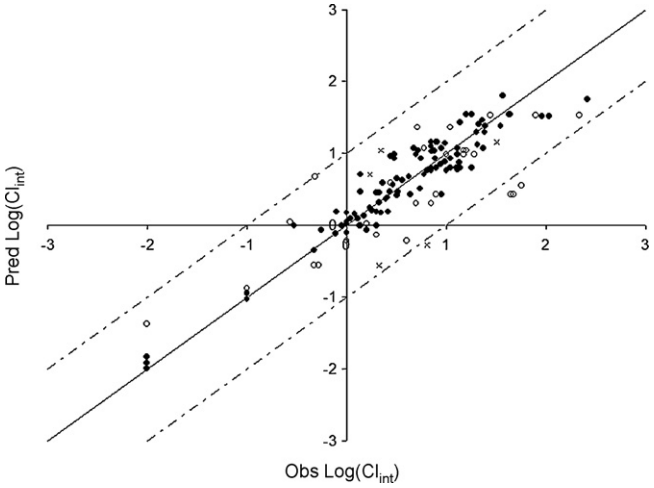

Fig. 1. Plot of the *in vitro* observed vs *in silico* predicted $\log(\mathrm{CL}_{\mathrm{int}})$ values for the ANN model. Solid line represents the line of unity and the dashed lines the ±1 log tolerance value. Closed marks represent drugs that were included in the train set. Open marks are indicative of drugs in the test set. Cross marks represent drugs in the external validation group.

The best neural network presented an architecture of 21-3-2-2-1 and its performance is presented in Table 5 and Fig. 1. The model produced good correlations both in the training and test groups. Additional statistical comparison of the ANN for the training and

Table 5
Statistical evaluation of the performance of the ANN model to predict *in vitro* $\mathrm{CL}_{\mathrm{int}}$ values based on the molecular descriptors for the train and test data.

	RMSE	ME	Correlation	% Within 10-fold difference	% Within 2-fold difference
Train	0.236	−0.001	0.953	100	79
Test	0.544	−0.173	0.804	91	53
Validation	0.747	−0.233	0.315	80	0

Table 6
Statistical comparison between the use of *in vitro* data and the ANN model to predict *in vivo* CL_{int} values. *In vivo* CL_{int} values were determined by using the "well-stirred" model either considering blood binding for all drug classes or just to acid drugs. Group A consisted of 30 drugs from Table 2 with *in vitro* CL_{int} data that were used in the training process of the ANN model. Group B includes the 112 drugs from Table 3 for which only *in silico* CL_{int} were used.

Data set	RMSE	ME	Correlation	% Within 10-fold difference	% Within 2-fold difference
f_B in all drugs					
Group A (*in vitro*)	1.042	−0.838	0.636	57	16
Group B (*in silico*)	1.450	−0.748	0.527	56	19
f_B in acids					
Group A (*in vitro*)	0.643	−0.073	0.579	93	52
Group B (*in silico*)	1.197	−0.249	0.508	63	16

test groups revealed a similar behaviour, either in RMSE (precision of the predicted values) and ME (bias in the predicted values), indicating that overfitting was not significant. Similar values were also obtained when considering the quantitative and qualitative ability of the network. An Y-scrambling analysis was also performed, by randomly permuting the output values in the training data set, and no statistically significant models were achieved (Fig. 2), indicating that the occurrence of chance correlations was not present.

Evaluation of the ANN performance in the external validation group of 5 drugs provided similar RMSE and ME when compared to the test group of data (Table 5). Correlation was not so evident but, considering the data range of the external validation group (0.23–1.51 log CL_{inf} values) and the RMSE, the obtained value is still acceptable. None of the drugs were predicted within the 2-fold difference, but only marginally in the case of 4-methylumbelliferone. When considering the within 10-fold difference acceptance interval, 4 of the drugs were correctly predicted. In the original work (Jacobson et al., 2007), the CL_{int} value for Irbesartan was not able to predict CL_H within a reasonable limit. Because of this, we removed this drug from the external validation group and found that it significantly improved the group statistics to a RMSE = 0.635, ME = −0.020, r = 0.467 and 100% drugs within the 10-fold error difference.

3.2. Model applicability

Pharmacokinetics for a total of 290 drug molecules is described in Goodman et al. (2006). In order to include only drugs with reliable *in vivo* intrinsic clearances, all drugs with the following characteristics were removed prior to analysis: CL/F determination, CL with high variability, liposomal formulations, isomers with different pharmacokinetics, known metabolization by multiple organs, CL data obtained in cancer patients, non-linear elimination pharmacokinetics, pro-drug data, re-conversion of the metabolite, endogenous substances, unreliable pharmacokinetic data or due to impossibility to calculate all the required molecular descriptors. This was done because these facts would impair the correct quantification of *in vivo* CL_{int}. The remaining 142 drugs included 30 drugs (Table 2) presented in the ANN optimization process, used to establish the ability of the *in vitro* hepatocytes suspension to predict the *in vivo* CL_{int} determined by "well-stirred" model and the human *in vivo* CL_H values. This was done by comparing the *in vitro* values in this group to the *in vivo* determined values. The remaining 112 drugs (Table 3), for which only *in silico* predictions were available, were used to evaluate the prediction ability of the ANN model by itself.

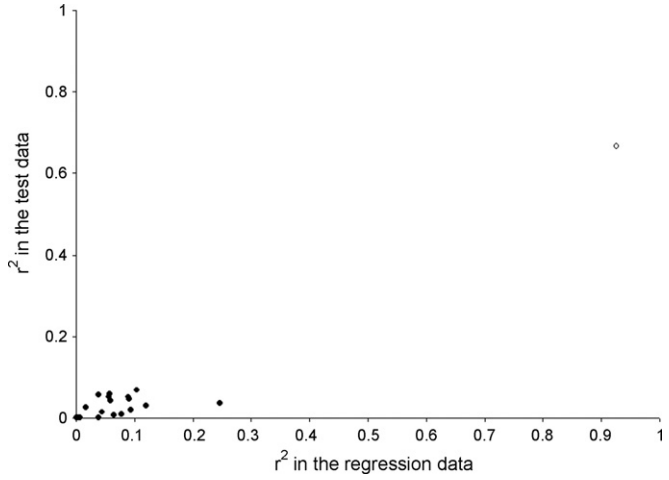

Fig. 2. Evaluation of the occurrence of chance correlations by a y-randomization test. Open mark indicates the real data performance. Close marks indicates the model performance when the *in vitro* CL_{int} values in the train data set were randomly permuted in their position.

The *in vivo* CL_{int} values used in the validation process were calculated by Eq. (3) as described in methods. Since some authors pointed out a better correlation when drug binding is considered only in acidic drugs at pH 7.4, the *in vivo* values were also calculated by considering f_B equal to 1 for the remaining classes of drugs. Statistical performance of these two approaches is presented in Table 6 and Fig. 3. When considering drug binding (Fig. 3a), a significant correlation is obtained, but both the *in vitro* and *in silico* values resulted in underpredictions. A low precision is observed, with only 19% of the *in silico* estimations and 16% of the *in vitro* estimations within a 2-fold difference of the *in vivo* observed value, indicating a poor ability to quantitatively estimate this parameter. In the situation that drug binding is neglected for basic, neutral and zwitterionic drugs (Fig. 3b), a significant correlation is also obtained. A smaller underprediction is observed but again only 16% of the *in silico* determined drugs in the group B are predicted within a 2-fold difference of the *in vivo* observed value. For the *in vitro* data, however, a significant improvement in prediction was observed with 52% of the predictions within a 2-fold difference of the *in vivo* observed value.

When considering a 10-fold difference, the prediction ability is considerably increased in all situations, indicating that the ANN model may be used to qualitatively predict the *in vivo* CL_H. In order to test this hypothesis, both the *in vitro* and the *in silico* CL_{int} values were used to predict the *in vivo* CL_H by using the "well-stirred" model of the liver (Fig. 4). Again, drug binding was either considered to all drugs or just to acidic drugs as previously. A successful match was considered when the predicted value is not different from the observed one within a ±4 ml min^{-1} kg^{-1}, assuming also a 50% error in the *in silico* CL_{int} value. When drug binding is considered, 67% of the *in vitro* based predictions and 69% of the *in silico* based prediction are correctly predicted (Fig. 4a). When drug binding is applied

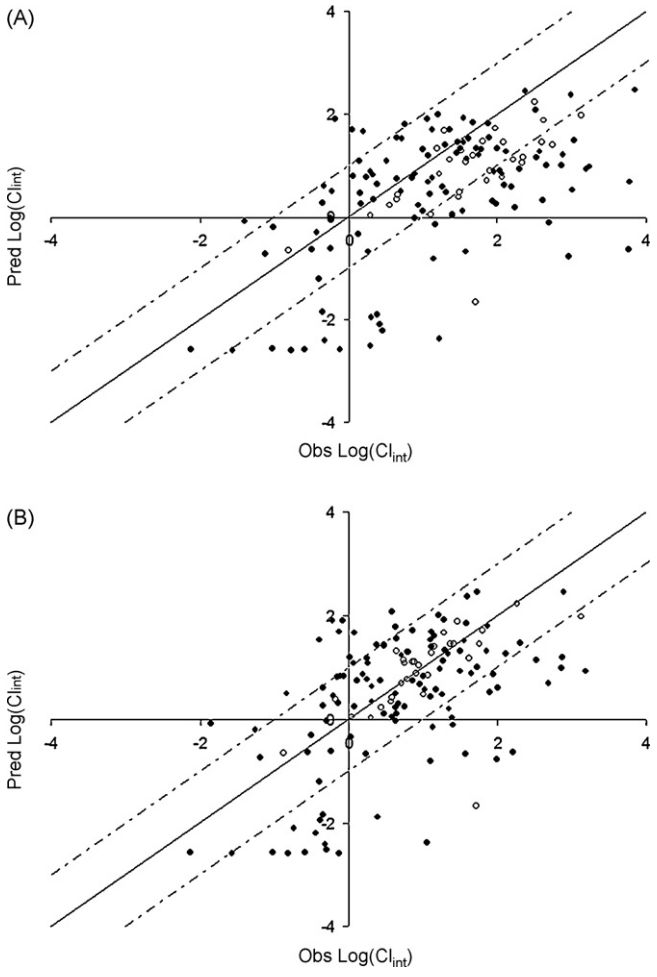

Fig. 3. Plot of the *in vivo* observed vs *in vitro* and *in silico* predicted log(CL_{int}) values for the ANN model in unities of ml min^{-1} kg^{-1}. Solid line represents the line of unity and the dashed lines the ±1 log tolerance value. Open marks are indicative of drugs from Table 2 with *in vitro* CL_{int} data. Closed marks represent drugs from Table 3 with *in silico* CL_{int} data. The *in vivo* observed values were determined by the "well-stirred" model based on the *in vivo* CL_H and including f_B in all drugs (A) or considering f_B only in acid drugs (B).

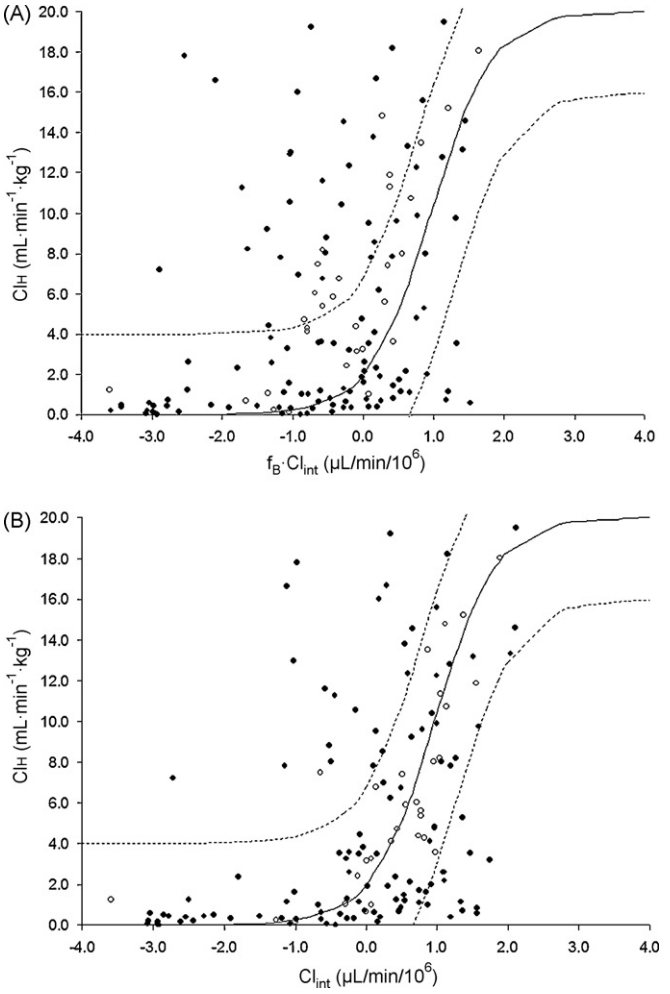

Fig. 4. Plot of the *in vitro* and *in silico* predicted log(CL_{int}) vs *in vivo* observed CL_H values for the ANN model. Solid line represents the predicted CL_H values based in the introduction of the *in vitro* and *in silico* CL_{int} values in the "well-stirred" model considering f_B in all drugs (A) or only in acid drugs (B). The dashed lines represent the tolerance interval. *In vivo* observed CL_H are represented by open circles for the Table 2 drugs were *in vitro* CL_{int} data was used. Closed circles are used for Table 3 drugs were *in silico* CL_{int} data was used. The closer a mark is to the solid line the better the prediction.

to acidic drugs only, 97% of the *in vitro* based predictions and 72% of the *in silico* based prediction are within the acceptance limits (Fig. 4b).

4. Discussion

4.1. Model development

To our knowledge, this is the first attempt to model *in silico* the CL_{int} obtained in suspensions of human hepatocytes. Previous *in silico* works were done based on *in vitro* microssomes (Chang et al., 2008; Ekins, 2003; Ekins and Obach, 2000; Lee et al., 2007; Li et al., 2008), combining *in vitro* and *in vivo* data (Schneider et al., 1999; Zuegge et al., 2001), *in vitro* and *in silico* data (Jolivette and Ward, 2005; Wajima et al., 2002) and relating *in silico* with human *in vivo* pharmacokinetics (Li et al., 2008; Turner et al., 2004).

CL_{int} as determined by using suspensions of isolated hepatocytes presents advantages over other *in vitro* methods. Hepatocytes are intact cells with a complete set of phase I and II metabolizing enzymes that mimic the *in vivo* metabolization of drugs (Gomez-Lechon et al., 2003). With the optimization of the cryopreservation protocols, an increased pool of liver sources is now available, with a minimal loss of metabolic activity (Blanchard et al., 2005; Griffin and Houston, 2004; McGinnity et al., 2004; Naritomi et al., 2003). Due to these facts, it appears to be the most promising tool to predict CL_H in the development phase of new drug entities (Fagerholm, 2007) making it also an ideal target for QSAR models.

This ANN model was trained based in a data set of 71 different drugs, with data from different laboratories. Although similar experimental conditions were considered, some intra-drug variability was observed between laboratories, as observed previously (Nagilla et al., 2006). This variability may have a physiologic meaning (Shibata et al., 2002), and was introduced in the model by including all individual determinations with a weighting related to the number of different determinations per drug. ANN models are prone to overfitting problems. In order to reduce this possibility, we used an early stop procedure, a frequently used approach to minimize data memorization (Cataltepe et al., 1999), with a test group of 18 drugs. Additionally, the ratio between the number of patterns to the number of connections was maintained above 1 as this could

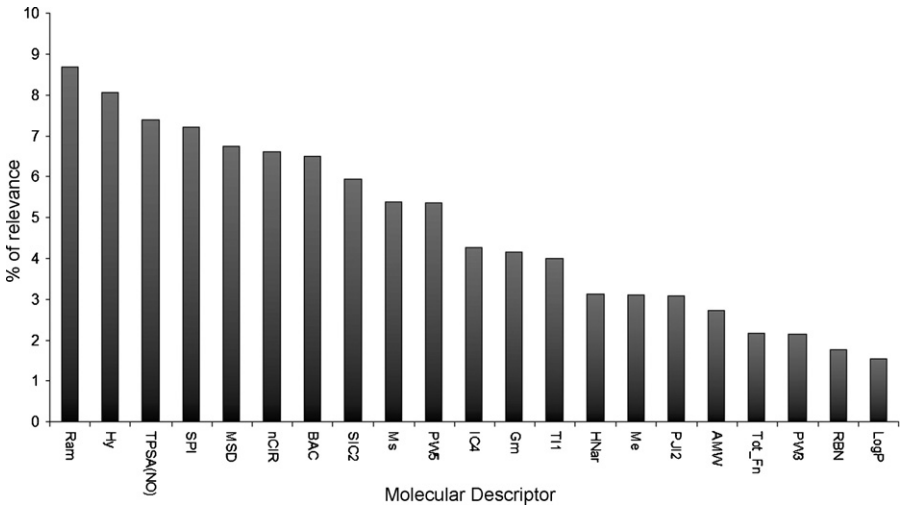

Fig. 5. Relevance of each molecular descriptor in the prediction of CL_{int} by the ANN, obtained by cycling each input for all training patterns and computing the effect on the network's output response at a time.

also reduce the ability of the model to memorise the data (Turner et al., 2004). Comparison of the model performance in the train and test groups resulted in similar statistics which indicates that network training was stopped before overfitting did occur. Evaluation of the model performance in the external validation group of data is also indicative of the model's predictive abilities, and an indication that it may be used to predict the CL_{int} value in new drugs.

4.2. Model inputs

Interpretation of the relationships between the input and output variables are difficult to make when using ANN models. One possible approach is to evaluate the relevance of each input on the final output by cycling each input for all training patterns and computing the effect on the network's output response. The percent contribution of the input descriptors for the output response is presented in Fig. 5. As can be seen, the molecular descriptors encode information related to electronic (Ms, Me, Ram, TPSA(NO), RBN), physical-chemical properties (Hy, log *P*, Tot_Fn), branching, compactness and symmetry (SPI, HNar, SIC2, BAC, Gm, IC4, MSD, TI1), size and shape (AMW, PJI2, PW3, PW5) and aromaticity (nCIR) (Todeschini and Consonni, 2000). The top relevant descriptors also indicate the significance of structural considerations at an atomic and molecular level in determining the metabolic behaviour of a compound. Ram is a σ-electron descriptor and a measure of atom connectedness for acyclic graphs within the molecule (Araujo and De la Pena, 1998). Hy is an empirical index related to the hydrophilicity of the compounds. TPSA(NO) is a descriptor related to the hydrogen-bonding ability of compounds and has been previously used to predict membrane permeability (Linnankoski et al., 2006; Zhao et al., 2007). SPI is a molecular descriptor derived from the H-depleted molecular graph proposed to enhance the role of terminal vertices in QSAR studies and it has been used to predict the antiulcer activity in a set of 128 molecules (Gupta et al., 1999). MSD and BAC are molecular descriptors known to be related to the branching in a structure (Caputo and Cook, 1989). nCIR counts the number of walks with non-repeated paths in the graph representation of the molecule. It is a direct indication of cycle structures in the molecule. SIC2 is a topological information index representing a measure of the structural complexity. Ms is a measure of the average electronic accessibility of the atoms in the molecule and can be interpreted as a probability of interaction with another molecule. PW5 is a molecular shape descriptor and is related to transport phenomena and interaction capabilities between ligands and receptors (Todeschini and Consonni, 2000).

4.3. Model applicability

The applicability of the proposed model was evaluated by testing the ability of the ANN model to predict the *in vivo* intrinsic clearance with the latter being estimated based on the pharmacokinetic values of the drugs. Different classes of drugs with a broad range of CL_H values were included but, since extrahepatic CL is neglected, there is a possibility of overestimation when estimating the CL_H. Use of known drugs with reliable intravenous data and comparison with the E_H estimated by bioavailability may reduce this effect when extremely evident.

The liver model may also influence the estimation of CL_{int}, especially in high CL drugs (Houston and Carlile, 1997) and the "dispersion" and "parallel tube" models usually present the best results (Iwatsubo et al., 1997). However, due to its simplicity and minor differences in the prediction on *in vivo* CL_H, the use of the "well-stirred" model is still often recommended (Ito and Houston, 2004). Use of f_B is also a question of debate. Neglecting this parameter in basic, zwitterionic and neutral drugs seems to improve CL_H estimations both when using microsomes (Obach, 1999) or hepatocytes suspensions (Jacobson et al., 2007; Lau et al., 2002; McGinnity et al., 2004; Reddy et al., 2005).

We tested the predictive ability of the ANN model either by using f_B in all classes of drugs or just in acidic drugs using the "well-stirred" model. Similar correlations were observed when comparing both the *in vitro* and the *in silico* predictions with the *in vivo* CL_{int} observed values either using f_B to all drugs or just for acids, and comparable to previously observed values for *in vitro–in vivo* extrapolations (McGinnity et al., 2004). It has also been proposed to evaluate the performance of a method by determining the percentages of predicted values below a 2-fold error (Obach et al., 1997). When applied to CL_{int} data our results were unsatisfactory either using the *in vitro* or the *in silico* predicted values (Table 6). These values were, however, in accordance with previously described values for *in vitro–in vivo* extrapolation with human hepatocytes (Naritomi et al., 2003; Riley et al., 2005) and reasons for this lack of prediction may be either lower activity of the hepatocytes after cryopreservation or inappropriate *in vivo* CL_{int} estimation

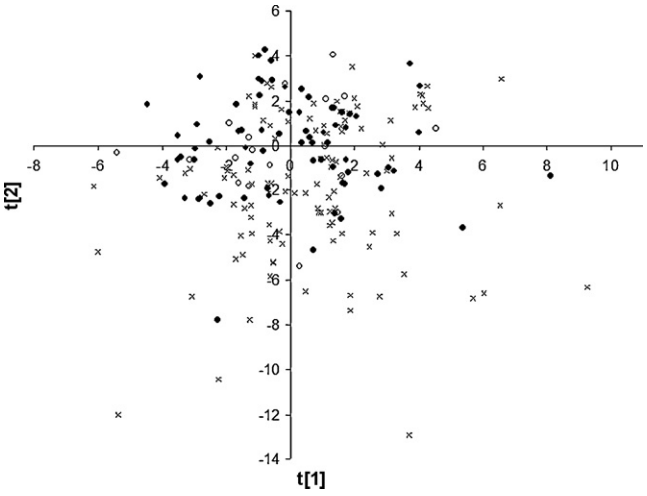

Fig. 6. PCA scores t[1] and t[2] for the train (close marks) and test (open marks) drugs from Table 1. Table 3 drugs, used to test the ANN model applicability, are represented as cross marks. The analysis resulted that the first 2 components explained 50% of the descriptors variance.

methods (Fagerholm, 2007). However, considering the described inter-individual (Shibata et al., 2002) and inter-laboratory (Nagilla et al., 2006) variability of the *in vitro* CL_{int} in various drugs, also visible in data from Table 1, probably the 2-fold error is too restrict. When using a less strict limit of a 10-fold error, previously used in another *in silico* approach (Ekins and Obach, 2000), the final performance improved considerably particularly when including the f_B term only in acids.

Final evaluation of the method was made by establishing the ability to use the *in silico* CL_{int} to predict the *in vivo* CL_H by using the "well-stirred" model of the liver. As can be seen in Fig. 4, overall predictions are good with 69 (Fig. 4A) and 72% (Fig. 4B) well predicted values within the accepted values. Low clearance drugs are generally well predicted either using f_B for all drugs or just for acidic drugs. For high clearance drugs, however, neglecting f_B for basic, neutral and zwitterionic drugs results in better predictions. To mimic the *in vivo* situation, it is assumed in the "well-stirred" model that the distribution within the liver is perfusion rate limited with no diffusion barriers, only unbound drug crosses the cell membrane and occupies the enzyme site, and there is a homogenous distribution of metabolic enzymes in the liver (Houston and Carlile, 1997). Removal of f_B could contradict the model assumptions and was not recommended (Fagerholm, 2007). These are, however, gross approximations of the complex physiology of the liver. Several active drug transporters are present in human hepatocytes, resulting that the unbound intracellular concentration may differ from that in the external medium (Hewitt et al., 2007; Pelkonen and Turpeinen, 2007). Non-specific binding inside the cell is expectably higher in neutral, basic and zwitterionic drugs, and this could cancel the effects of plasma protein binding (Obach, 1999). Lipoprotein uptake in hepatocytes may also promote the entry of basic and neutral hydrophobic drugs, as these are highly bound to these proteins (Ohnishi et al., 2002). There is also evidences of albumin conformational changes when in contact with hepatocytes that may accelerate drug dissociation at the cells surface (Horie et al., 1988). All these facts are of considerable influence when extrapolating from CL_{int} to CL_H, especially in drugs with high CL_H values, but are still prone to investigation. It is also visible that using f_B in all drugs results in a marked underprediction. This underprediction is partially reduced when f_B is considered only for acids. Other reasons for this underprediction may also be: (i) lower metabolic activity of the *in vitro* hepatocytes, (ii) the assumption that Vd in hepatocytes equals that of the physical volume of the cells and/or (iii) overestimation of the *in vivo* CL_H (Fagerholm, 2007).

When considering the use of *in vitro* CL_{int} values to predict the *in vivo* CL_H, similar performance to the *in silico* data was observed with 67% well predicted values within the accepted values (Fig. 4A). Neglecting f_B for basic, neutral and zwitterionic drugs resulted again in significative better predictions with 97% well predicted values within the accepted values (Fig. 4B). In this case, the *in vitro* data presented better performance when compared to the *in silico* based CL_{int} values. When evaluating the data chemical space by means of a principal component analysis (Fig. 6) both train and test data sets, used in the ANN model optimization process, share the same descriptor space. Drugs used in the *in silico* model applicability (Table 3), however, present some molecules that are outside the model molecular space and for which an increased uncertainty of the prediction is expected.

5. Conclusions

In conclusion, we present an *in silico* method to predict the *in vitro* CL_{int} obtained in human hepatocytes suspensions, based only in calculated molecular descriptors. Model applicability in a large group of data not previously used in the model building procedure demonstrated a reasonable ability to predict human *in vivo* CL_H from the *in silico* CL_{int} for a wide range of drugs, only marginally inferior to the performance of the *in vitro* data from which the model was build, with similar correlations and less than the double RMSE. Nevertheless, for optimal use, the model predictive performance should be improved possibly by including more drugs, when available, in the ANN train process. This model may be a valuable tool in early drug discovery by providing a relevant pharmacokinetic parameter. Additionally, by estimating an *in vitro* determined parameter, it is also prone for easy confirmation which is fundamental in the drug development process in order to reduce costs and utilization of large animal data.

Acknowledgment

This work was partially supported by project number SFRH/BD/28545/2006 from Fundação para a Ciência e a Tecnologia.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2009.12.007.

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