Punt et al.:

Predictive Performance of Next Generation Human Physiologically Based Kinetic (PBK) Model Predictions Based on *In Vitro* and *In Silico* Input Data

Supplementary Data

Supplementary methods

Chemicals

Adenosine 3'-phosphate 5'-phosphosulfate lithium salt hydrate (PAPS; CAS 109434-21-1), antipyrine (CAS 60-80-0), bisphenol A (CAS 80-05-7), bosentan hydrate (CAS 157212-55-0), buspirone (CAS 36505-84-7), caffeine (CAS 58-08-2), coumarin (CAS 91-64-5), curcumin (CAS 458-37-7), daidzein (CAS 486-66-8), dipotassium hydrogenphosphate (CAS 7758-11-4), genistein (CAS 446-72-0), L-ascorbic acid (CAS 50-81-7), lidocaine (CAS 137-58-6), metoprolol tartrate (CAS 56392-17-7), ochratoxin A (CAS 303-47-9), omeprazole (CAS 73590-58-6), potassium dihydrogen phosphate (CAS 7778-77-0), nifedipine (CAS 21829-25-4), prazosin hydrochloride (CAS 19216-56-9), prednisolone (CAS 50-24-8), quinidine (CAS 56-54-2), resveratrol (CAS 501-36-0), tolbutamide (CAS 64-77-7) and uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA; CAS 63700-19-6), verapamil hydrochloride (CAS 52-53-9) and warfarin (CAS 81-81-2) were purchased from Sigma-Aldrich (Steinheim, Germany). Dimethyl sulfoxide (DMSO; CAS 67-68-5) was purchased from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Magnesium chloride hexahydrate was purchased from Merck (Darmstadt, Germany). Clozapine (CAS 5786-21-0), diltiazem hydrochloride (CAS 33286-22-5), disopyramide (CAS 3737-09-5), fluvastatin sodium (CAS 93957-55-2), imipramine hydrochloride (CAS 50-49-7), midazolam (CAS 59467-70-8), naloxone (CAS 465-65-6), propranolol (CAS 525-66-6), rosuvastatin calcium (CAS 147098-20-2) and timolol (CAS 26839-75-8) were purchased from European Pharmacopoeia Reference (Strasbourg, France). Dextromethorphan (CAS 125-71-3) was purchased from United States Pharmacopeia Reference (Rockville, USA). Diazepam (CAS 439-14-5) was purchased from Duchefa (Haarlem, The Netherlands). Nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH; CAS 2646-71-1) was purchased from Roche Diagnostics (Mannheim, Germany). Imazalil (CAS 35554-44-0) was purchased from HPC chemicals. Diclofenac (CAS 15307-86-5) was purchased from Fluka and sildenafil (CAS 139755-83-2) was purchased from Cerilliant (Round Rock, USA).

Caco-2 permeability studies

For 30 out of the 44 model compounds of the study, Caco-2 Papp values were obtained from *in vitro* Caco-2 transwell experiments (Tab. S1). For 11 of these compounds the data were obtained from Punt et al. (2022). For the remaining 19 compounds Caco-2 P_{app} values were measured in the current study. For the remaining 30 compounds, Caco-2 P_{app} values were measured in the current study. For these experiments, Caco-2 cells (ATCC, Manassas, VA, USA; passage 30-41) were cultured in DMEM (Gibco, Life technologies; New York, USA) containing 4.5 g/L D-glucose, L-glutamine, and supplemented with 10% (v/v) FBS (Gibco, Life Technologies, New York, USA), 1% (v/v) minimal essential medium non-essential amino acids (Gibco, Life technologies; New York, USA), and 10,000 U/mL penicillin and 10 mg/mL streptomycin (Sigma-Aldrich, Steinheim, Germany). Cells were seeded at a density of 4.0 x 10^4 cells/ cm^2 onto 12-well transwell inserts containing a polycarbonate membrane (12 mm insert, 0.4 µm pore size, Corning Incorporated, New York, USA). The seeded cells were maintained for 21-22 days in a 5% CO_2-humidified atmosphere at 37°C during which the medium in the apical and basolateral compartments (0.5 and 1.5 mL, respectively) was changed every 2 or 3 days and always 1 day before exposure.

The procedure of the transport experiments is based on the procedure described in Hubatsch et al. (2007). Prior to the start of the transport experiment, the cell culture medium was removed and the cells were equilibrated in HBSS without phenol red (pH 7.4, Sigma, Steinheim, Germany) supplemented with 25 mM HEPES (Sigma, Steinheim, Germany) and 0.35 g/L $NaHCO_3$ (Sigma, Steinheim, Germany) at 37°C for 30-45 min. The test compounds were diluted to a final concentration of 10 µM in the same pre-warmed HBSS buffer (donor solution), with a final DMSO level of 0.2%. In apical-to-basolateral experiments, 0.45 mL donor solution was added to the apical compartment, followed by 1.2 mL pre-warmed HBSS to the basolateral compartment. Samples of 50 µL were taken immediately from the apical compartment at 0 min and placed on ice. After 15 min, 50 µL was sampled from the basolateral compartment and the volume was replaced with the same amount of pre-warmed HBSS to keep the compartment volume constant. 50 µL sample was taken from both the apical and the basolateral compartment after 30 min and stored on ice. In basolateral-to-apical experiments, 1.25 mL donor solution was added to the basolateral

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compartment, followed by 0.4 mL HBSS to the apical compartment. A sample of 50 µL was taken immediately from the basolateral compartment at 0 min and placed on ice. After 15 min, 50 µL was sampled from the apical compartment and the volume was replaced by the same amount of pre-warmed HBSS. After 30 min, 50 µL sample was taken from both the apical and the basolateral compartment and stored on ice. Cells were kept in a 5% CO_2-humidified atmosphere at 37°C during the transport experiment. Samples were analysed by LC-MS as described in the LC-MS analysis section in this appendix.

Permeability coefficient P_{app} was calculated according to: $P_{app} = (dQ/dt)(1/(AC_0))$, where dQ/dt is the steady-state flux (µmol/s), A is the surface area of the insert membrane and C_0 is the initial concentration in the donor compartment (µM). The P_{app} values obtained were scaled in the PBK model as described by equations 1-4 in the Materials and Methods section of this study.

Tab. S1: Compounds for which the Caco-2 permeability was obtained

Antipyrine
Bisphenol A
Buspirone
Caffeine
Clozapine
Curcumin
Dextromethorphan
Diazepam
Diclofenac
Diltiazem
Disopyramide
Fluvastatin
Genistein
Imipramine
Metoprolol
Midazolam
Naloxone
Nifedipine
Ochratoxin A
Omeprazole
Prazosin
Prednisolone
Propranolol
Quinidine
Rosuvastatin
Sildenafil
Timolol
Tolbutamide
Verapamil
Warfarin

Human liver S9 clearance

To newly generate S9 clearance data, stock solutions of 19 compounds (Tab. S2) of 1 mM were prepared in DMSO (Mallinckrodt Baker B.V., Deventer, The Netherlands) and further diluted to 100 µM in 100 mM potassium phosphate buffer (Sigma-Aldrich, Steinheim, Germany) with 5 mM magnesium chloride (Sigma-Aldrich, Steinheim, Germany) (pH 7.4). The final incubations contained 1 µM substrate (0.1% DMSO) in 100 mM potassium phosphate buffer with 5 mM magnesium chloride (pH 7.4), enriched with 0.025 mg/mL alamethicin (Sigma-Aldrich, Steinheim, Germany), human liver S9 and 1 mM L-ascorbic acid (Sigma-Aldrich, Steinheim, Germany) to increase the stability of the substrate (see Tab. S2 for the optimized liver S9 concentrations for each chemical).

After 5 min of pre-incubation, the reaction was started by adding a mix of three cofactors: 3 mM NADPH, 5 mM UDPGA and 0.2 mM PAPS to allow both phase I and phase II reactions to take place. All (pre-)incubations were carried out in Eppendorf tubes (Safe-Lock 1.5 mL, Eppendorf) in a shaking incubator (300 rpm) at 37°C (Eppendorf Thermomixer C). The final reaction volume was 100 µL. Reactions were stopped by adding 100 µL ice cold methanol after 0, 5, 10, 20, 40 or 60 min. Samples were vortexed, put on ice, and stored at -20°C. Two types of controls were included: incubations without human liver S9 fractions and incubations without the cofactor mix. To determine suitable S9 concentrations, a pilot study was executed first. Compounds with three S9 concentrations (0.1, 1 and 2 mg/mL) were incubated for 60 min. When there was no substrate depletion after 60 min, metabolic clearance was considered zero and a study with the other time points was not included.

The incubations were measured with LC-MS analysis to quantify the (parent) compounds in the incubations. To that end, samples were thawed and centrifuged at 14,000 rpm at room temperature for 10 min. Supernatant was transferred to glass insert vials suitable for LC-MS/MS injection (BGB Analytik Benelux B.V., Harderwijk, The Netherlands). More details on the LC-MS analysis can be found in the LC-MS analysis section in this document. A total of four replicates of the incubations were carried out on two independent days (two replicates per day).

CLint values were determined by plotting the natural logarithm (ln) of substrate concentrations against time. The slope of the linear part of these ln-transformed substrate depletion curves represents the elimination rate constant (k, min^{-1}). After calculation of the half-life of each compound ($t_{1/2}$ (min) = ln(2)/k (min^{-1})) and incubation volume (V (µL/mg) = 1000 / [liver S9] (mg/mL), CLint was calculated by: CLint (µL/min/mg protein) = V (µL/mg) * ln(2)/$t_{1/2}$ (min).

Tab. S2: Studied compounds in human liver S9 clearance studies with corresponding S9 concentrations

compound	[human liver S9] (mg/mL)
Bisphenol A	0.5
Caffeine	4
Coumarin	0.5
Curcumin	0.1
Dextromethorphan	1
Diazepam	4
Diclofenac	0.125
Fluvastatin	3
Genistein	0.5
Metoprolol	4
Midazolam	0.5
Naloxone	1
Ochratoxin A	2
Propranolol	1
Resveratrol	0.5
Rosuvastatin	3
Tolbutamide	2
Verapamil	0.25

LC-MS analysis

Samples from the Caco-2 permeability and liver S9 clearance experiments were analysed on a Waters Acquity I UPLC (Milford, USA) system. The system was equipped with a Waters Acquity UPLC BEH C18 (100 x 2.1 mm, 1.7 µm) column. The column heater was kept at 60°C and the temperature of the autosampler was kept at 10°C. Bisphenol A, daidzein, genistein and resveratrol were eluted from the column using a gradient of 0.05% ammonia in water (A) and 0.05% ammonia in acetonitrile/water (90:10%) (B). The gradient started at 0% B, was kept at 0% B for 2 min, and then linearly increased to 50% B in 1 min. After 1 min 50% B, the gradient linearly increased to 100% B in 2 min and was kept at 100% B for another 2 min. The gradient decreased to 0% B in 0.5 min and was kept at this condition for 2.5 min.

For all the remaining compounds, mobile phase A was water and B was 95% methanol; both contained 1 mM ammonium formate and 0.1 % formic acid. The gradient started at 0% B, was kept at 0% B for 1 min, was then linearly increased to 50% B in 2 min and was kept at 50% B for 1 min. Then, the gradient linearly increased to 100% B in 2 min (for curcumin and ochratoxin A: in 5 min) and was kept at 100% B for 2 min (for curcumin and ochratoxin A: for 1 min). The gradient returned to 0% B in 0.5 min and was kept at this condition for 2.5 min, followed by the next injection. The injection volume of all samples was 5 µL.

Mass spectrometric detection was performed with a Micromass Quattro Ultima mass spectrometer (Waters, Milford, USA), which was equipped with an electrospray ionization interface (ESI). A capillary voltage of 2.50 kV, a source temperature of 120°C, a desolvation temperature of 350°C, a cone gas flow of 194 L/h, and a desolvation gas flow of 564 L/h was used. Argon was used as collision-induced dissociation gas. Cone voltage and collision energy were optimized by direct infusion for each compound. Specific ion mode, cone voltage, collision energy, and mass charge (m/z) transitions for each compound are described in Table S3.

Calibration curves from the liver S9 studies were prepared in 100 mM potassium phosphate buffer with 5 mM magnesium chloride enriched with the same liver S9 concentration and methanol:buffer ratio as used in the incubations. Calibration samples were analysed before and after analysis of the incubation samples. Standards for the Caco-2 permeability studies were prepared in HBSS with 25 mM HEPES and 0.35 g/L $NaHCO_3$.

Tab. S3: Settings of Micromass Quattro Ultima mass spectrometer

compound	CAS	ion mode	cone (V)	m/z parent	m/z daughter	collision energy (eV)
Antipyrine	60-80-0	ESI+	20	189.07	56.47	30
					77.45	40
					189.07	30
Bisphenol A	80-05-7	ESI-	3030	227.45	212.39	25
					133.32	15
Bosentan	147536-97-8	ESI+	30	552.51	205.18	30
					246.43	20
Buspirone	36505-84-7	ESI+	30	386.22	122.28	30
					222.27	30
Caffeine	58-08-2	ESI+	30	195	110	23
					138	18
Clozapine	5786-21-0	ESI+	30	327.12	270.21	20
					296.47	30
					192.45	35
Coumarin	91-64-5	ESI+	30	147	65	22
					91	21
Curcumin	458-37-7	ESI+	25	369.47	145.33	30
					177.13	20
					285.19	15
Daidzein	486-66-8	ESI-	20	253.37	194.5	30
					209.19	30
					224.46	30
Dextromethorphan	125-71-3	ESI+	30	272.3	147	30
					171	40
Diazepam	439-14-5	ESI+	30	285	154	30
					193	30
Diclofenac	15307-86-5	ESI+	30	296	214	30
					151	30
					215	30
Diltiazem	42399-41-7	ESI+	30	415.28	178.39	30
Disopyramide	3737-09-5	ESI+	30	340.18	239.39	20
					195.39	35
					194.45	35
Fluvastatin	93957-54-1	ESI+	15	411.9	266	30
					224	30
Genistein	446-72-0	ESI-	30	269.41	133.16	30
Imipramine	50-49-7	ESI+	30	281.3	58.68	40
					86.54	20
					208.51	30
Lidocaine	137-58-6	ESI+	30	235.32	58.49	30
					86.36	20
Metoprolol	51384-51-1	ESI+	30	268.14	133.18	25
					116.19	20
Midazolam	59467-70-8	ESI+	30	326	209	30
					223	35
					291	25
Naloxone	465-65-6	ESI+	30	328.27	212.24	35
Nifedipine	21829-25-4	ESI+	30	347.22	210.88	20
Ochratoxin A	303-47-9	ESI+	20	404.4	239.15	25
					358.44	15
Omeprazole	73590-58-6	ESI+	30	346	198.1	20
Prazosin	19216-56-9	ESI+	30	384.1	247.1	20
Prednisolone	50-24-8	ESI+	30	361	147	25
Propranolol	525-66-6	ESI+	30	260	116	30

Quinidine	56-54-2	ESI+	30	325.19	160.07	25
					117.05	25
					172.02	25
Resveratrol	501-36-0	ESI-	25	227.39	143.41	30
					185.31	25
Rosuvastatin	287714-41-4	ESI+	20	482.44	258.09	30
Sildenafil	139755-83-2	ESI+	30	475.21	99.51	30
					100.39	30
					100.58	30
Timolol	26839-75-8	ESI+	30	317.2	261	20
Tolbutamide	64-77-7	ESI+	30	271	91	25
					155	20
					172	30
Verapamil	52-53-9	ESI+	30	455.4	150.1	25
					165.1	25
Warfarin	81-81-2	ESI-	30	307.11	161.04	20
					250.06	20

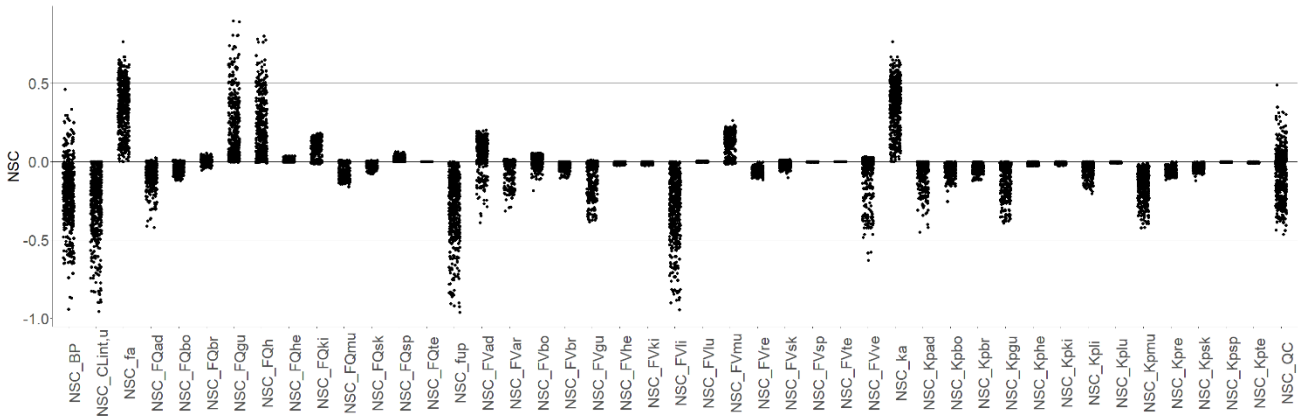

Fig. S1: Normalized sensitivity coefficients (NSCs) of the C_{max} predictions to different input parameters for the different compounds

The datapoints in the figures correspond to the NSCs for a random selection of 12 C_{max} simulations based on different input approaches per chemical. BP, blood plasma ratio; CLint,u, unbound intrinsic liver clearance; fa, fraction absorbed; FQ[tissue], fraction of the blood flow to a specific tissue; fu_p, fraction unbound in plasma; FV[tissue], volume fraction of a specific tissue; ka, intestinal uptake rate; Kp[tissue], plasma partition coefficient of a specific tissue; QC, cardiac output; [tissue]: ad (adipose), bo (bone), br (brain), gu (gut), h (hepatic), he (heart), ki (kidney), mu (muscle), sk (skin), sp (spleen), or te (gonads).

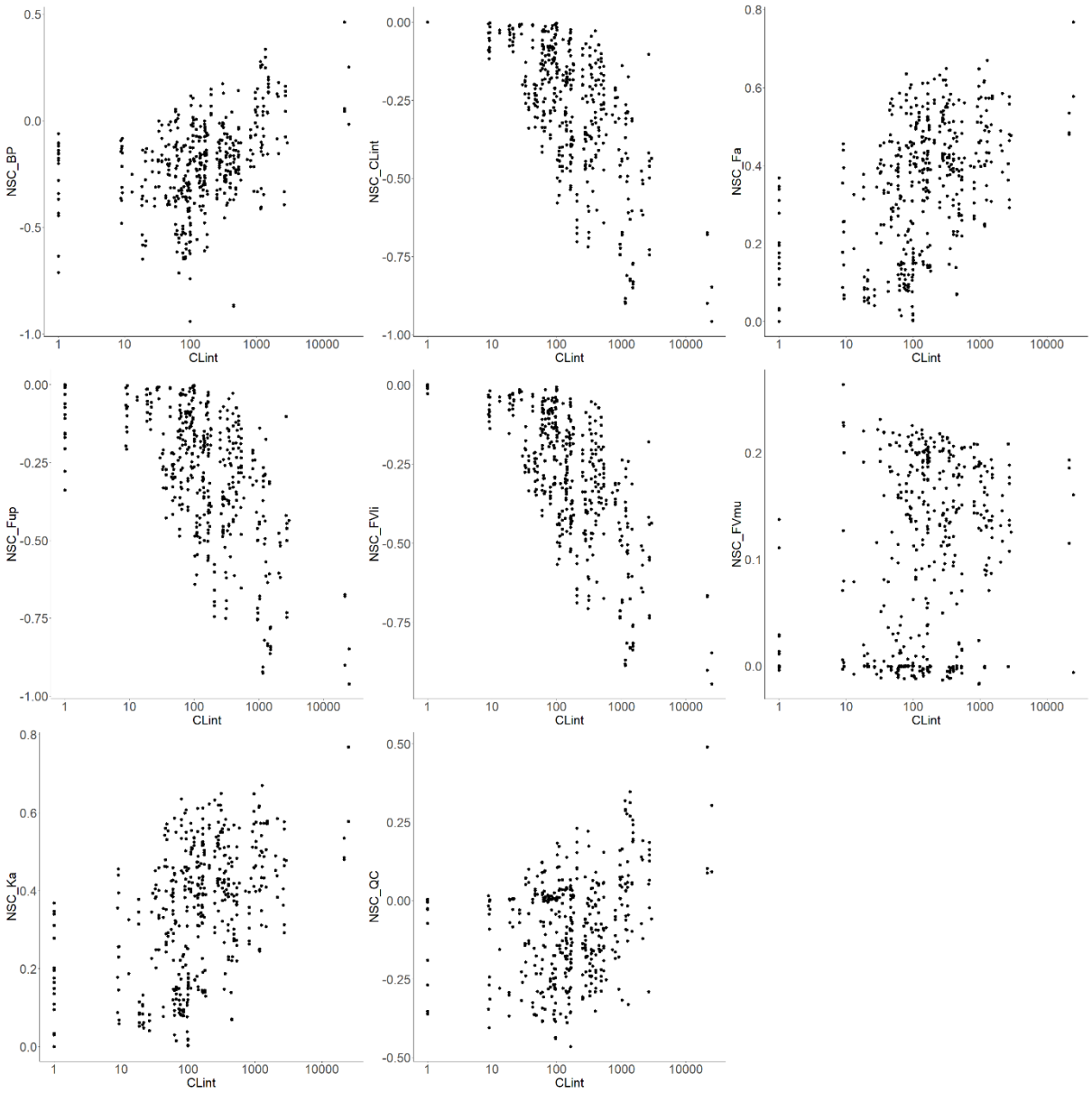

Fig. S2: Correlations between the extent of CLint,u and normalized sensitivity coefficients (NSCs) for different parameters

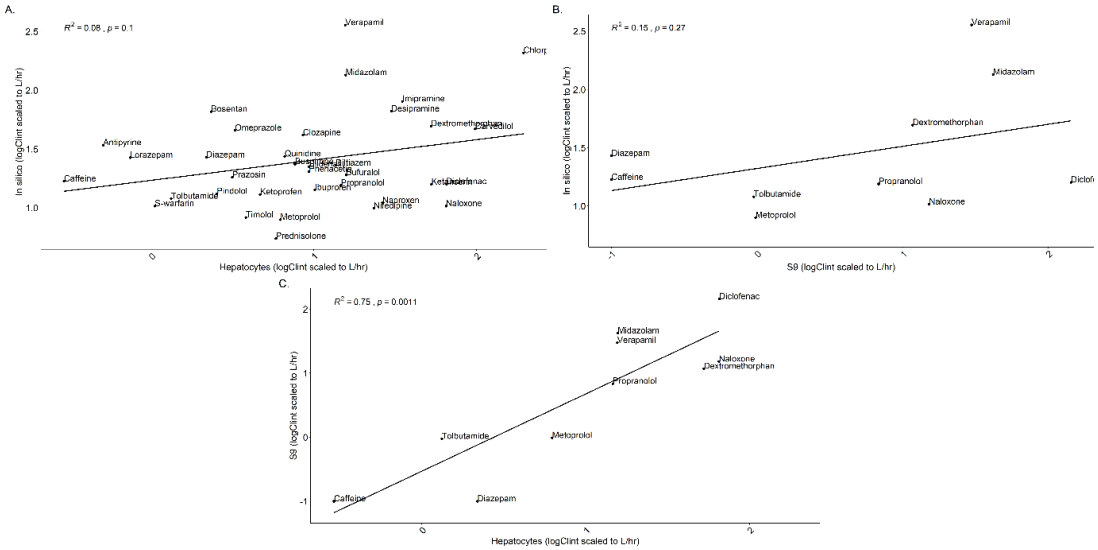

Fig. S3: Correlations between *in vitro* (hepatocytes or S9) and *in silico* calculated intrinsic hepatic clearance values

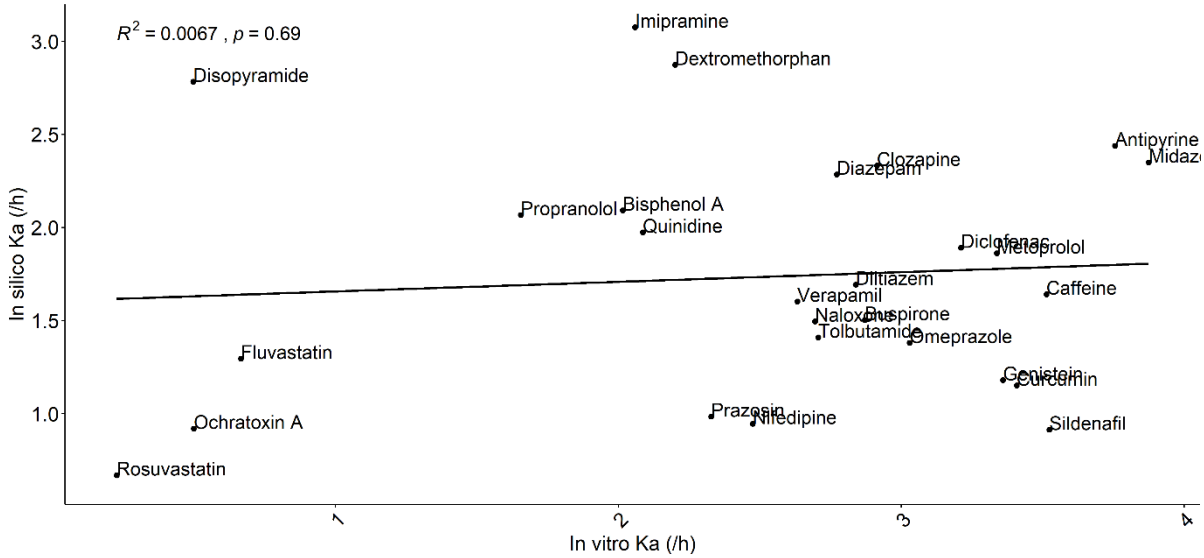

Fig. S4: Correlation between the *in vitro* Caco-2 permeability results and the *in silico* calculated P_{app} values

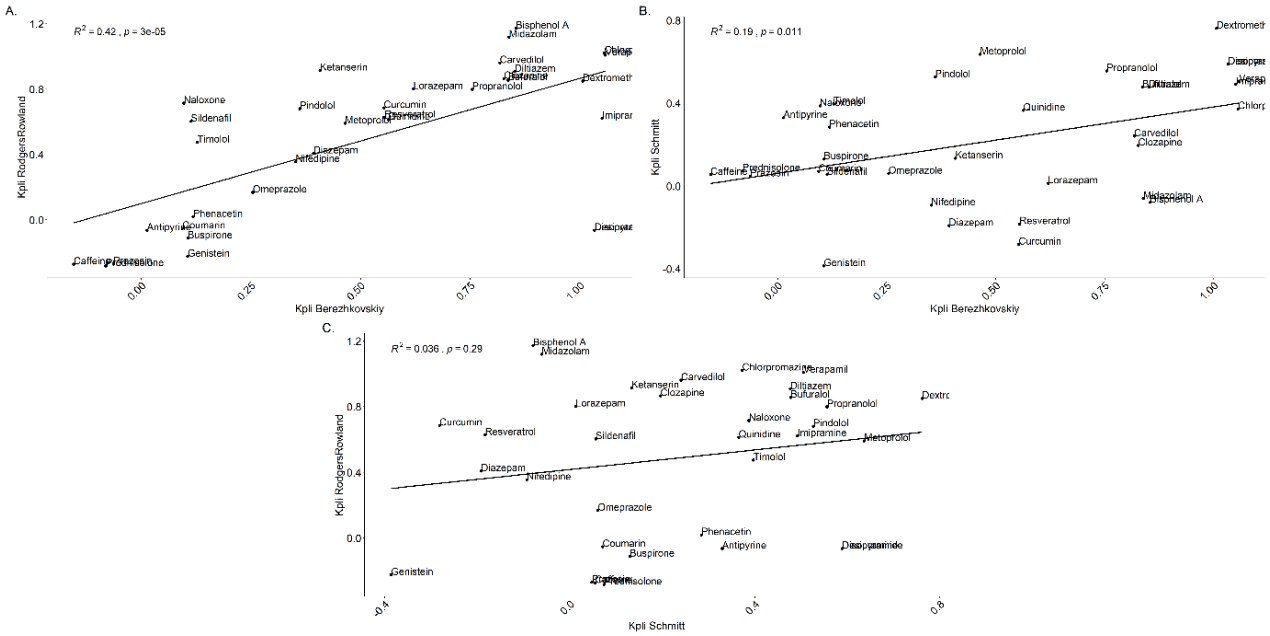

Fig. S5: Correlations between predicted Kpli with the different calculation methods

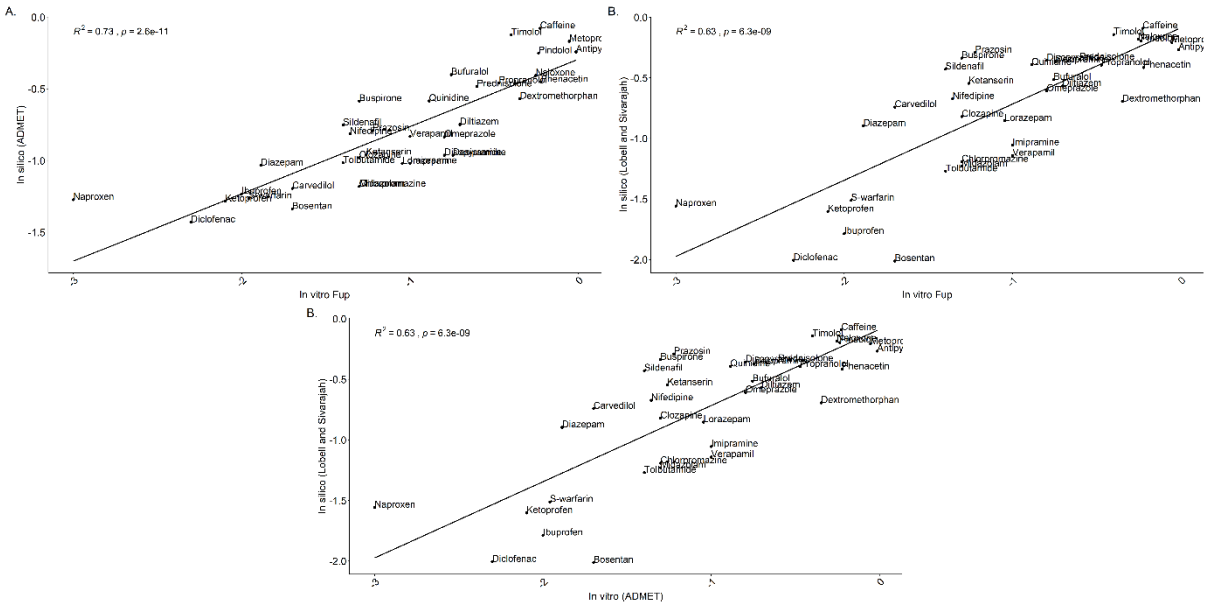

Fig. S6: Correlation between the *in vitro* and *in silico* calculated fraction unbound in plasma

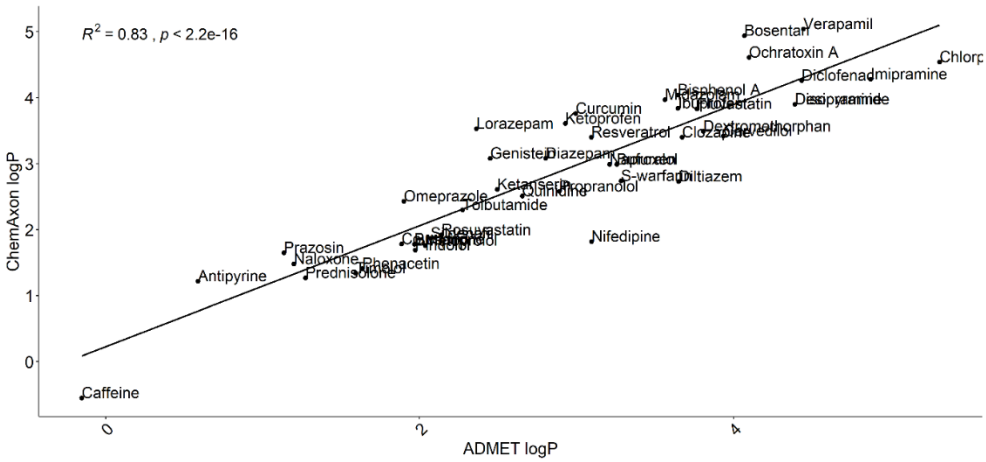

Fig. S7: Correlation between the logP values calculated with ChemAxon and ADMET Predictor

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