Supplementary Material

Experimental exposure assessment for *in vitro* cell-based bioassays in 96- and 384-well plates

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S1: Test chemicals

Text S1: pK_a measurement.

The acidity constants (pK_a) of six of the test chemicals were measured using a Sirius T3 titrator (Pion). A detailed description of the experimental procedure can be found in the literature (Niu et al, 2022). Briefly, 5 µL of a 10 mM DMSO stock solution of each test chemical and 25 µL of a phosphate buffer (14.4 mM K_2HPO_4 and 0.15 M KCl) were added to a Sirius T3 test vial. A reference vial containing 5 µL DMSO (Roth, A994-100 ML) and 25 µL phosphate buffer was measured with each sample. The pK_a measurement was performed using the automated UV-metric pK_a protocol of the Sirius T3 Control software (version 2.0.0.0.). Three sequential titrations were performed with the same sample and a constant ionic strength of 0.15 M KCl ranging from pH 2 to 12 by adding 0.5 M HCl or 0.5 M KOH while UV absorbance was measured. Data analysis was performed using Sirius T3 Refine software (version 2.0.0.0.). All pK_a values were reported as the mean of the three titrations (or six for 2,4-D). The pK_a of ibuprofen was measured in the presence of 25 – 52 % methanol with 0.15 M KCl. The measured pK_a was extrapolated to the pK_a at 0 % methanol with Yasuda-Shedlovsky extrapolation (Shedlovsky, 1962; Yasuda, 1959).

Table S1: Test chemicals, their chemical class, CAS number, purity, supplier and SPME desorption solution.

Chemical	***Chemical class***	***CAS***	***Purity***	***Supplier***	***SPME desorption solution***
caffeine	neutral	58-08-2	≥99%	Sigma-Aldrich	10/90 ACN/H_2O
lamotrigine	base	84057-84-1	≥98%	Cayman Chemical Company	50/50 ACN/H_2O
diclofenac sodium	acid	15307-79-6	≥99%	Cayman Chemical Company	90/10 ACN/H_2O
2,4-dichloro-phenoxyacetic acid (2,4-D)	acid	94-75-7	≥97%	Cayman Chemical Company	50/50 MeOH/H_2O
(*S*)-naproxen	acid	22204-53-1	≥99%	Cayman Chemical Company	50/50 MeOH/H_2O
ibuprofen	acid	15687-27-1	≥99.6%	Euro OTC Pharma	50/50 MeOH/H_2O
torasemide	acid	56211-40-6	≥98%	Sigma-Aldrich	50/50 MeOH/H_2O
warfarin	acid	81-81-2	≥99%	Sigma-Aldrich	50/50 MeOH/H_2O
telmisartan	multifunctional	144701-48-4	≥98%	Cayman Chemical Company	90/10 ACN/H_2O

S2: Instrumental analysis

Table S2: LC parameters for the test chemicals used in this study. The eluents used are composed as follows: A) 5 % acetonitrile and 95 % water, B) 100 % acetonitrile, C) 5 % acetonitrile and 95 % water with 0.1 % formic acid, D) 95 % acetonitrile and 5 % water with 0.1 % formic acid.

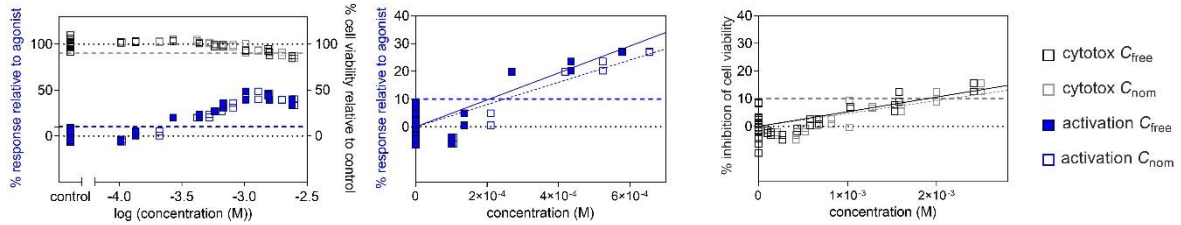
Chemical	***Column***	***Eluent***	***Retention time [min]***	***Flow rate [mL/min]***	***Injection volume [µL]***
caffeine	Phenomenex Luna Omega Polar C18 1.6 µm 50 × 2.1 mm, 25°C	95 % A 5 % B	1.75	0.5	1
lamotrigine	BioZen peptide PS-C18 1.6 µm 50 × 2.1 mm, 25°C	90 % C 10 % D	0.79	0.5	1
diclofenac	Phenomenex Kinetex C18 1.7 µm 50 × 2.1 mm, 30°C	50 % C 50 % D	1.27	0.5	1
2,4-D	Phenomenex Kinetex C18 1.7 µm 50 × 2.1 mm, 40°C	60 % C 40 % D	0.96	0.5	1
naproxen	Phenomenex Kinetex C18 1.7 µm 50 × 2.1 mm, 40°C	55 % C 45 % D	0.83	0.5	PBS: 2 Desorption solution: 15
ibuprofen	Phenomenex Kinetex C18 1.7 µm 50 × 2.1 mm, 40°C	45 % C 55 % D	0.98	0.5	5
torasemide	Phenomenex Kinetex C18 1.7 µm 50 × 2.1 mm, 40°C	75 % C 25 % D	0.95	0.5	1
warfarin	Phenomenex Kinetex C18 1.7 µm 50 × 2.1 mm, 30°C	55 % C 45 % D	1.2	0.5	1
telmisartan	Phenomenex Kinetex C18 1.7 µm 50 × 2.1 mm, 40°C	90 % C 10 % D	0.82	0.5	1

Table S3: MS parameters for the test chemicals used in this study.

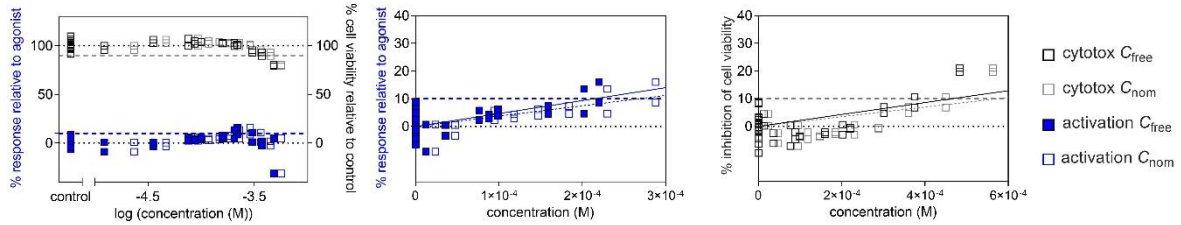
Chemical	***Ion source***	***Frag-mentor voltage [V]***	***MRM transitions (Collision energy [V])***	***Source parameters: capillary voltage [V]; gas flow [ml/min]; gas temperature [°C]; nebulizer [psi]***
caffeine	ESI+	110	195.2 → 138/110.1 (17/25)	5000; 13; 310; 60
lamotrigine	ESI+	150	256 → 211/145 (26/45)	3000; 13; 290; 50
diclofenac	ESI-	80	294 → 249.8/213.9 (9/21)	2000; 13; 350; 25
2,4-D	ESI-	80	218.96 → 160.9/124.9 (12/28)	2000; 13; 350; 25
naproxen	ESI-	80	229.08 → 185/168.9 (1/33)	5000; 13; 290; 25
ibuprofen	ESI-	80	205.12 → 161.1 (4)	4000; 13; 320; 25
torasemide	ESI+	120	349.14 → 263.9/183 (12/36)	2000; 13; 350; 50
warfarin	ESI+	120	309.12 → 162.9/250.9 (12/16)	4500; 13; 350; 30
telmisartan	ESI+	230	515 → 497/276 (38/40)	2000; 8; 350; 60

S3: Bioassay results

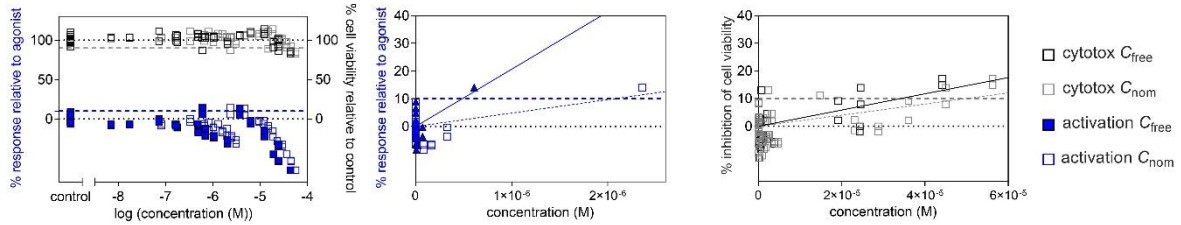
Caffeine



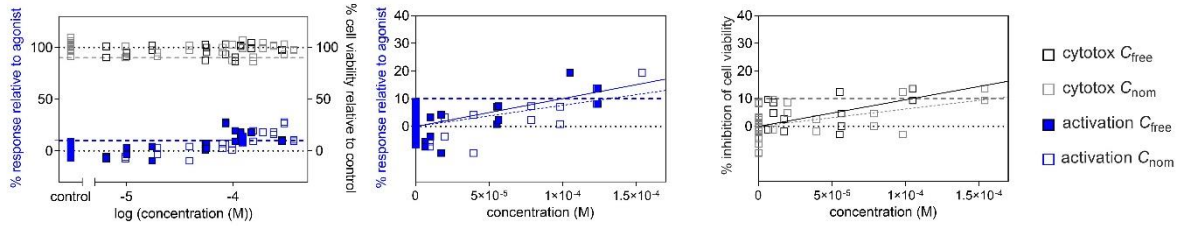
Lamotrigine



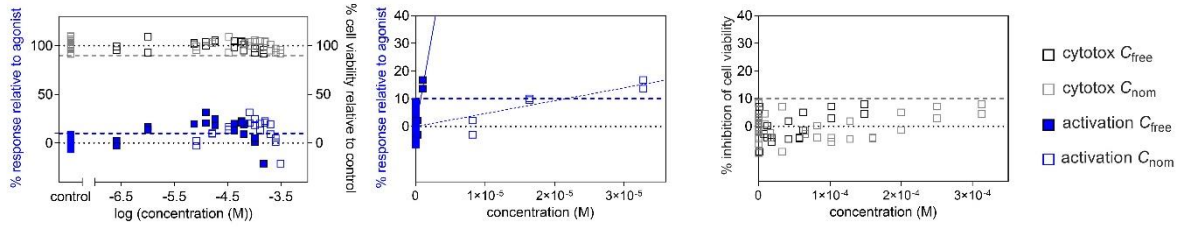
Diclofenac



2,4-D



Naproxen



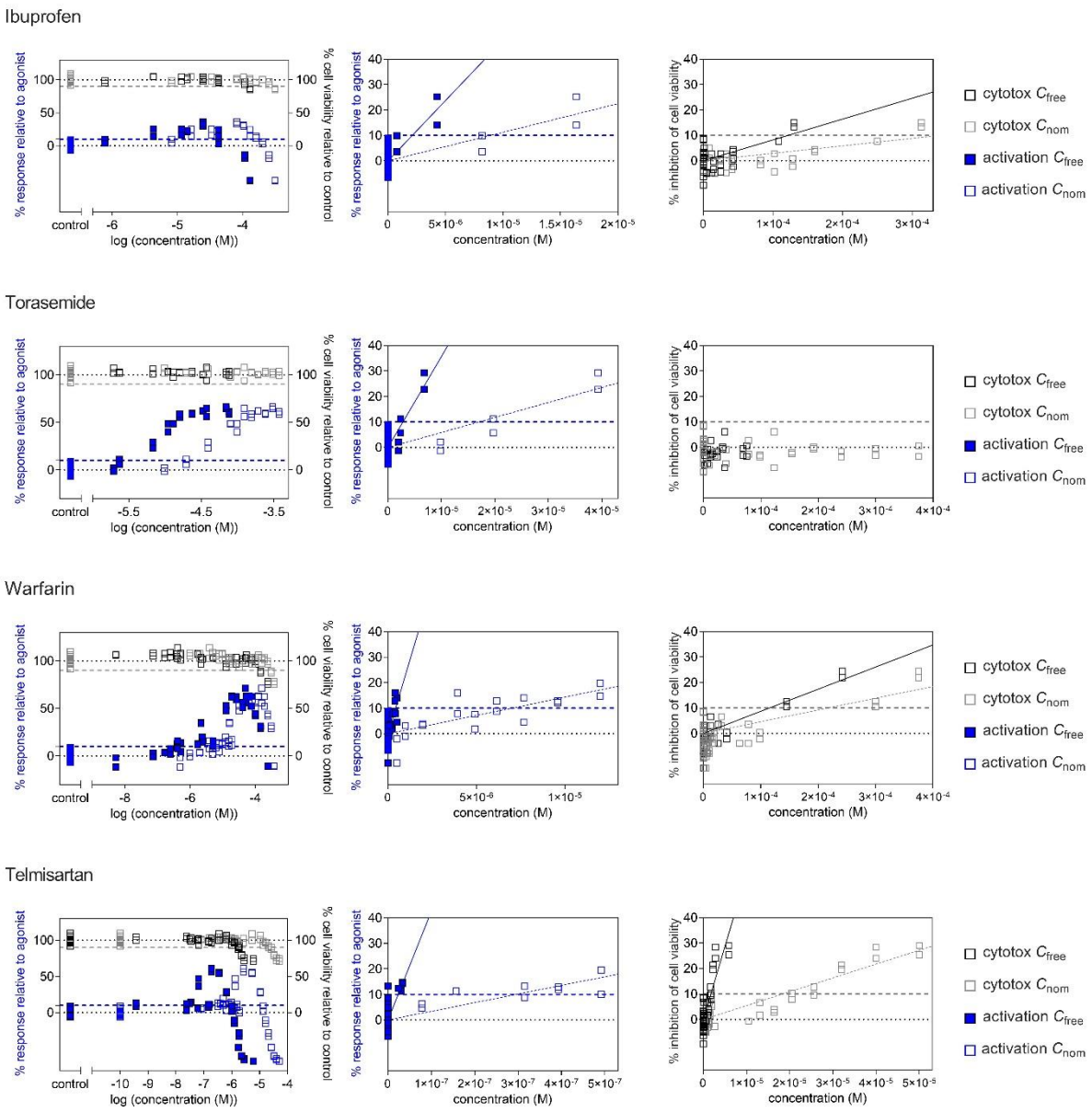


Figure S1: Nominal and freely dissolved concentration-response curves of all chemicals obtained from 384-well plates. [Left] Full concentration-response curve for cell viability and induction of the PPARγ. [Middle] Linear range of the concentration-response curve for the induction of the PPARγ at low concentration levels. [Right] Linear range of the concentration-response curve for cytotoxicity

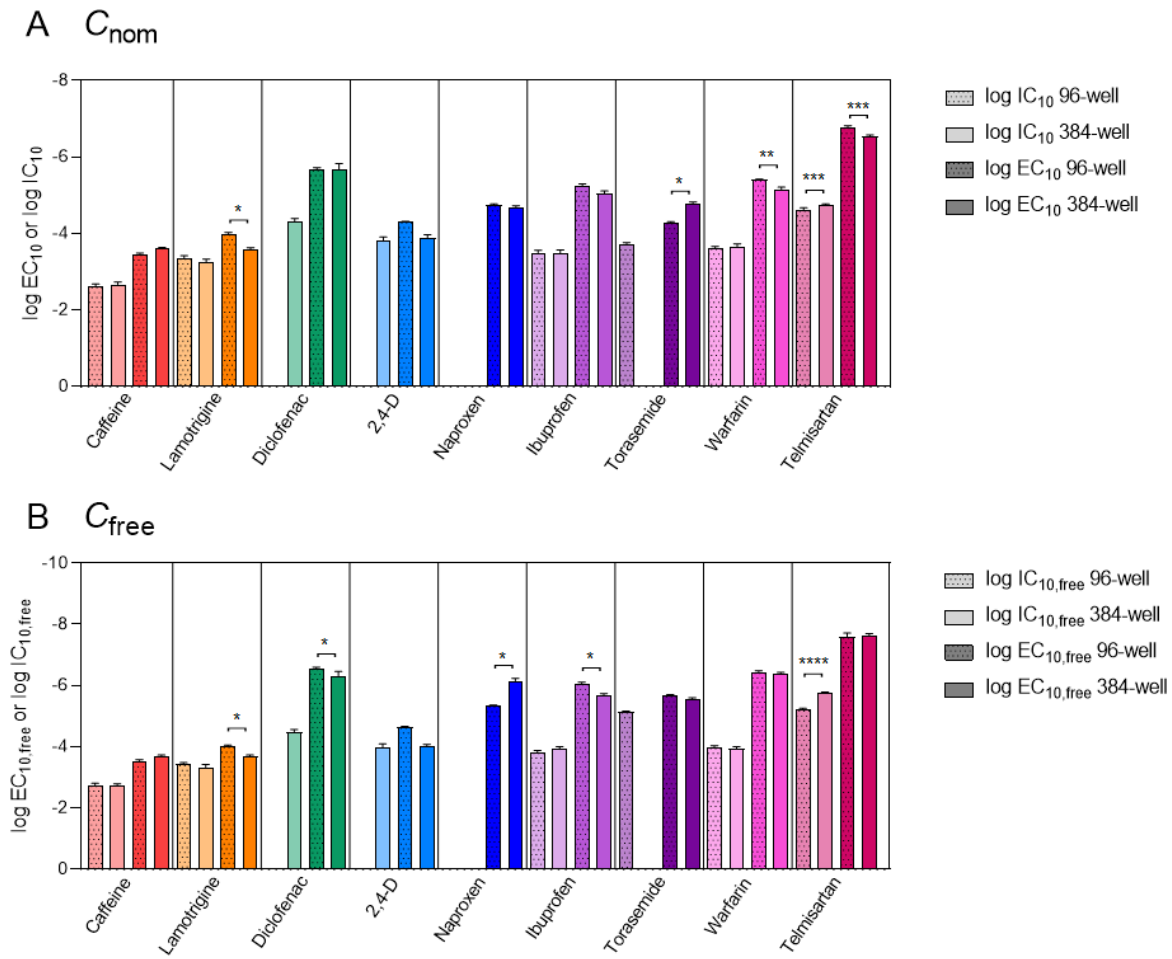


Figure S2: Unpaired t-test of log IC_{10} and log EC_{10} of the test chemicals obtained from 96-well plates (Huchthausen et al, 2020) compared to 384-well plates (A). Unpaired t-test of log $IC_{10,free}$ and log $EC_{10,free}$ of the test chemicals obtained from 96-well plates (Huchthausen et al, 2020) compared to 384-well plates (B).

Table S4: P-values of unpaired t-test of log IC_{10}, $IC_{10,free}$ and log EC_{10} or $EC_{10,free}$ of the test chemicals obtained from 96-well plates (Huchthausen et al, 2020) compared to 384-well plates.

Chemical	***P-value unpaired t-test 96 vs. 384 of IC_{10}***	***P-value unpaired t-test 96 vs. 384 of EC_{10}***	***P-value unpaired t-test 96 vs. 384 of $IC_{10,free}$***	***P-value unpaired t-test 96 vs. 384 of $EC_{10,free}$***
caffeine	0.3601	0.0839	0.7278	0.0572
lamotrigine	0.2814	0.0374	0.3713	0.0492
diclofenac		0.9048		0.0380
2,4-D		0.0924		0.0517
naproxen		0.3017		0.0405
ibuprofen	0.7462	0.0910	0.3761	0.0325
torasemide		0.0272		0.1509
warfarin	0.3457	0.0016	0.3059	0.0960
telmisartan	0.0003	0.0004	0.0001	0.4752

S4: References

Huchthausen, J., Mühlenbrink, M., König, M., Escher, B. I. & Henneberger, L. (2020) Experimental Exposure Assessment of Ionizable Organic Chemicals in In Vitro Cell-Based Bioassays. *Chemical Research in Toxicology*, 33(7), 1845-1854. doi 10.1021/acs.chemrestox.0c00067

Niu, L., Henneberger, L., Huchthausen, J., Krauss, M., Ogefere, A. & Escher, B. I. (2022) pH-Dependent Partitioning of Ionizable Organic Chemicals between the Silicone Polymer Polydimethylsiloxane (PDMS) and Water. *ACS Environmental Au*, 2(3), 253–262. doi: 10.1021/acsenvironau.1c00056

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