Supplementary Information and Data

Donor information on primary human hepatocyte lots:

Supplementary Table 1. Donor information on primary human hepatocyte lots.

	Age, sex, ethnicity	Alcohol Use	Tobacco Use	Diabetes	Verified Spheroid formation
Donor HUM201221	51, female, African American	no	no	no	yes
Donor HUM183121	49, male, African American	no	no	no	yes

HPLC-MS/MS conditions:

(1) The high-performance liquid chromatography (HPLC) analysis of 2-MPA was performed using an InfinityLab Poroshell 120 CS-C18 column (2.1 × 50 mm, 2.7 µm, Agilent, Santa Clara, CA, USA; 699775-942), an Agilent 1100 G1379A degasser (Agilent), and an Agilent 1200 Series Gradient HPLC system (Agilent) with an autosampler and an Agilent 1100 G1312A binary solvent pump (Agilent). Samples were injected with a volume of 1 µL and at an oven temperature of 50 °C. Milli-Q-water containing 0.2 mM ammonium fluoride was used as mobile Phase A (A), whereas 100 % methanol was used as mobile Phase B (B). The flow rate was 0.4 mL/min and the following gradient was applied: 96 % A / 4 % B for 1 min, followed by 90 % A / 10 % B 3 min, ramp up to 5 % A / 95 % B in 0.5 min and kept for 1 min. The MS analysis was done in the positive ion mode using the Agilent Triple Quadrupole 6470 mass spectrometer (Agilent) with an Agilent jet stream electrospray ionization source (AJS ESI; G1958-65638). The source parameters multiplier voltage, gas temperature, gas flow, nebulizer, sheath gas temperature, and sheath gas flow were set at - 300, 250 °C, 8 L/min, 60 psi, 375 °C, and 11 L/min, respectively. The capillary spray voltage was set to 3,000 V Positive and Negative, and the nozzle voltage to 500 V positive and

negative. The Dwell time was set for 35 ms. Analytical compound conditions are reported in Supplementary Table 2. A 15-point external calibration curve was with $R^2 > 0.995$ was built for each analytical run in the range of approximately 0.02 - 34 ng/µL, thus covering the concentration ranges of the samples. Agilent MassHunter LCMS Acquisition Console (Agilent) was used for control of the equipment and data acquisition. Data evaluation was performed using Agilent MassHunter Quantitative Analysis 10.1 (Agilent).

Supplementary Table 2. Retention time, percursor ion, product ions, collision energy, and fragmentor voltage for 2-MPA.

Compound	Retention time (RT, min)	Percursor ion (m/z)	Product ions (m/z)	Collision energy (CE, eV)	Fragmentor Voltage (V)
2-MPA	0.37	103	70.8*	9	40

*Quantifier ion.

(3) The analysis of diclofenac and testosterone was performed with an Agilent 1260 Infinity II HPLC system (Agilent Technologies, Waldbronn, Germany), including a quaternary pump (G7104C), an autosampler (G7167A) and a column oven (G7167A). The jet weaver mixer in the quaternary pump was set to bypass to reach a low delay volume. Samples were injected with a volume of 1 µL and at an oven temperature of 40 °C onto a ZORBAX SB-C8 column (4.6 × 50 mm, 1.8 µm, Agilent; 822975-906). Milli-Q-water with 2 mM ammonium fluoride plus 0.1 % formic acid was used as mobile Phase A (A), whereas 100 % methanol was used as mobile Phase B (B). The flow rate was 0.4 mL/min. The gradient started at 1 min with 40 % of B, from 1 - 6.5 min, 100 % B, from 6.5 - 7 min, 100 % B, from 7 - 7.01 min, 40 % B and re-equilibration for 3 min. Mass spectrometry was done in the positive ion mode with an Agilent 6465BA Ultivo triple quadrupole equipped with a classic electrospray ionization source (G1948B). The source parameters gas temperature, gas flow, nebulizer were set at 350 °C, 10 L/min, 50 psi, respectively. The capillary spray voltage was set to 3,500 V Positive and the Dwell time was set for 50 ms. Analytical compound conditions are reported in Supplementary Table 3. A 13-point external calibration

curve for testosterone and diclofenac with $R^2 > 0.995$ was built for each analytical run in the range of approximately 0.6 - 290 ng/mL, thus covering the concentration ranges of the samples. Production of 4-OH-diclofenac and 6β-OH-testosterone was qualitatively assessed by determining the area under the curve. The LC-MS system was controlled under MassHunter Acquisition for Ultivo version 1.2, and the data analysis was done with MassHunter Quantitative Analysis version 10.2 (Agilent) and Microsoft Excel 2016.

Supplementary Table 3. Retention time, percursor ion, product ions, collision energy, and fragmentor voltage for diclofenac, 4-hydroxy-diclofenac, 6β-hydroxy-testosterone, and testosterone.

Compound	Retention time (RT, min)	Percursor ion (m/z)	Product ions (m/z)	Collision energy (CE, eV)	Fragmentor Voltage (V)
diclofenac	3.6	296	250*	10	100
			214.9		
4-OH-diclofenac	2.8	312	266	10	100
			231*		
6β-OH-testosterone	3.9	305	269.2*	20	100
testosterone	2.5	289	253.1	25	100
			109*		

*Quantifier ion.

GC-MS/MS conditions:

(2) The gas chromatography (GC) analysis of β-PGME was performed using the 6890N Network GC system (Agilent) coupled to a 5973 Network mass selective detector (Agilent) and equipped with a Rxi-624Sil MS GC capillary column (60 m, 0.25 mm ID, 1.4 µm, Restek, Bad Soden, Germany). Samples were liquid injected with a volume of 1 µL in splitless mode (200 °C, 8.4 psi) using a PAL auto sampler system. Column conditions: pressure 8.4 psi, 1.1 mL/min flow rate. GC conditions: carrier gas helium, 60 °C for 1 min, 10 °C/min up to 200 °C and hold 3.5 min, then 40 °C/min up to 250 °C and hold 1.25 min. Total run: 21 min. MS conditions: positive ion polarity, ion

source 230 °C. The total Dwell time was set for 300 ms. Analytical compound conditions are reported in Supplementary Table 4. A 6-point external calibration curve using cyclohexanol as internal standard (IS) with $R^2 > 0.995$ was built in the range of approximately 1 - 100 ng/µL covering the concentration ranges of the samples. The samples were diluted with an IS solution before analysis. Data evaluation was performed using Agilent MassHunter Quantitative Analysis 10.0 (Agilent).

Supplementary Table 4. Retention time, percursor ion, and product ions for β-PGME and the internal standard (IS) cyclohexanol.

Compound	Retention time (RT, min)	Percursor ion (m/z)	Product ions (m/z)
β-PGME	7.589	152	31*
			43*
			59
cyclohexanol (IS)	11.069	152	57*
			67*
			82

*Quantifier ion.

Determination of protein amount per incubation:

Supplementary Table 5. Protein per incubation for the 3D HepaRG and 3D primary human hepatocyte (pHH) metabolism studies. The protein was determined using the Pierce BCA Protein assay kit in 1 - 2 independent measurements for N = 3 independent biological repeats (3D HepaRG) and N = 2 independent biological repeats using one donor each (3D pHH). Values represent means ± SD.

Protein/incubation (µg)			
	Experiment 1	**Experiment 2**	**Experiment 3**
3D HepaRG	115	60.7 ± 4.19	71.8 ± 3.10
3D pHH	94.1 ± 30.1 (HUM201221)	27.6 (HUM183121)	-

The amount of metabolite formed in the biological experiment in pmol was normalized for each biological repeat separately to the determined protein content (µg).

Uncertainty analysis:

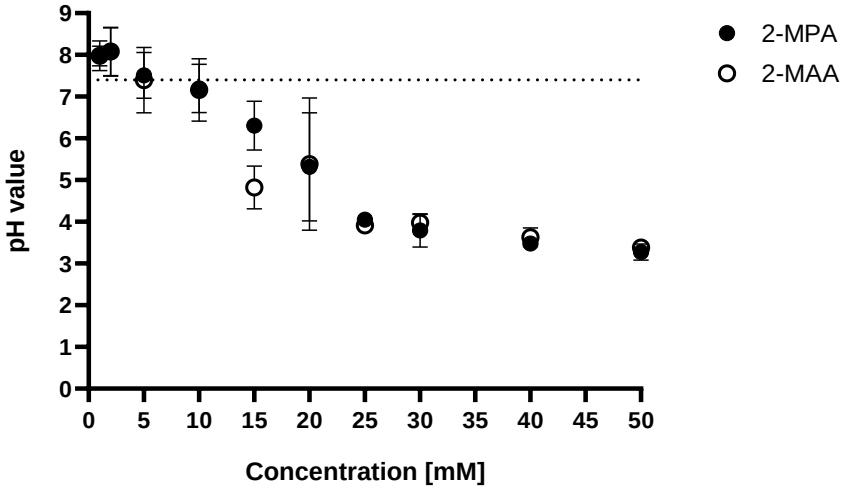
Supplementary Table 6. Results of the uncertainty analysis for the area under the curve (AUC) outputs of β-PGME blood concentration, and 2-MPA urine/blood concentration.

Parameter	AUC unbound PGME in blood	AUC unbound 2-MPA in urine	AUC unbound 2-MPA in blood	Uncertainty
Physiological parameters				
BW	-0.29013341	-0.11163982	-0.28996405	L
QCC_{rest}	0.99973559	0.99968382	0.99977783	L
Biochemical parameters				
R_{pulm}	1.00004304	0.99968382	1.00034750	L
K_{ur}		-0.99031544		L
K_{MPA}			-0.92344151	H
Fu_p	0.99963311			L
Rb	-0.98969212			H
$P\_ba_{\_MPA}$			0.99862141	H
$P\_ca_{\_MPA}$			-0.98893149	H
$Rb_{\_MPA}$			-0.98895402	H
$Fu\_p_{\_MPA}$			0.99920815	H
Physico-chemical parameters				
$MW_{\_MPA}$	1.00004304	0.99968382	1.00034750	L
Beta_percent	1.00004304	0.99968382	1.00034750	L
High impact	L = Low uncertainty / M = Medium uncertainty / H = High uncertainty			

Medium impact

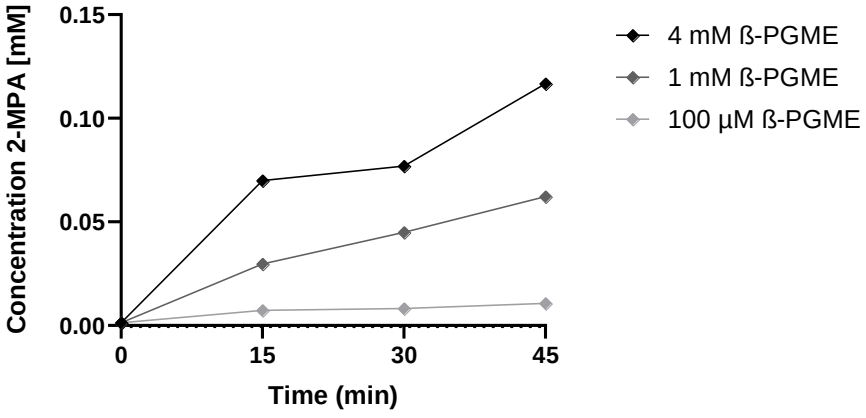
Abbreviations: body weight (BW), cardiac output (QCC_{rest}), β-PGME pulmonary retention (R_{pulm}), urinary excretion rate constant (K_{ur}), urinary excretion rate of 2-MPA (K_{MPA}), β-PGME unbound fraction in plasma (Fu,p), β-PGME blood-to-plasma ratio (Rb), blood-air partition coefficient (PC) of 2-MPA (Pba_{MPA}), central-air PC of 2-MPA (Pca_{MPA}), 2-MPA blood-to-plasma ratio (Rb_{MPA}), 2-MPA unbound fraction in plasma ($Fu,_{p\_MPA}$), molecular weight (MW), percentage of β-PGME present in the mixture used for the exposure experiment (Beta_percent).

Effect of 2-MAA and 2-MPA on the pH:



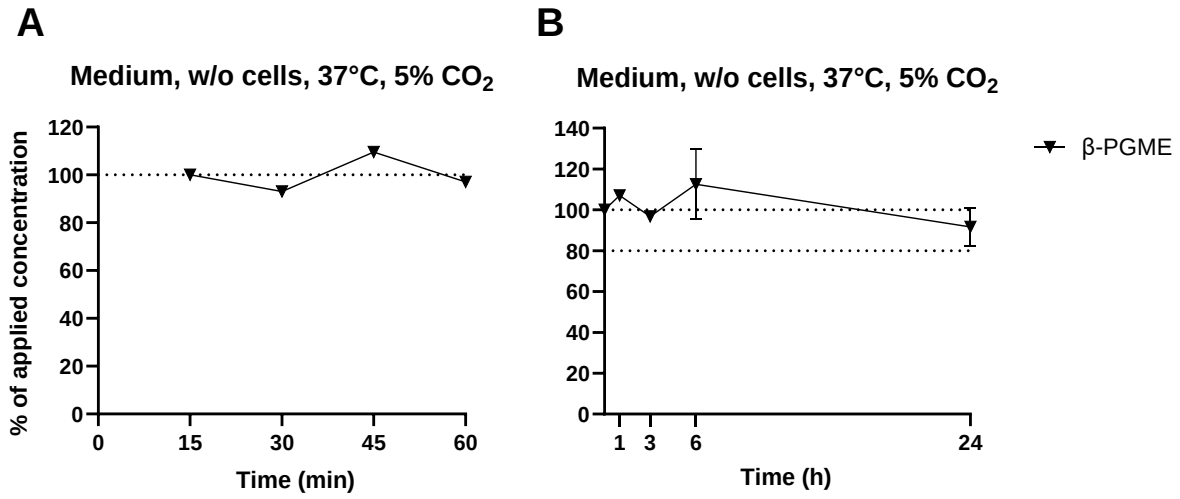
Supplementary Figure 1. Measured pH values for the corresponding 2-MAA and 2-MPA compound solutions. The pH of treatment solutions containing either 2-MAA or 2-MPA was measured using a pH electrode. Data points represent means ± SD of N = 1 incubation with 2 - 4 technical replicates. The dotted line represents a neutral pH 7.4.

Determination of linear incubation conditions for S9:

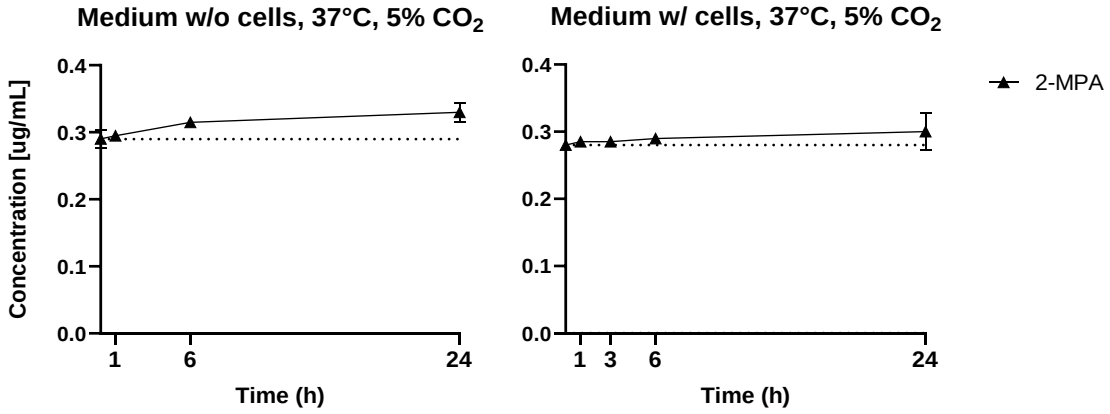


Supplementary Figure 2. Metabolite formation-time profiles in human liver S9 incubations. 2-MPA was quantified for three different β-PGME concentrations (low, mediate, high) after sampling at time-points 15 min, 30 min, and 45 min. Data points represent N = 1 incubation with 1 technical replicate.

Evaporation / Adsorption / Stability tests:



Supplementary Figure 3. Evaporation, adsorption, and stability tests for β-PGME (5 mM) in the elplasia plate in absence of cells. **(A)** Concentrations were quantified in samples collected at 15 min, 30 min, 45 min, and 60 min. Data points represent N = 1 experiment with 1 technical replicate. **(B)** Concentrations were quantified in samples collected at 1 min, 1 h, 3 h, 6 h, and 24 h. Data points represent means ± SD of N = 2 experiments with 2 technical replicates. The dotted lines represent 100 % and 80 % of the applied β-PGME concentration.



Supplementary Figure 4. Evaporation, adsorption, and stability tests for the metabolite standard 2-MPA in the elplasia plate without (w/o) and with (w/) cells. Concentrations were quantified in samples collected at 1 min, 1 h, 3 h (only with cells), 6 h, and 24 h. Data points represent means ± SD of N = 1 experiment with 2 technical replicates. The dotted lines represent the nominal 2-MPA concentration.