Title: DNA methylation and histone acetylation changes to cytochrome P450 2E1 regulation in normal aging and impact on rates of drug metabolism in the liver.

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Supplementary Methods

Western Blot Analysis

Cell protein lysates were created from homogenization of 20mg liver tissues in 200µL Pierce RIPA buffer (89900, Thermo Fisher, Waltham, MA) with final concentration of 1% v/v (2 µL) Halt Protease Inhibitor buffer (78430, Thermo Fisher, Waltham, MA). The Pierce BCA assay (23227, Thermo Fisher, Waltham, MA) was used to measure protein concentrations using manufacturer's standard protocol. Absorbance at 562nm was measured on a Synergy HT plate reader and the unknown protein concentration was determined by interpolating absorbance of unknown samples on an 8-point standard curve created from diluted albumin standards.

10µg of total protein in 30µL β-Mercaptoethanol (βME)-Laemmli buffer (1610710, 1610747, Bio-Rad) were separated on 10% Mini-PROTEAN precast SDS-PAGE gels (4568033, Bio-Rad) at constant 200V for 35min and transferred onto PVDF membrane using a TransBlot Turbo (1704150, Bio-Rad) at 1.3A for 5 min. Membranes were blocked for 30 min in 5% milk in 1X TBST (Tris Buffered Saline, Tween20). Blots were probed using anti-CYP2E1 primary antibody (1:2500, ab28146, Abcam) in 10mL 5% Milk in 1X TBST at 4ºC overnight and washed three times with 1X TBST the next day. The blots were incubated with HRP-coupled rabbit IgG secondary antibody (1:10000, Cell Signaling, Danvers, MA) and 0.5µL Precision Protein StrepTactin-HRP conjugate (Bio-Rad) for 1hour at RT in 10mL 5% Milk in 1X TBST. Membranes were washed five times with 1X TBST. Blots were treated with 2mL Clarity Western ECL (1705060, Bio-Rad) at 1:1 clarity western luminol enhancer: peroxide solution ratio to start chemiluminescence and imaged 2min afterwards on the ChemiDoc Touch (Bio-Rad). Blots were washed with 1X TBST and stripped

for 30min using Restore stripping buffer (21059, Thermo Fisher). After stripping, the membrane was incubated with GAPDH primary antibody (1:5000, MA5-15738, Thermo Fisher) and then mouse IgG secondary antibody (1:20000, 7076S, Cell Signaling, Danvers, MA). The bands were visualized at 49 and 37 kDa for CYP2E1 and GAPDH respectively using Image Lab software v 6.0 (Bio-Rad, Hercules, CA). For each sample, CYP2E1 band intensity were normalized to GAPDH.

Chromatin Immunoprecipitation (ChIP) – qPCR

80 mg of mouse liver was minced into 1 mm3 fragments with -20 C precooled scalpel and transferred to 2mL Eppendorf tube. TruChIP tissue shearing kit (520237, Covaris, Woburn, MA) was used to prepare sheared chromatin. Centrifugations were at 200g for 5 min at 4ºC unless otherwise noted. The minced tissue was washed with 1 mL 1X cold PBS and centrifuged at 200g for 5min at 4ºC. The supernatant was discarded and 1mL of fixing buffer A was used to resuspend the pellet. 100µL of freshly prepared 11.1% methanol-free formaldehyde (1% final concentration) was added and the fixation was quenched after 2 min with 58µL quenching buffer E. The suspension was centrifuged, and the supernatant discarded. Pellet was washed twice with 1mL 1X cold PBS and centrifuged. Pellet was transferred to tissueTUBE (TT05M XT) provided in the kit and flash frozen in liquid nitrogen for 45sec and pulverized into powder by a precooled pestle. Pulverized tissue was transferred by inverting to a screwed-on milliTUBE-2mL and stored at -80ºC until nuclei separation and shearing step the next day. Pulverized tissue was transferred to a 2mL Eppendorf tube using two successive transfers by 500µL lysis buffer B. The 2mL tube was incubated on a rotor at 4ºC for 20min to complete lysis. Nuclei

were pelleted by centrifugation at 1700g for 5min at 4ºC. Supernatant was discarded and pellet was resuspended in 1mL wash buffer C and incubated on a rocker for 10min at 4ºC. Nuclei were pelleted again at 1700g for 5min at 4ºC and supernatant discarded. Pellet was resuspended with 1mL wash buffer C and centrifuged at 1700g for 5min at 4ºC. Pellet was resuspended with 1mL shearing buffer D2 and sheared on M220 (Covaris, Woburn, MA) for 8min at 75 PIP, 10% duty factor, 200CPB, 7C set point temperature (4/10 Min/Max), and no degassing. Time course trials were conducted to determine optimal shearing (2-20 min) and fixation times (2 and 5 min) of 8 and 2 min respectively which yielded the highest percentage (>75%) of fragment sizes between 150 and 700 bp and the lowest percentages (<25%) of fragment sizes less than 150 and higher than 701 bp combined. Shearing and fixation time course trials were analyzed on the Agilent 2100 Bioanalyzer using the Agilent DNA 12000 chip on Agilent 2100 expert software. 25µL of sheared chromatin was incubated with 1µL 10mg/ml RNAse (EN0531, Thermo Fisher, Waltham, MA) at 37ºC for 30min, then it was treated with 4µL 10mg/ml Proteinase K (17916, Thermo Fisher, Waltham, MA) at 65ºC overnight (16 hours). DNA was purified using QIAquick PCR purification Kit (Qiagen, Hilden, Germany). The concentration of eluted DNA was measured on Qubit 4.0 fluorometer and used to calculate the volume required to have 2µg sheared chromatin as starting material for the Chromatin immunoprecipitation (ChIP) step (1:1 ratio of DNA to chromatin was used to calculate chromatin concentration). The sheared chromatin was diluted 1:2 with 3X Covaris IP dilution buffer in order to decrease final SDS concentration to 0.083% and prevent SDS interference with epitope and antibody binding.

Each ChIP had 2µg of sheared chromatin. 2% of the volumes of sheared chromatin per IP was set aside at 4ºC as input control and was not processed through ChIP step. ChIP was carried out by incubating 5 µL of H3K9ac (39137, Active Motif, Carlsbad, CA), or 5µL of H3K27ac (39133, Active Motif, Carlsbad, CA), or 5µL of Rabbit IgG (ab171870, Abcam) with 2µg sheared chromatin from each sample overnight (16 hours) at 4ºC. Hence, three worth of ChIP volumes were used from each sample. The formed complex was incubated with 50 µL Dynabeads Protein G (10003D, Thermo Fisher, Waltham, MA) for 4 hours at 4ºC. The bead linked complex was inserted on the DynaMag (Thermo Fisher, Waltham, MA) magnet rack for 2 min and the supernatant discarded. The bead coupled complex was removed from the magnet and washed 3 times with 500 µL with cold 0.05X Tween 20 in PBS PH7.4 (10010023, Thermo Fisher, Waltham, MA) for 3 min each at room temperature on HulaMixer (Thermo Fisher, Waltham, MA). Finally, 50 µL of IP elution buffer (Aq. 1% SDS, 0.1 M NaHCO3, pH 9.0) was added to the bead coupled complex and incubated on a heat block for 1hour at 65ºC with 15sec vortexing every 15 min. The samples were then inserted back on the DynaMag for 2 min and 50 µL of the supernatant was transferred to a 96 well PCR plate. The input control was diluted 1:2 with 3X Covaris IP dilution buffer to mimic the dilution done to the samples and preserve its percentage (2%). The ChIP'ed samples and their input controls were incubated each with 2 µL RNAse for 30 min at 37C and then 8 µL of Proteinase K was added and incubated overnight (16 hours) at 65^{ºC}. The next day, DNA was purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) to final elution volume of 50 µL. 2 µL of each DNA elution (H3K9ac, H3K27ac, IgG, and input control) per sample was used per qPCR reaction.

In each qPCR reaction, 2 µL of DNA was amplified in triplicate on the Quant Studio 3 instrument (Applied Biosystems). Reaction volumes was 20µL containing final concentration of 1X of PowerUp SYBR Green Master Mix (A25742, Applied Biosystems) and 0.2 µM of forward (0.4 µL) and reverse (0.4 µL) primers and 7.2 µL molecular biology grade H2O. The qPCR run conditions are as follows: 2min hold at 50$^{\circ C}$ followed by 2min hold at 95$^{\circ C}$ followed by 40 cycles of the following: 15sec at 95$^{\circ C}$ followed by 30sec at annealing temp of 59$^{\circ C}$ and by 1min at 72$^{\circ C}$, followed melt curve analysis stage. This stage is as follows: 15sec hold at 95$^{\circ C}$ followed by 1min hold at 59$^{\circ C}$ and then the continuous fluorescence acquisition starts while increasing the temp by 0.15$^{\circ C}$/s to end at 95$^{\circ C}$ followed by a 15sec hold at 95$^{\circ C}$. Primer specificity and efficiency were tested on 2% agarose gel and a 5-point 1:2 serial dilution standard curve respectively. No secondary amplification or primer dimers were detected indicating primer specificity. A singular melt peak was shown by the qPCR melt curves indicating formation of a single product. Primer efficiency outside of accepted ranges [90-110] were discarded until reaction conditions were optimized. The Cq values for each plate were downloaded from ThermoFisher Cloud. The mean threshold cycle (Cq) values were obtained for each sample and normalized to the dilution factor (2% = 1/50) corrected Cq value (Log2 (50) =5.6438) of the input control to obtain Delta Cq. Percentage of input was calculated by multiplying 100 with 2 raised to the exponent of Delta Cq. For each sample, three “percentage of input” values were obtained and are H3K9ac, H3K7ac, and IgG.

LINE1 High-Resolution Melt (HRM) analysis

HRM LINE1 assay was performed as described previously (Newman et al. 2012) with slight adjustments. MeltDoctor HRM MasterMix (Applied Biosystems, Foster City, CA) on a Quantstudio 3 instrument was used to measure 5mC levels at the 193 base pair LINE1 region reported in Newman et al encompassing 11 CpGs. Samples were amplified by qPCR as follows 10min hold at 95ºC followed by 45 cycles of: 15sec at 95ºC, 60sec at 60ºC, followed by a melt curve stage with temperature range of 60ºC to 95ºC. Each reaction included 20ng bisulfite-converted DNA and 0.75µM each of unmethylated forward and reverse primer (Newman et al. 2012) in 1X MeltDoctor HRM MasterMix. Standards of 0, 25, 50, 75, 100% methylation (EpigenDx, Hopkinton, MA) were run in duplicate while the liver samples were run in triplicate. The Net Temperature Shift (NTS) values (Newman et al. 2012) of the liver samples were interpolated on the standard curve to yield their 5mC percentage.

Pyrosequencing Assays

The first pyrosequencing assay amplified a 257 base pair product at *Cyp2e1* 5'UTR (chr7:147,949,576-147,949,850/mm9) that encompasses 7 CpGs (Position 2-8) chr7: 147,949,679 - 147,949,684 - 147,949,743 - 147,949,754 - 147,949,770 - 147,949-791 - 147,949,806, mm9. The second assay amplified a 157 base pair product at *Cyp2e1* promoter (chr7:147,942,437-147,942,593, mm9) that encompass 1 CpG (Position 1) chr7: 147,942,492, mm9. PyroMark PCR Kit (978703, Qiagen, Hilden, Germany) was used to amplify a PCR product for each of the two pyrosequencing assays with final concentrations in the PCR reaction mixture as follows: 1x

PyroMark PCR Master Mix, 1x CoralLoad Concentrate, 1.5mM MgCl2, 1x Q-Solution, 10 ng of bisulfite modified genomic DNA, and 0.2µM of Forward, 0.2µM Reverse, and 0.2µM biotinylated M13 (Royo et al. 2007) primers (Sup. Table 2). 2µL of the PCR product was separated by 2% agarose gel electrophoresis to confirm correct product size and absence of primer dimers and secondary amplification. The 2% agarose gel was made with the following ratio to obtain 0.75mm thickness in the Owl EasyCast B2 Mini Gel Electrophoresis System cast (Thermo Fisher Scientific, Waltham, MA): 2.52 g of agarose powder (BP2410-100, Fisher Scientific, Hampton, NH) and 1.89 µL ethidium bromide (BP1302-10, Fisher Scientific, Hampton, NH) in 126 mL 0.5X Tris-Borate-EDTA (TBE) buffer (95:5, H2O:10XTBE (BP1333-4, Fisher Scientific, Hampton, NH)) mixed until complete gelling of agarose. The gel was poured onto cast and let to solidify for 30 min at RT. The cast was filled with 400 µL 0.5X TBE buffer. Each gel had 2µL of 100 bp DNA ladder in the first and last well of the gel. The gel was run on constant voltage of 100V for 1 hour. The gel was imaged on ChemiDoc Touch (Bio-Rad, Hercules, CA) using default settings of auto optimal exposure for ethidium bromide stained gels.

5µL PCR product was allowed to bind by shaking for 5 min with 2µL of Streptavidin Sepharose High Performance beads (45-000-279, Fisher, Hampton, NH) in 40 µL Binding buffer (979006, Qiagen, Hilden, Germany) and 33µL of molecular biology grade water. The complex was transferred to a 96-well HS plate (979101, Qiagen, Hilden, Germany) containing 12µL per well of final concentration of 0.3µM of sequencing primer in Annealing buffer (979009, Qiagen, Hilden, Germany) on the PyroMark Q96 vacuum preparation station. The HS plate was inserted on the PyroMark Q96 MD and the PyroMark Gold Q96 reagents (972804, Qiagen, Hilden, Germany) were used for each assay to sequence the PCR product. Each pyrosequencing assay was run duplicate for each

sample. A standard consisting of 0, 5, 10, 25, 50, 75, and 100% methylated mouse genomic DNA was included on each plate (808060M, EpigenDx, Hopkinton, MA). Both the standard and the liver test samples were pyrosequenced in duplicates.

CYP2E1 intrinsic clearance

Mouse Liver Microsomes (MLM) were prepared as previously described with mild adjustments (Knights et al. 2016). 500 mg of liver tissue was homogenized using the Fisher 150 homogenizer (15340167, Fisher Scientific) on high speed in cold 10 mL microsome preparation buffer 5 times for 30 sec each with 30 sec breaks in between on ice. Homogenate was centrifuged at 700g for 10 min at 4ºC, directly followed by centrifugation at 10,000g for 10 min at 4ºC. Supernatant was transferred to precooled 10mL ultracentrifuge tubes and ultracentrifuged at 100,000g (25,000 rpm) for 75 min at 4ºC on the Optima l-90K ultracentrifuge using the SW41 rotor. The microsome pellet was transferred using 2 mL microsome preparation buffer to a precooled 5mL Potter-Elvehjem grinder connected to an electric pivot driver. The pellet was ground for 1 min at low speed with approximately 10 strokes and transferred back to the ultracentrifuge tube with four successive 2mL transfers using microsome preparation buffer to prevent residual loss in the grinder. The homogenate was ultracentrifuged again at 100,000g (25,000 rpm) for 75min at 4ºC. Afterwards, the supernatant was discarded, and the pellet was transferred using 1mL microsome storage buffer to 5mL grinder and ground for 1min at low speed. The suspension containing the microsomes was stored at -80ºC. 10µL of the microsome suspension was used to measure

microsomal protein concertation by the microplate procedure of the BCA assay (23227, Thermo Fisher). Average microsome yield was 1.07% w/w [0.55-1.69].

Hydroxylarion of Chlorzoxazone (CZ) to 6-hydroxychlorzoxazone (6-OH-CZ) was measured to determine the catalytic activity of CYP2E1 using MLM reactions. Each sample was run in 8 MLM reactions that vary by the final concentration (10, 20, 40, 80, 160, 320, 640, 1000 µM) of the parent probe drug (CZ) to determine Michaelis-Menten kinetic constants. Reaction rates (pmol/min/mg) was plotted against CZ concentration (µM) and fit by the Michaelis-Menten equation (GraphPad Prism v 8.0, San Diego, CA) to estimate Km and Vmax, where Vmax is the maximal rate of hydroxylation and Km is CZ concentration at the half-maximal reaction rate. The Michaelis-Menten kinetic constants were used to estimate the intrinsic clearance CL_{int} (CL_{int} = Vmax/Km). Standards for CZ (#18869) and (6-OH-CZ) (#10009029) were purchased from Cayman Chemical (Ann Arbor, MI, USA). The reactions were repeated twice except for the18 and 32 months ages due to lower MLM yield in these groups. The final concentrations of the MLM reaction components were 50mM Potassium Phosphate buffer PH 7.4, 10mM $MgCl_2$, 1mg/ml MLM protein, 1mM EDTA (E4884, Sigma, St. Louis, MO), 1mM NADPH (AK1395, AkronBiotech, Boca Raton, FL), 0.5% DMSO (16785, Acros Organics), with incubation time of 25min. The reaction was stopped with equal volume (150µL) of HPLC grade methanol (A452-4, Fisher Scientific, Hampton, NH) and centrifuged for 10 min at 20,000g. The supernatant was transferred to 96-well plate and 80 µL was injected into the High-Performance Liquid Chromatography (HPLC) system by Series 200 Autosampler (PerkinElmer, Waltham, MA).

A HPLC separation coupled with ultraviolet detection method was used to determine CZ and 6-OH-CZ concentration as previously described with mild adjustments to optimize assay conditions (Court et al. 1997). The HPLC system composed of a binary pump, C18 BDS hypersil 50 mm x 4.6 with 5µm particle size (28105-054630, Thermo Fisher, Waltham, MA) and diode array detector. Detection wavelengths were 299 and 280 nm for 6-OH-CZ and CZ respectively. Isocratic gradient elution was used with aqueous mobile phase (A) containing 2% acetic acid (BDH20108.292, VWR, Radnor, PA), 1 % Triethylamine (A12646 Alfa Aesar, Tewksbury, MA), while the organic phase (B) was HPLC grade methanol (A452-4, Fisher Scientific, Hampton, NH) (Sup. Table 3). Linear standard curves with $R^2 \geq 0.999$ were obtained from 0.46-240 µM for 6-OH-CZ and 2.34-1200 µM for CZ. Total run time was 6.5 min with retention time of 1.86 and 5.17 min for 6-OH-CZ and CZ respectively. Km, Vmax, and CL_{int} were determined by GraphPad Prism through the fit estimates by the Michaelis-Menten equation

Supplementary Tables

Table S1. Summary of human EWAS findings for phase I drug metabolism genes

The human ADME genes list from pharmaADME (www.pharmaadme.org) was contrasted with the top findings from epigenome-wide association studies and genome-wide gene expression studies of normal aging in human blood DNA. Genes encoding phase I drug metabolizing enzymes that showed significant association in the top findings of these studies are marked with an "X". The total number of studies where a specific gene was a top association in the reported results is shown in the last column of the table.

	Longevity Map(Human Ageing Genomic Resources)	**Peters (Peters et al. 2015)**	**Heyn (Heyn et al. 2012)**	**Hannum (Hannum et al. 2013)**	**Horvath (Horvath 2013)**	**Reynolds (Reynolds et al. 2014)**	**Martilla (Marttila et al. 2015)**	**Steegenga (Steegenga et al. 2014)**	
	Exp	Exp	Meth		Meth	Meth	Both	Both	Total
CYP2E1	X	X			X			X	4
CYP1B1	X	X	X				X		4
CYP2J2		X		X					2
CYP1A1	X							X	2
ADHFE1		X			X				2
PDE3B		X				X			2
GPX7		X				X			2
DHRS9		X					X		2
CYP2D6	X								1
CYP3A4									0
CYP1A2									0

Table S2. HRM and ChIP primers and qPCR product sequences

Primers used in the High-Resolution Melt and Chromatin Immunoprecipitation Quantitative Polymerase Chain Reaction assays with size of PCR product, genomic coordinates, and assembly with CpG count when appropriate.

Gene	**Primers (5'→ 3')**		**Product size/coordinates/assembly**	
	Forward Primer	**Reverse Primer**		
	High-Resolution Melt			**CpG count**
Cyp2e1	AATTAGTATTTTAGGTTAAGGGAGATGAGTGG	TCCCTTACCTTAATTAAAAACTTAAAAATATCCTTC	319bp/chr7:147,949,616-147,949,934/mm9	7
Cyp1b1	TGTTTTGTTGTATTAGGGTTTGGTGGATGG	CCTTTATATCCCCAACATAACCACCAAC	350bp/chr17:80,113,318-80,113,667/mm9	21
	Chromatin Immunoprecipitation Quantitative Polymerase Chain Reaction			
Cyp2e1				
Region 1	ATGCTGAGCCAGCTGTGA	CCACATGCAAAGACAATCCT	145bp/ chr7:147,950,223-147,950,367/mm9	
Region 2	ATTTGCTGCCTAGCTGCTTC	CAGCACTCTGAGACCCCAGT	119bp/ chr7:147,942,350-147,942,468/mm9	
Gapdh				
Positive control	AGAGAGGGAGGAGGGGAAATG	AACAGGGAGGAGCAGAGAGCAC	200bp/ chr6:125,115,604-125,115,803/mm9 [a]	
Negative control	CTCCAGATGCTGAGAGAAAAAC	AGGCATACCAAGCACAGAAA	100bp/ chr17:95,049,080-95,049,179/mm9	

[a] blasted from Pace et al., 2018 to obtain coordinates

Table S3. Pyrosequencing primer sequences

Primers used in the Pyrosequencing assays of region 1 and 2 with size of pyrosequenced PCR product, coordinates, and assembly with CpG count.

Region	***Cyp2e1* Region 1**	***Cyp2e1* Region 2**
Product size/coordinates/assembly	275/chr7:147,949,576-147,949,850/mm9	157/ chr7:147,942,437-147,942,593/mm9
Forward primer	5'GGGGGTAGGTTTTAATTTTTATAGAT3'	5'TTAGTATTTTTATTGGGGTTTTAGAGTG3'
Reverse primer	5'CGCCAGGGTTTTCCCAGTCACGAC ATCAACCTTTAAAATAATAACCAACTACA3'	5'CGCCAGGGTTTTCCCAGTCACGAC ATAACCTCCAAATCTAAACTTCTATTTAAC3'
Sequencing primer	5'ATTTTTATAGATTTGTTTTTAGATG3'	5'GTTGGAGTTTAATGGGA3'
M13 primer [a]	5'biotin-CGCCAGGGTTTTCCCAGTCACGAC3'	
CpG count	7	1

[a]M13 primer sequence published in Royo et al. 2007.

Table S4. HPLC parameters

Chromatography parameters of the isocratic gradient co-elution and detection method of Chlorzoxazone and 6-hydroxychlorzoxazone. A is the aqueous mobile phase (2% acetic acid and 1% trimethylamine v/v). B is organic mobile phase (HPLC grade methanol).

Chromatographic conditions		
Column	BDS Hypersil C18 (50 x 4.6 mm, 5µm)	
Column temperature	35 ºC	
Flow rate	1.0 mL/min	
Wavelength	CZ at 280 nm, 6-OH-CZ at 299 nm	
Injection volume	80 µL	
Run time	6.5 min	
Time (minute)	A (%)	B (%)
0	85	15
1.5	85	15
1.6	75	25
5	75	25
5.1	85	15

Table S5. Pearson correlation tests p-values for epigenetic and drug metabolism variables

Table reporting Pearson correlation test p-value for each pair of variables. Age: Chronological age, Pos1-8, Position 1-8: chr7: 147,942,492-147,949,679 - 147,949,684 - 147,949,743 - 147,949,754 - 147,949,770 - 147,949-791 - 147,949,806, mm9. Vmax: maximal rate of 6-hydroxylation reaction of chlorzoxazone by CYP2E1, Km: chlorzoxazone concentration at half maximal rate, CL.Int: intrinsic clearance, K9: Histone 3 Lysine 9 acetylation in *Cyp2e1* intron 1 (chr7:147,950,223-147,950,367, mm9), R2K9 Histone 3 Lysine 9 acetylation in *Cyp2e1* promoter (chr7:147,942,350-147942468, mm9).

	Age	Pos1	Pos2	Pos3	Pos4	Pos5	Pos6	Pos7	Pos8	K9	R2K9	Vmax	Km	CL.Int
Age	0													
Pos1	0.065395	0												
Pos2	0.081807	7.70E-14	0											
Pos3	0.07162	3.97E-14	3.56E-15	0										
Pos4	0.092281	3.79E-12	1.55E-17	9.63E-15	0									
Pos5	0.0705	9.53E-14	2.54E-17	8.87E-19	1.48E-16	0								
Pos6	0.082019	5.76E-11	9.50E-15	1.47E-12	1.25E-15	1.01E-13	0							
Pos7	0.18272	6.65E-08	1.19E-09	1.08E-08	2.97E-10	3.22E-09	2.62E-10	0						
Pos8	0.15685	1.93E-09	5.77E-12	2.06E-10	4.16E-13	3.93E-11	1.94E-12	2.02E-13	0					
K9	0.080837	0.822009	0.991494	0.868065	0.93381	0.926034	0.888193	0.611609	0.699176	0				
R2K9	0.132699	0.308984	0.197871	0.227448	0.158191	0.211515	0.153698	0.055628	0.084396	0.002332	0			
Vmax	0.031566	0.386084	0.545814	0.528949	0.633683	0.552158	0.636751	0.992157	0.826633	0.025017	0.000729	0		
Km	0.367772	0.102387	0.068177	0.067084	0.049134	0.06689	0.056673	0.025507	0.029863	0.062959	5.46E-05	0.021432	0	
CL.Int	0.808532	0.003069	0.002033	0.001539	0.001285	0.001781	0.002045	0.000891	0.000806	0.518669	0.005073	0.268212	1.79E-05	0

Table S6. Pryosequencing and HRM 5mC correlation

Pearson correlation test result rho (r) and p-value of each CpG methylation values investigated by Pyrosequencing and the High-Resolution Melt (HRM) methylation result of the 5'UTR region of *Cyp2e1*. The pyrosequencing assays confirmed the HRM data with all CpG positions positively correlated with HRM methylation. Pos 1-8: Position 1-8 chr7: 147,942,492-147,949,679 - 147,949,684 - 147,949,743 - 147,949,754 - 147,949,770 - 147,949-791 - 147,949,806, mm9.

	High Resolution Melt Methylation	
Pyrosequencing Methylation	Pearson r	p-value
Pos1	0.51063431	0.001380327
Pos2	0.490981863	0.000314928
Pos3	0.512258323	0.001592567
Pos4	0.485952536	0.000289392
Pos5	0.522468882	0.000715259
Pos6	0.518541845	0.001105784
Pos7	0.398590327	8.58E-08
Pos8	0.403590987	6.49E-06

Table S7. Michaelis-Menten Constants per age group

Table reporting mean (+-SD) of the pharmacokinetics constants Vmax (pmol/min/mg protein), Km (uM), and CL_{int} (uL/min/mg protein) per age group (months).

Age (months)	Vmax (pmol/min/mg protein) Mean (+-SD)	Km (uM) Mean (+-SD)	CL_{int} (uL/min/mg protein) Mean (+-SD)
4	626.05 (297.1)	391.44 (196.7)	1.59 (1.51)
18	602.96 (493.9)	1009.54 (1662.1)	0.59 (0.29)
24	915.07 (774.7)	377.59 (405.2)	2.42 (1.91)
32	604.8 (434.3)	732.24 (891.9)	0.82 (0.48)

Supplementary Figures

Fig. S1. LINE 1 methylation percentage per age

Box plots with regression line (blue) of age-associated changes to LINE 1 methylation percentage (n=20). Data represent median (middle hinge), 25% (lower hinge) and 75% (upper hinge) quantile. Data points beyond upper or lower 1.5 * Inter Quantile Range are represented as individual black dots.

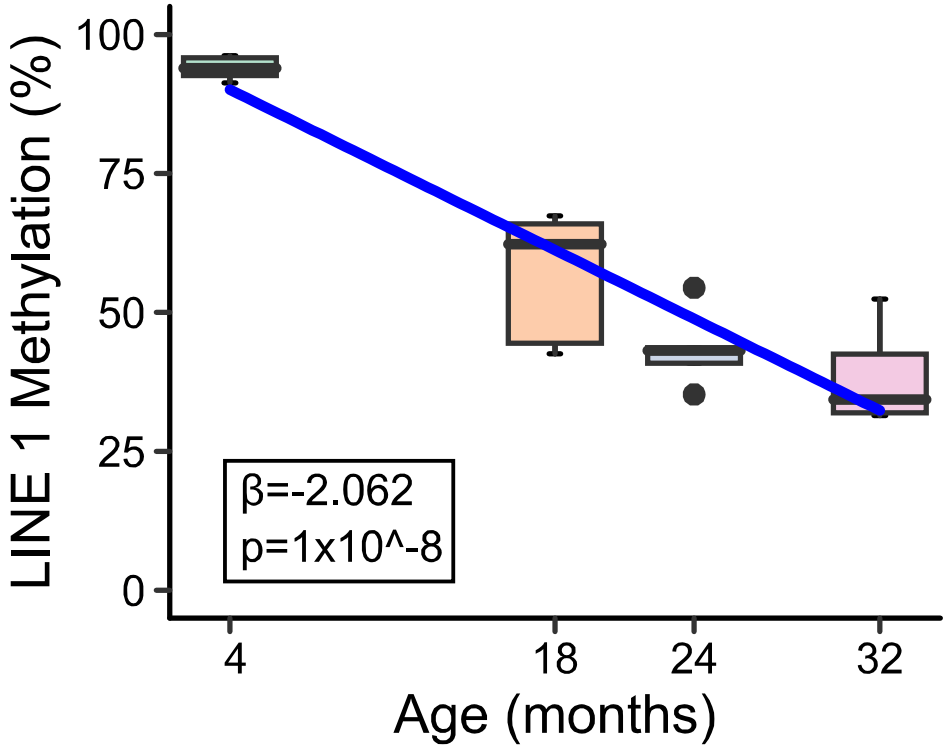


Fig. S2 Representative LINE1 melt curves by age

Aligned melt curve from High resolution melt assay showing representative curves for ages 4, 18, 24, and 32 months. Melt curves of the 0 and 100 % methylation standard are shown for reference.

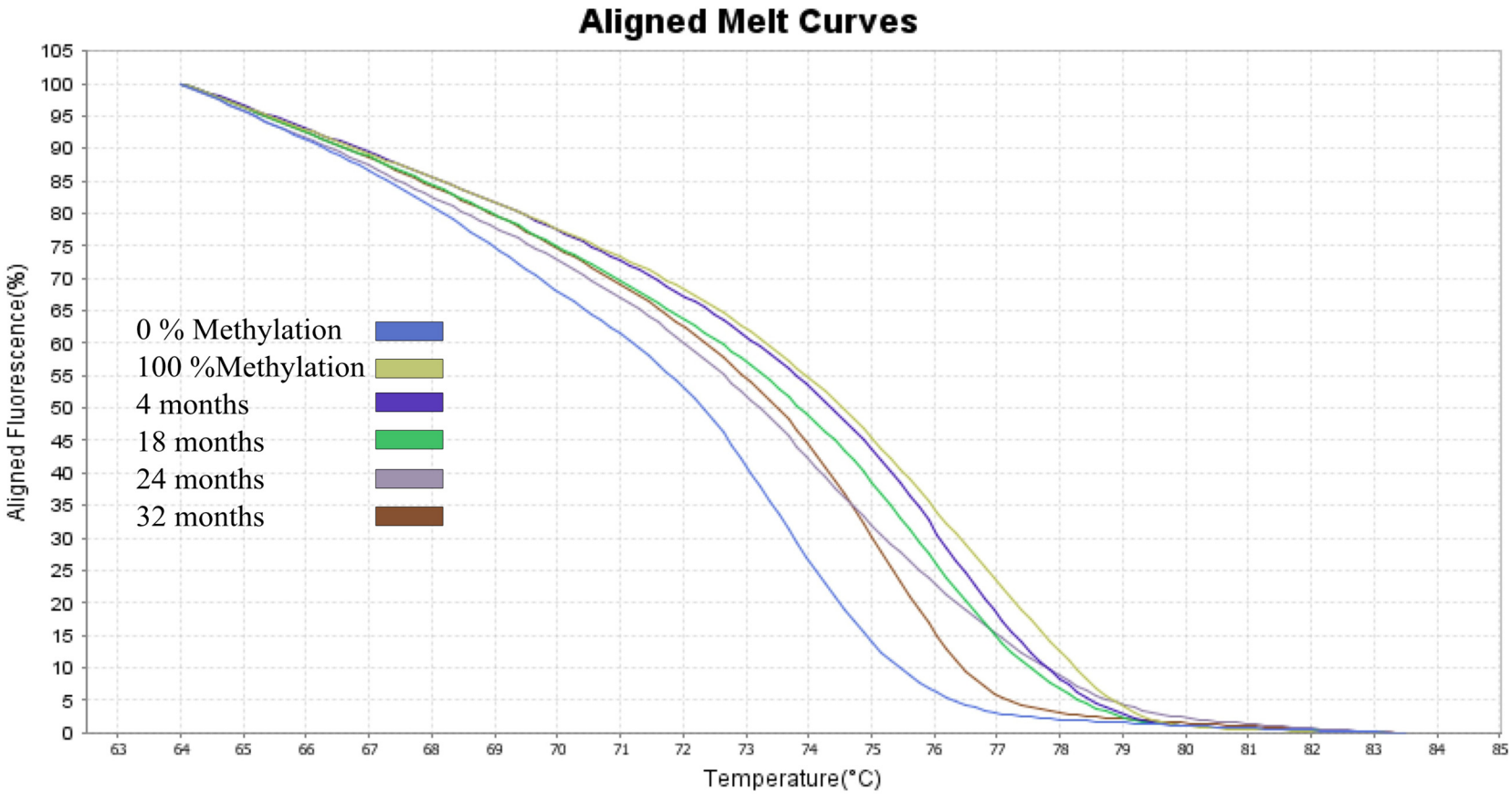


Fig. S3 *Cyp1b1* 5mC with age

Boxplot showing no changes to methylation at *Cyp1b1* as age increases from 4 to 32 months

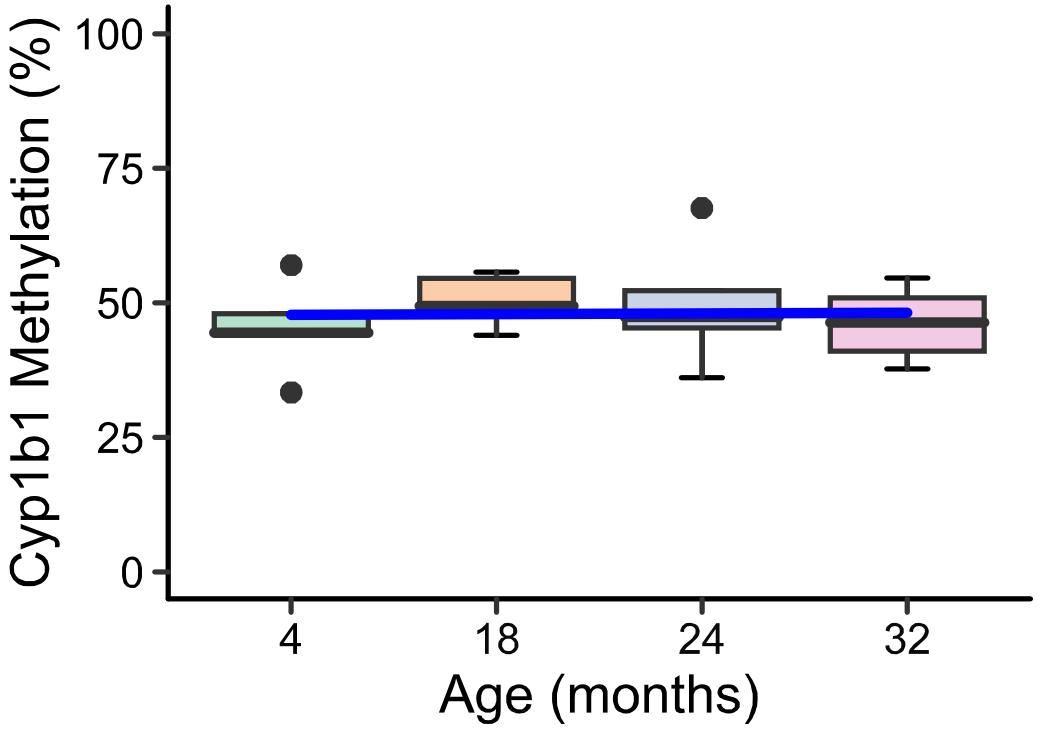


Fig. S4 Chlorzoxazone standard curve

High performance liquid chromatography coupled with Ultraviolet detection method linearity for parent probe drug Chlorzoxazone. Linear range established at 2.34-1200 µM. Scatter plot with linear regression line of peak area intensity (mAU.s) against chlorzoxazone concentration (µM).

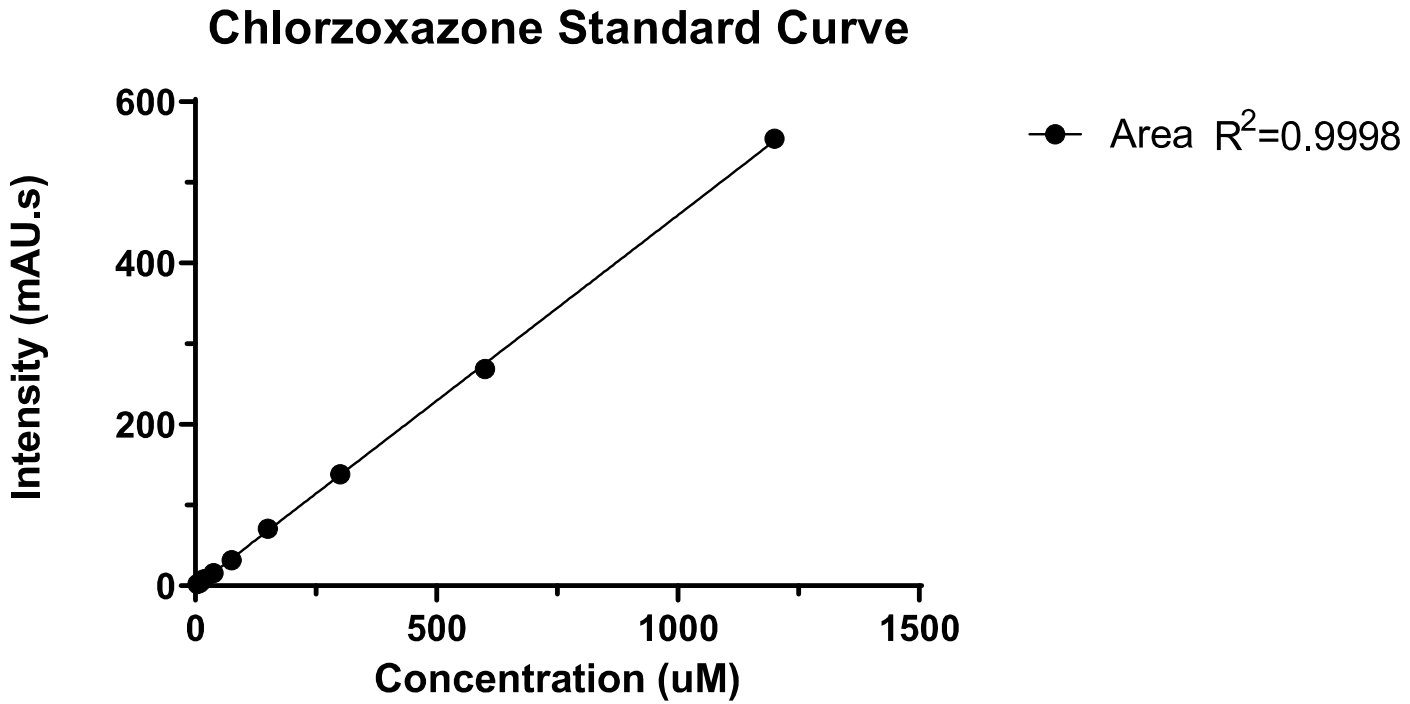


Fig. S5 6-hydroxychlorzoxazone standard curve

High performance liquid chromatography coupled with Ultraviolet detection method linearity for metabolite 6-hydrozychlorzoxazone. Linear range established at 0.47-240 µM. Scatter plot with linear regression line of peak area intensity (mAU.s) against 6-hydroxychlorzoxazone concentration (µM).

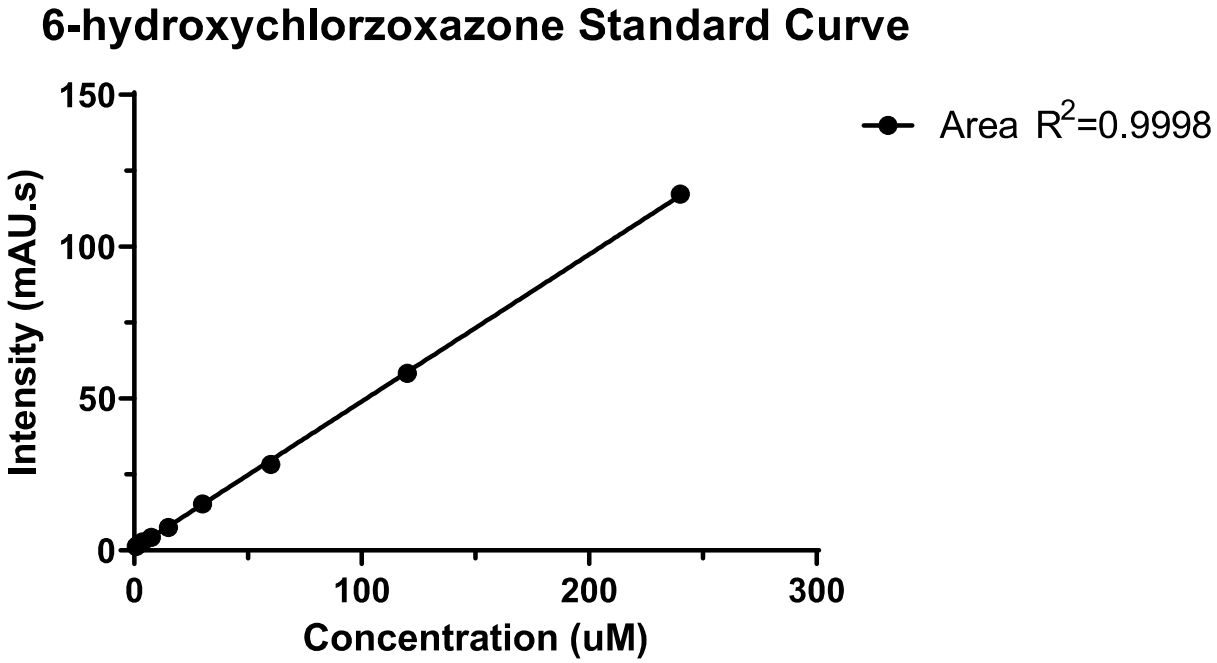


Fig. S6 Metabolite formation against time plot

Line plot of 6-hydroxychlorzoxazone (6OH-CZ) concentration (μM) against tested total microsome reaction time (min). n=3 for each time point.

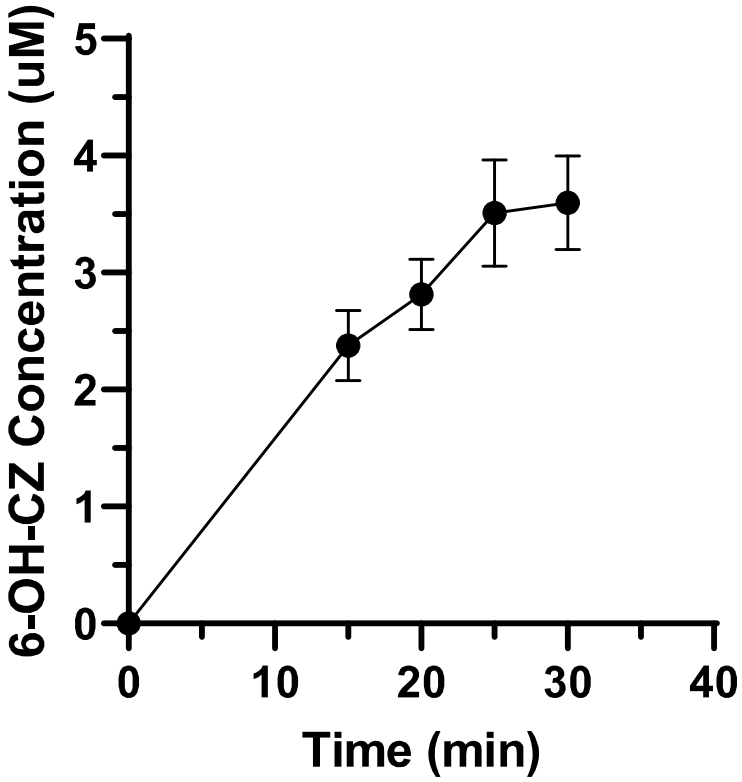


Fig. S7 Metabolite formation against microsome protein plot

Line plot of 6-hydroxychlorzoxazone (6OH-CZ) concentration (μM) against tested final Mouse Liver Microsome (MLM) total protein concentration (mg/mL). n=3 for each concentration.

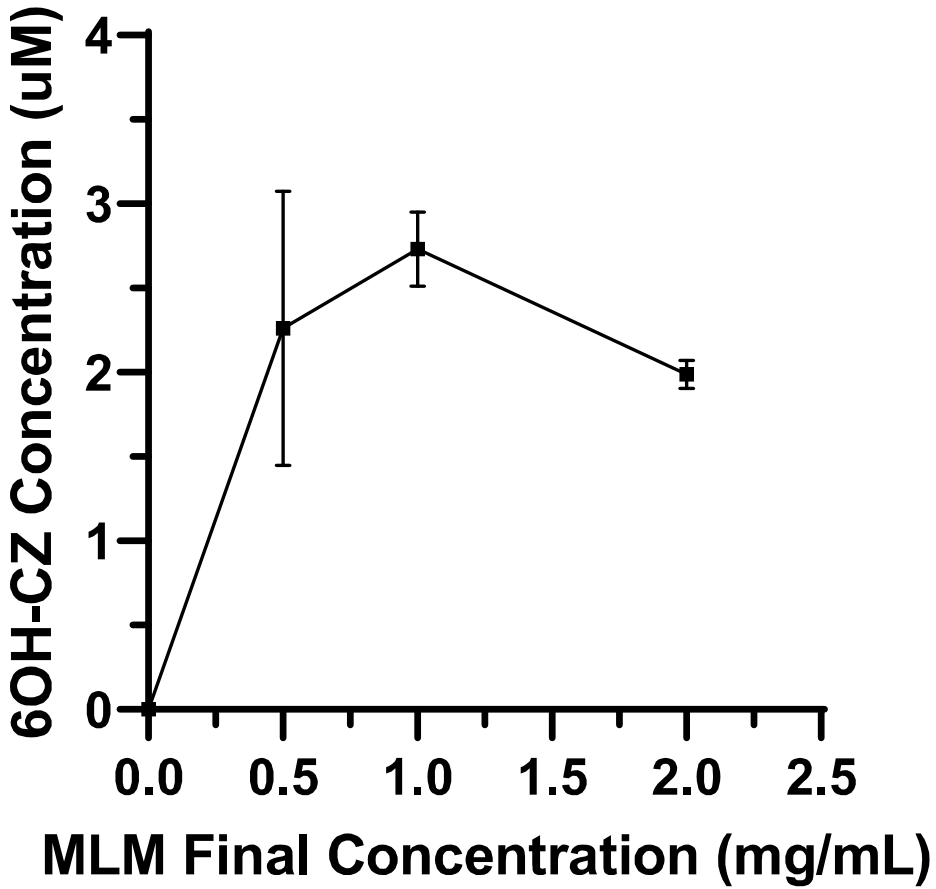
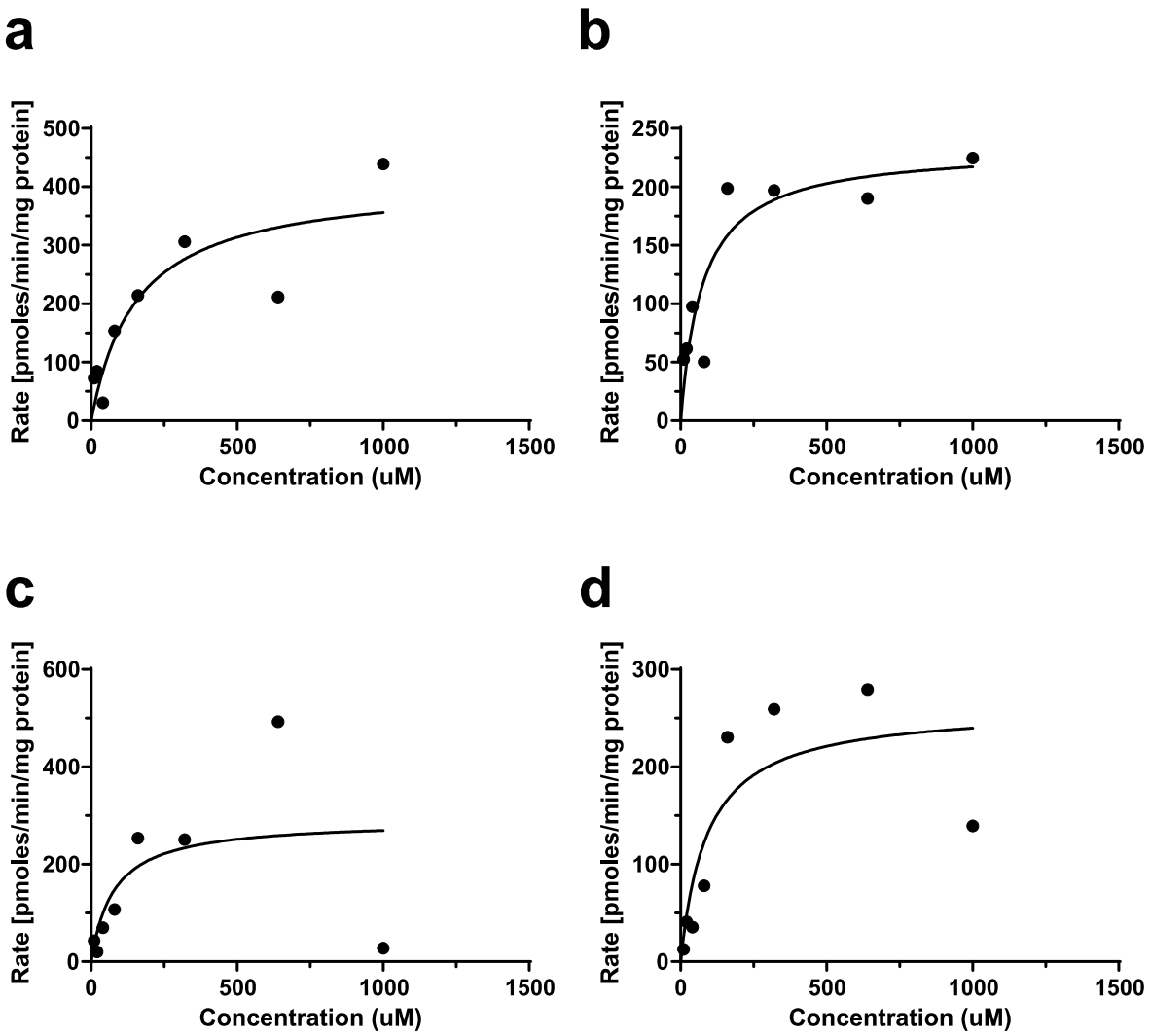


Fig. S8 Representative Michaelis-Menten curves of each age group

Representative Michaelis-Menten curves for (a) 4 months, (b) 18 months, (c) 24 months, and (d) 32 months ages. X-axis is parent drug chlorzoxazone concentration (μM). Y-axis is reaction rate (pmol/min/mg protein).



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