A Novel CD73 Inhibitor SHR170008 Suppresses Adenosine in Tumor and Enhances Anti-tumor Activity with PD-1 Blockade in a Mouse Model of Breast Cancer

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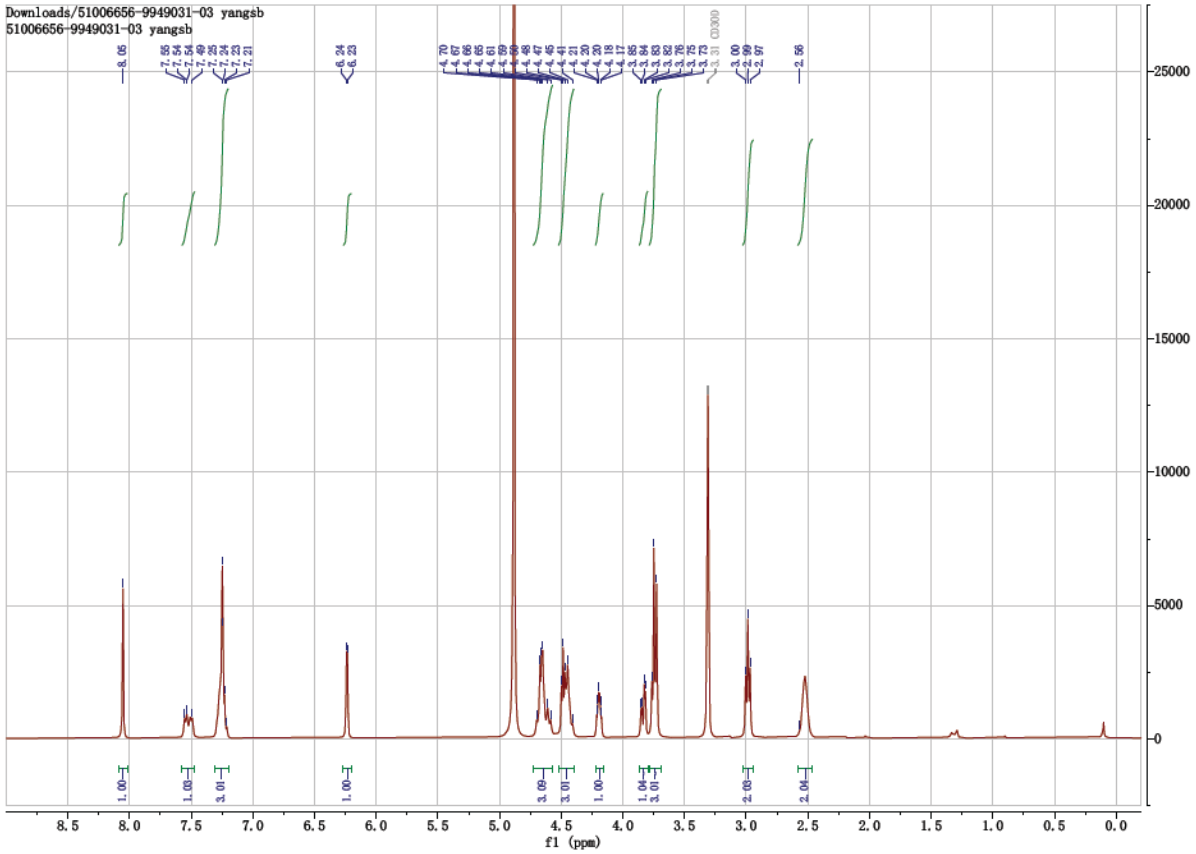
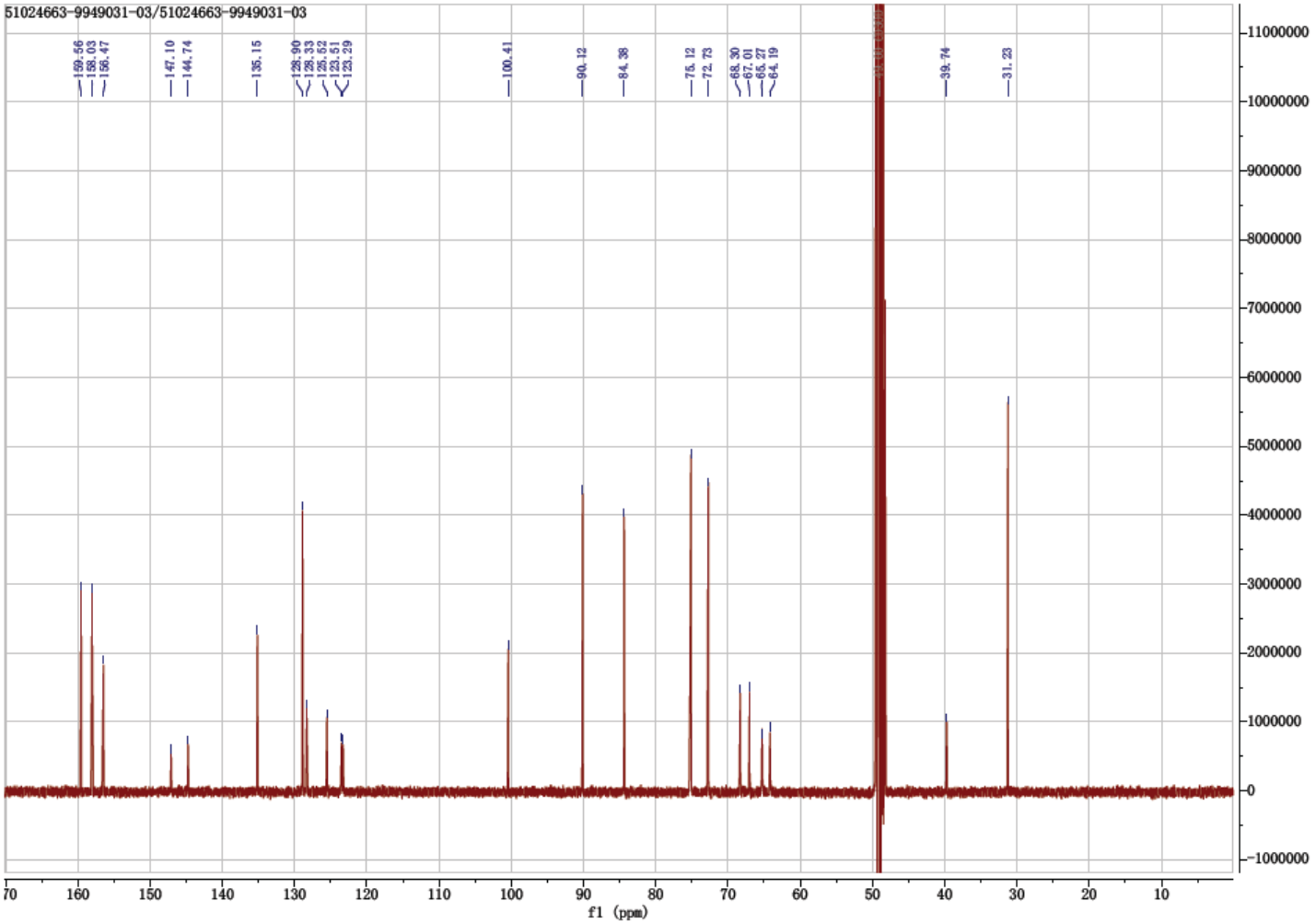
Supplementary Materials and Methods

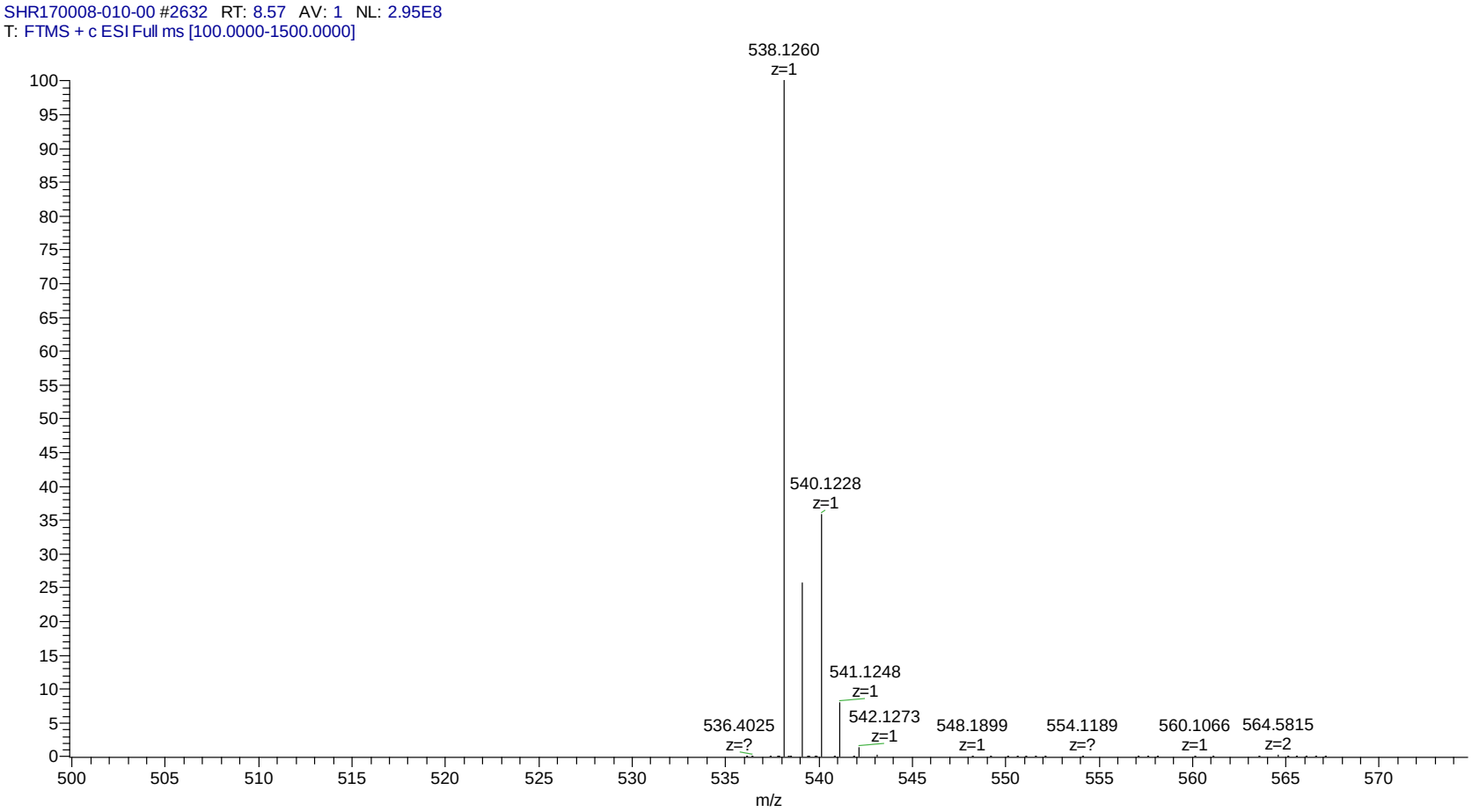
Figure S1 Characterization of SHR170008 by analyses of ^{1}H NMR, ^{13}C NMR, HRMS and HPLC. **Figure S1** Characterization of SHR170008 by analyses of ^{1}H NMR, ^{13}C NMR, HRMS and HPLC. (**A**) ^{1}H NMR spectrum of SHR170008 in Methanol-d_4 (500 MHz). (**B**) ^{13}C NMR spectrum of SHR170008 in Methanol-d_4 (126 MHz). (**C**) HRMS spectrum of SHR170008 ($[M+H]^+$ = 538.1260). (**D**) HPLC spectrum of SHR170008.



(A) ^{1}H NMR spectrum of SHR170008 in Methanol-d_4 (500 MHz).



(**B**) ^{13}C NMR spectrum of SHR170008 in Methanol-d_4 (126 MHz).



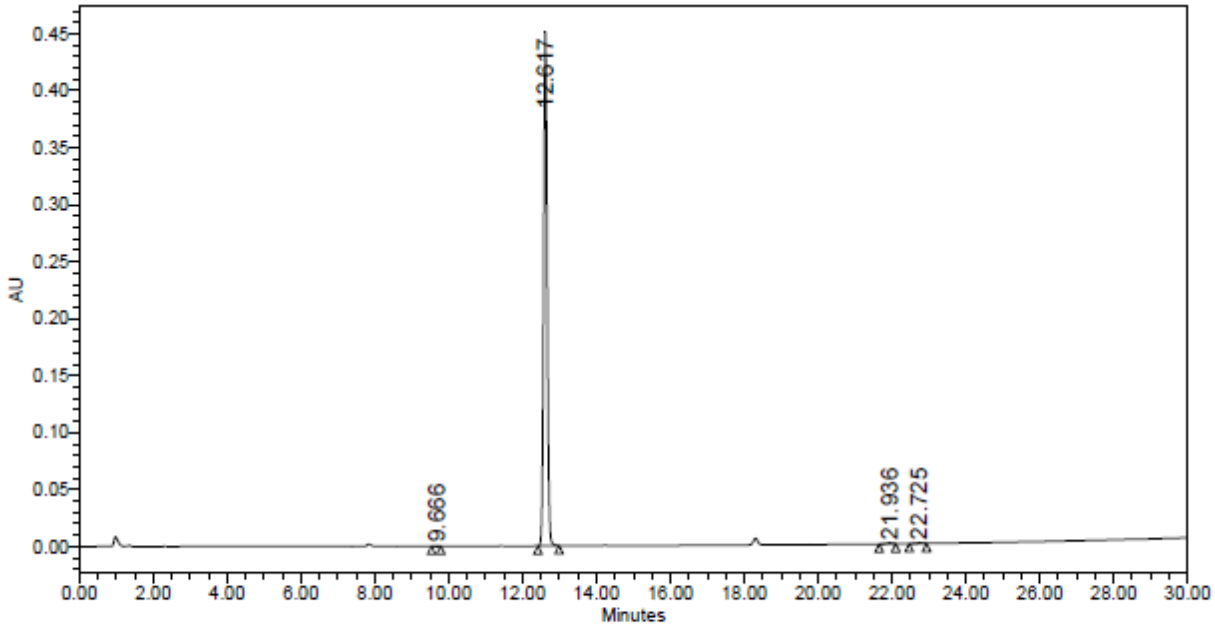
(**C**) HRMS spectrum of SHR170008 ($[M+H]^+$ = 538.1260).

Empower 2 software

HPLC

SAMPLE INFORMATION

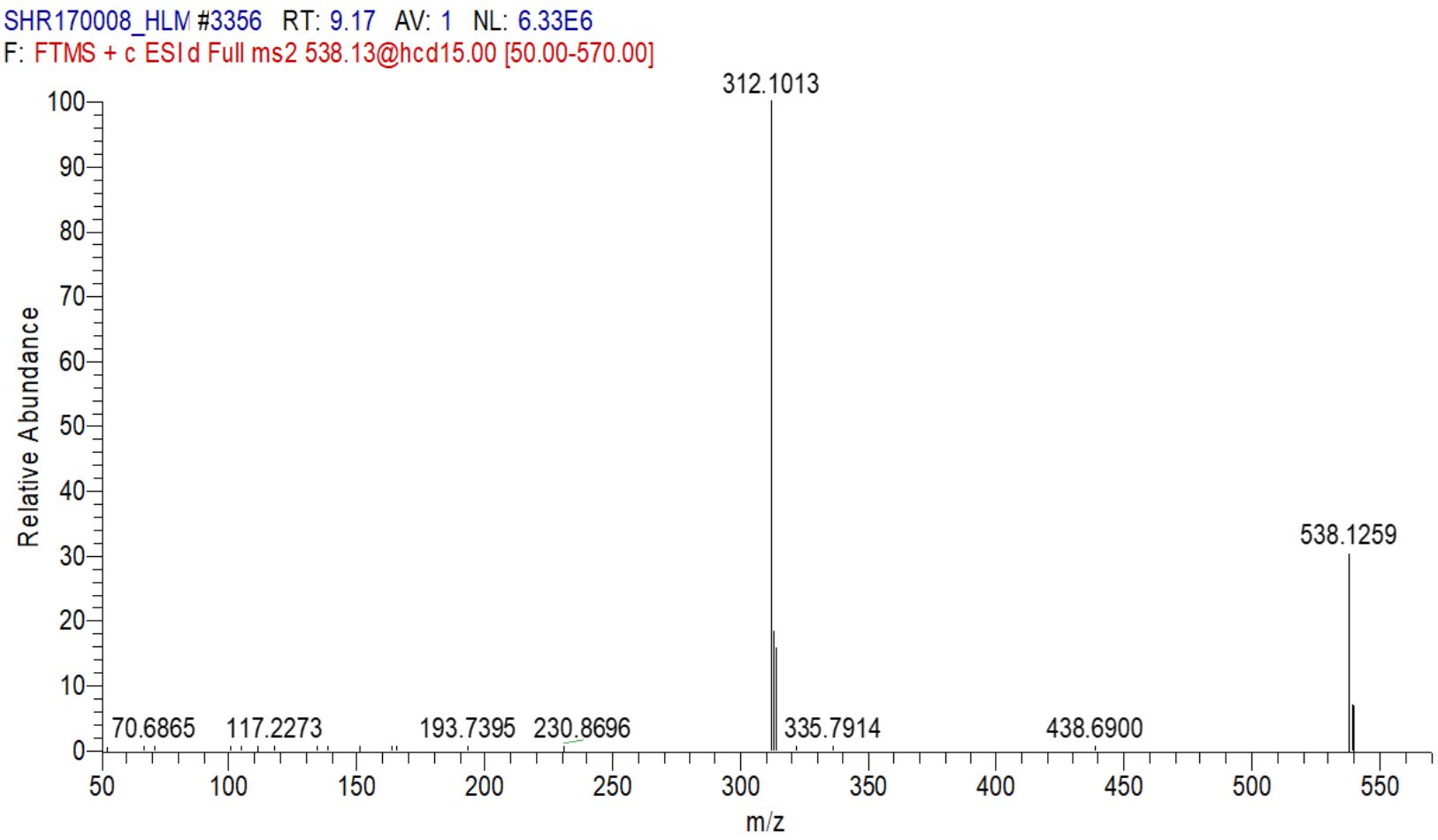
Sample Name:	70011225-9949020-01	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	20181031_1
Vial:	88	Acq. Method Set:	M_5_95_30MIN_BC
Injection #:	1	Processing Method	HPLC
Injection Volume:	5.00 ul	Channel Name:	W2489 ChB
Run Time:	30.0 Minutes	Proc. Chnl. Descr.:	W2489 ChB 254nm
Date Acquired:	10/31/2018 11:07:20 AM CST		
Date Processed:	10/31/2018 11:46:44 AM CST		



	RT	Area	% Area	Height	USP Resolution
1	9.666	1792	0.06	274	
2	12.617	2977273	99.55	448397	16.26
3	21.936	6753	0.23	614	37.38
4	22.725	4975	0.17	455	2.49

(**D**) HPLC spectrum of SHR170008.

Figure S2 MS/MS spectrum of SHR170008 in human liver microsomes.



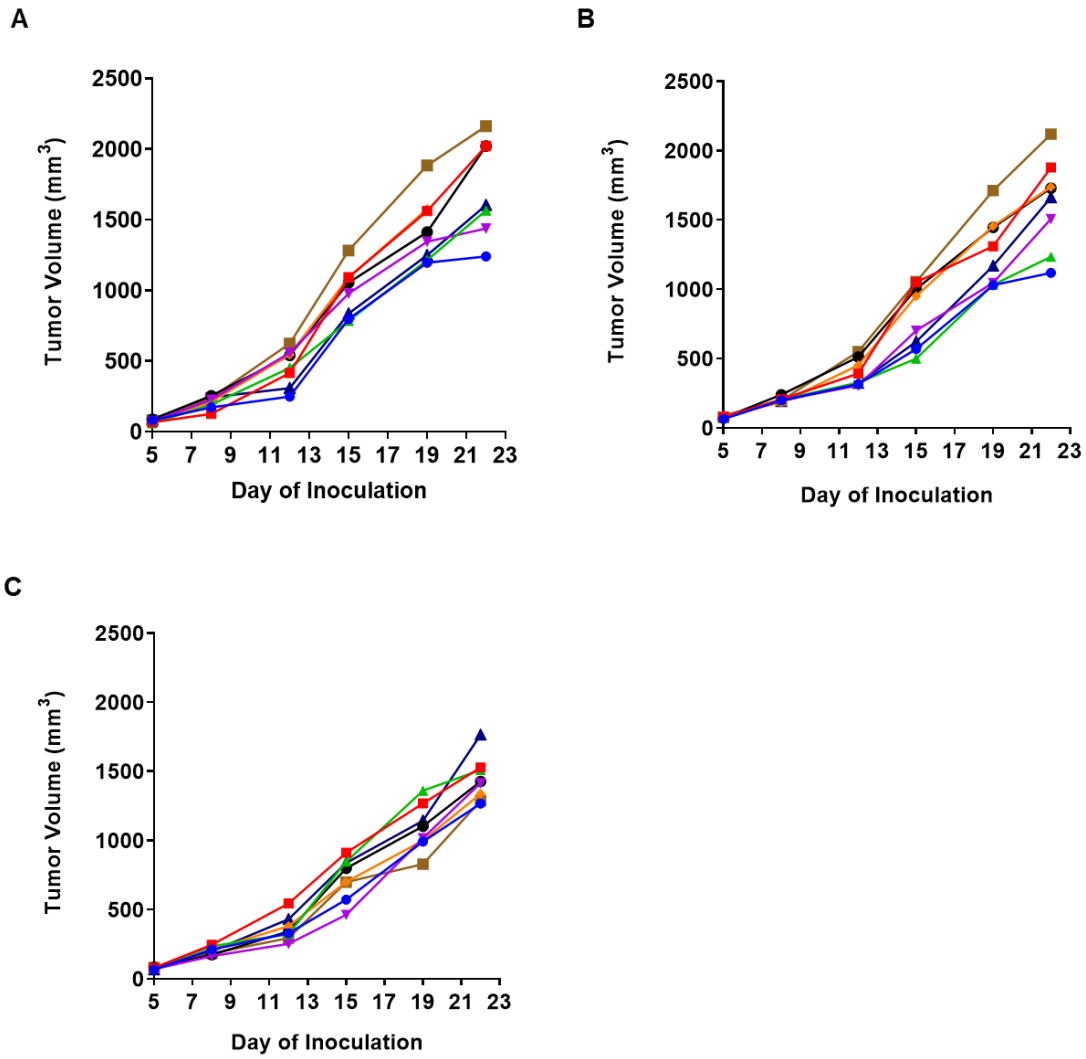


Figure S3 Tumor growth curves of individual mouse bearing EMT6 mouse breast tumor during treatment with 1 mg/kg (**A**), 3 mg/kg (**B**) and 10 mg/kg of SHR170008 (**C**).

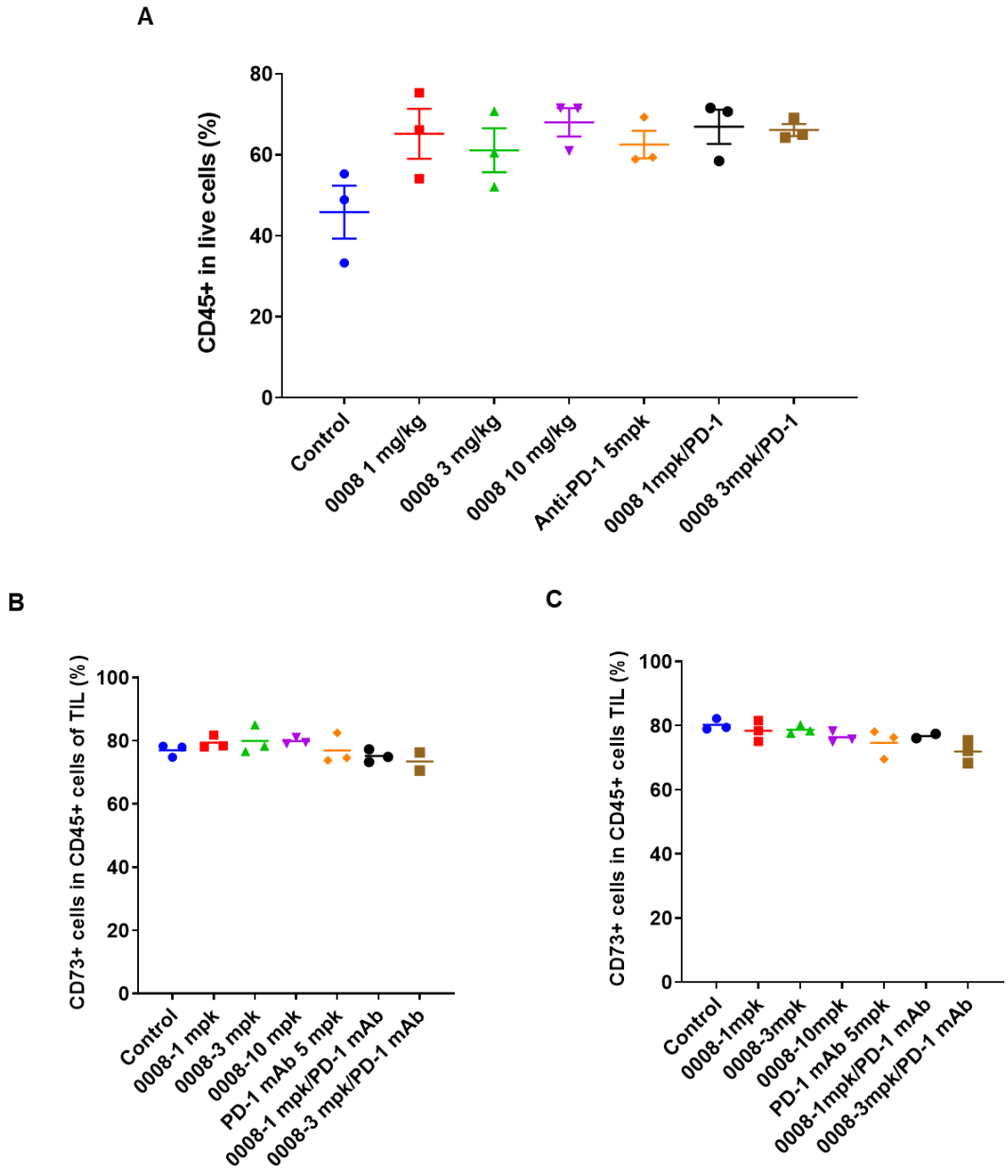


Figure S4 Tumor infiltrating leukocyte (TIL) in tumor tissues and CD73 expression on TIL. (**A**) TIL marker CD45 positive cells in tumor tissues collected at 2 hours post dosing of SHR170008 at the end of the study. Data show mean ± SEM. (**B and C)** The expression of CD73 on $CD45^{+}$ cells from tumor tissues at 2 hours post dosing (**B**) and 6 hours post dosing (**C**). mpk = mg/kg. 0008 = SHR170008.

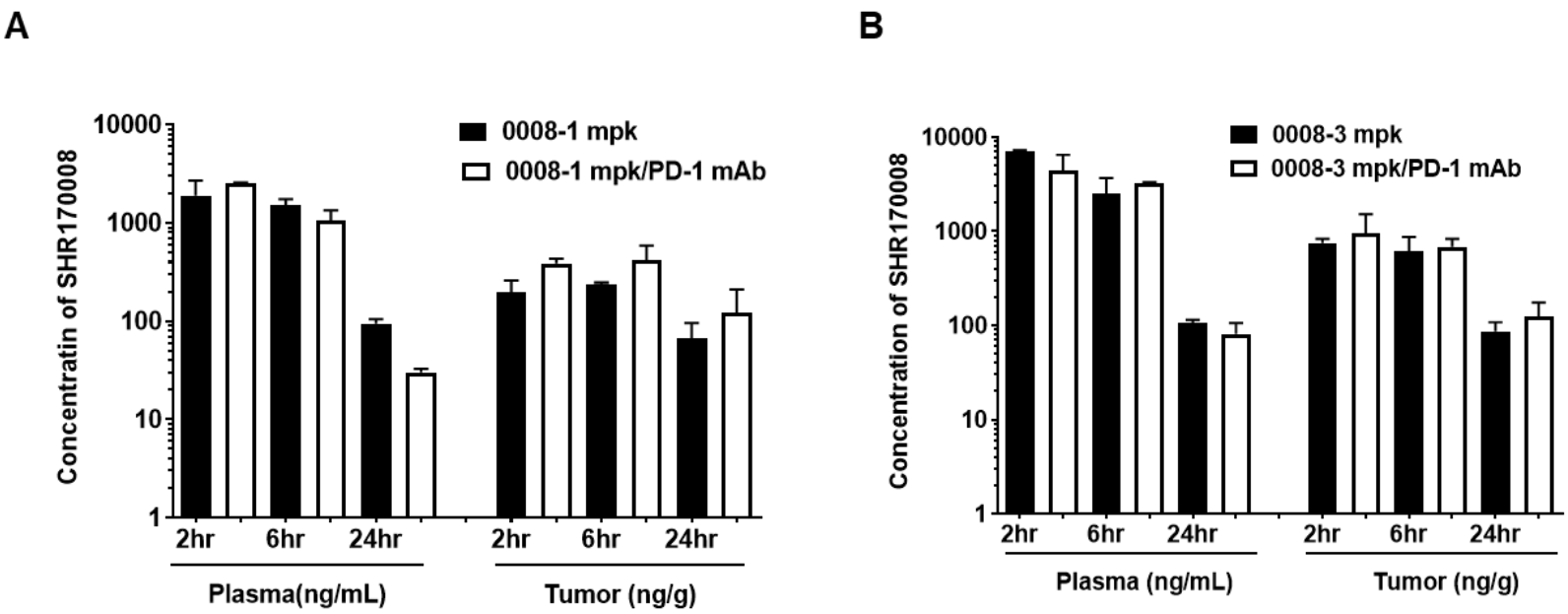


Figure S5 Exposures of SHR170008 in plasma and tumor tissues from syngeneic EMT6 mouse breast tumor model. (**A** and **B**) Exposures of SHR170008 at 1 mg/kg (**A**) or 3 mg/kg (**B**) as single agent (black bar) and in combination group (open bar) at the end of the study are shown. Symbols indicated mean ± SEM. 0008 = SHR170008.

Table S1 Stability of SHR170008 in rat plasma

Time (minute)	**SHR170008**		**Dierliuzuo**	
	%Remaining	$t_{1/2}$ (minute)	%Remaining	$t_{1/2}$ (minute)
0	100.0	154	100.0	26.0
30	80.6		45.7	
60	68.7		19.3	
90	67.3		8.07	
120	65.7		4.26	
240	65.8		0.17	

Table S2 Observed metabolites of SHR170008 in mouse, rat, dog, monkey, and human liver microsomes on LC-ddMS2 scan

Species	Peak ID	RT (min)	Metabolic pathway	Mass Shift	Formula Change	Theo. m/z	Meas. m/z	ppm	Peak Area
Mouse	SHR170008	8.795	SHR170008	0.0000000	-	538.1252888	538.1259766	1.3	362921538
Rat	SHR170008	8.888	SHR170008	0.0000000	-	538.1252888	538.1257324	0.8	390425194
Dog	SHR170008	8.973	SHR170008	0.0000000	-	538.1252888	538.1257324	0.8	405283279
Monkey	SHR170008	9.079	SHR170008	0.0000000	-	538.1252888	538.1259155	1.2	409237283
Human	SHR170008	9.119	SHR170008	0.0000000	-	538.1252888	538.1254272	0.3	430241899

Table S3 Observed metabolites of SHR170008 in mouse and rat plasma on LC- ddMS2 scan

Species	Peak ID	RT (min)	Metabolic pathway	Mass Shift	Formula Change	Theo. m/z	Meas. m/z	ppm	Peak Area
Mouse_ig	SHR170008	9.147	SHR170008	0.0000000	-	538.1252888	538.1264648	2.2	4955573
Mouse_iv	SHR170008	8.985	SHR170008	0.0000000	-	538.1252888	538.1260986	1.5	281494070
Rat_ig	SHR170008	9.175	SHR170008	0.0000000	-	538.1252888	538.1264038	2.1	13084565
Rat_iv	SHR170008	8.965	SHR170008	0.0000000	-	538.1252888	538.1258545	1.1	747277169
dog_ig	SHR170008	9.056	SHR170008	0.0000000	-	538.1252888	538.1267090	2.6	3329263
dog_iv	SHR170008	9.037	SHR170008	0.0000000	-	538.1252888	538.1263428	2.0	31159968
Monkey_ig	SHR170008	9.172	SHR170008	0.0000000	-	538.1252888	538.1262207	1.7	18803690
Monkey_iv	SHR170008	9.158	SHR170008	0.0000000	-	538.1252888	538.1259766	1.3	572366503

ig = intragastric adminisration; iv = intravenous administration.

Table S4 The screening results of SAFETYscan47 panel with 3 μM SHR170008

Assay Name	Assay Target	Mode	% Response		
			Replicate 1	Replicate 2	Average
Calcium Flux	ADORA2A	Agonist	0.0	3.9	2.0
		Antagonist	0.0	4.7	2.3
	ADRA1A	Agonist	0.0	0.3	0.1
		Antagonist	0.0	0.0	0.0
cAMP	ADRA2A	Agonist	9.5	8.6	9.0
		Antagonist	0.0	0.0	0.0
	ADRB1	Agonist	1.3	0.0	0.6
		Antagonist	0.0	0.3	0.1
	ADRB2	Agonist	0.0	0.0	0.0
		Antagonist	0.0	11.3	5.6
Calcium Flux	AVPR1A	Agonist	1.1	0.0	0.5
		Antagonist	0.0	0.0	0.0
	CCKAR	Agonist	0.7	0.7	0.7
		Antagonist	0.0	0.0	0.0
	CHRM1	Agonist	1.7	0.0	0.9

		Antagonist	0.0	0.0	0.0
cAMP	CHRM2	Agonist	0.0	0.0	0.0
		Antagonist	0.0	2.4	1.2
Calcium Flux	CHRM3	Agonist	0.0	0.0	0.0
		Antagonist	0.0	0.0	0.0
cAMP	CNR1	Agonist	1.7	5.4	3.5
		Antagonist	12.0	5.0	8.5
	CNR2	Agonist	3.9	11.6	7.8
		Antagonist	0.0	9.2	4.6
	DRD1	Agonist	0.0	0.0	0.0
		Antagonist	0.0	0.0	0.0
	DRD2S	Agonist	0.0	0.0	0.0
		Antagonist	0.9	1.7	1.3
Calcium Flux	EDNRA	Agonist	0.0	0.0	0.0
		Antagonist	0.0	0.0	0.0
	HRH1	Agonist	0.0	0.0	0.0
		Antagonist	0.0	0.0	0.0
cAMP	HRH2	Agonist	0.0	0.0	0.0

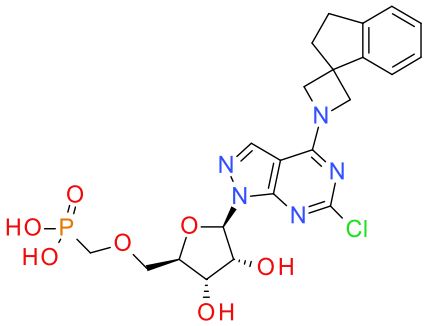
		Antagonist	0.0	0.0	0.0
	HTR1A	Agonist	1.6	4.3	2.9
		Antagonist	5.3	5.3	5.3
	HTR1B	Agonist	0.0	0.4	0.2
		Antagonist	8.6	2.2	5.4
Calcium Flux	HTR2A	Agonist	0.0	0.0	0.0
		Antagonist	0.0	0.0	0.0
	HTR2B	Agonist	0.0	1.4	0.7
		Antagonist	0.0	0.0	0.0
cAMP	OPRD1	Agonist	0.0	2.4	1.2
		Antagonist	3.5	0.0	1.8
	OPRK1	Agonist	0.0	0.0	0.0
		Antagonist	0.0	1.0	0.5
	OPRM1	Agonist	0.0	0.0	0.0
		Antagonist	0.8	2.4	1.6
NHR Nuclear Translocation	AR	Agonist	0.0	0.0	0.0
		Antagonist	0.0	4.2	2.1
NHR Protein	GR	Agonist	0.1	0.0	0.0

Interaction		Antagonist	6.0	0.0	3.0
Transporter	DAT	Blocker	0.0	0.0	0.0
	NET	Blocker	0.0	0.0	0.0
	SERT	Blocker	1.3	0.0	0.7
Ion Channel	CAV1.2	Blocker	0.0	2.5	1.2
	GABAA	Opener	0.0	0.0	0.0
		Blocker	0.2	0.0	0.1
	hERG	Blocker	0.0	0.0	0.0
	HTR3A	Opener	0.0	0.0	0.0
		Blocker	0.0	3.4	1.7
	KvLQT1/minK	Opener	0.0	0.0	0.0
		Blocker	6.7	3.0	4.9
	nAChR(a4/b2)	Opener	0.0	0.0	0.0
		Blocker	0.0	0.0	0.0
	NAV1.5	Blocker	1.5	0.0	0.7
	NMDAR (1A/2B)	Opener	0.0	0.0	0.0
		Blocker	0.0	0.0	0.0
Non-Kinase	AChE	Inhibitor	0.7	6.6	3.7

Enzymatic	COX1	Inhibitor	2.4	0.9	1.7
	COX2	Inhibitor	10.5	2.3	6.4
	MAOA	Inhibitor	13.2	9.5	11.4
	PDE3A	Inhibitor	1.6	1.3	1.4
	PDE4D2	Inhibitor	6.2	14.5	10.3
Kinase Binding	INSR	Inhibitor	18.5	16.8	17.7
	LCK	Inhibitor	8.4	3.3	5.8
	ROCK1	Inhibitor	3.3	11.1	7.2
	VEGFR2	Inhibitor	15.2	0.0	7.6

Supplementary Methods

Characterization of SHR170008 by analyses of ^{1}H NMR, ^{13}C NMR, HRMS and HPLC



SHR170008

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AVANCE II 400 MHz spectrometer. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker AVANCE NEO 500 MHz spectrometer. All chemical shifts are reported in parts per million (δ) relative to an internal standard tetramethylsilane. The following abbreviations are used to denote signal patterns: s = singlet, d = doublet, t = triplet, m = multiplet, and br = broad. The elemental composition was derived from the mass spectra acquired at the high resolution of about 140,000 on a Thermo Q Exactive mass spectrometer (Thermo Scientific). The high mass accuracy below 3 ppm was obtained by using a lock mass.

The purity of intermedia and SHR170008 were assessed by analytical HPLC. Purity data were collected by a HPLC with Waters e2695-2489 (uv/visible detector). The column was a Waters SunFire C18 (3.5 μm particle size, I.D. × L

4.6 mm × 75 mm, Waters, America) with a temperature of 30°C and a flow rate of 1.0 mL/min. Mobile phases A and B under an acidic condition were a mixture of 0.1% trifluoroacetic acid in H_2O and 0.1% trifluoroacetic acid in CH_3CN, respectively. The ratio of mobile phase B was increased linearly from 5% to 95% over 30 minutes.

^{1}H NMR (500 MHz, Methanol-d_4) δ (ppm) 8.05 (s, 1H), 7.58 – 7.47 (m, 1H), 7.35 – 7.21 (m, 3H), 6.24 (d, *J* = 4.0 Hz, 1H), 4.73 – 4.57 (m, 3H), 4.52 – 4.40 (m, 3H), 4.22 – 4.16 (m, 1H), 3.84 (dd, *J* = 10.9, 3.7 Hz, 1H), 3.76 – 3.72 (m, 3H), 2.99 (t, *J* = 7.0 Hz, 2H), 2.60 – 2.48 (m, 2H).

^{13}C NMR (126 MHz, Methanol-d_4) δ (ppm) 159.56, 158.03, 156.47, 147.10, 144.74, 135.15, 128.90, 128.33, 125.52, 123.51, 123.29, 100.41, 90.12, 84.38, 75.12, 72.73, 68.30, 67.01, 65.27, 64.19, 39.74, 31.23.

HRMS: calculated for $C_{22}H_{25}ClN_5O_7P$ $(M+H)^+$ 538.1258, found 538.1260.

HPLC purity: 99.55%.

SAFETYscan *In Vitro* Pharmacological Profiling

SAFETYscan testing was carried out by DiscoverX (San Diego, CA). SHR170008 was screened at 3 μM in duplicates.

Analysis of rat plasma stability:

Rat plasma and SHR170008 or control compound (Dierliuzuo) was mixed in individual wells of a 96-well plate. The final concentration of the compound was 1 μM (0.06% DMSO) in rat plasma. The plate was incubated at 37℃ in water bath with gentle agitation. During the incubation, aliquots were withdrawn at the time point indicated (0, 15, 30, 60, 90, 120, and 240 minutes, respectively). At timing start (0 minutes), internal standard glacial acetonitrile was added. At other time points, ACN solution containing internal standard was used to quench the reaction. After mixing, the quenched aliquots were centrifuged, and supernatant was withdrawn for analysis by LC-MS/MS. The slope of the ln (%remaining) versus time point line was used to calculate $t_{1/2}$ according to the following formula:

$$Half\ lie\ (t_{1/2}) = -\frac{\ln 2}{Slope}$$

Identification and Profiling of Metabolites

Liver microsomes of CD-1 mouse (8178001), Sprague-Dawley rat (8283002), Beagle dog (6116002), Cynomolgus monkey (0041001CNC) and human (0349001) were purchased from Corning Gentest. Microsomal incubation was carried out with liver microsomes of indicated (0.5 mg protein/mL), 10 μM SHR170008, 1 mM Nicotinamide adenine dinucleotide phosphate (NADPH, Acros Organics, A0354537) and 3 mM $MgCl_2$ (Sigma-Aldrich, M8266) in a total volume of 400 μL. The mixture was pre-incubated at 37˚C for 5 minutes and then NADPH was added to start the reaction. After incubation for 1 hour at 37˚C, an aliquot of 0.8 mL of acetonitrile was added to terminate the reaction and the mixtures were

then centrifuged at 20,879 × *g* for 10 minutes. The resulting supernatants were dried in vacuum. The residues were reconstituted with 200 μL of water-acetonitrile solution (*v/v*, 3:1) and centrifuged. 5 μL of the supernatant was submitted to UPLC-QE-HRMS for metabolite identification and profiling.

To identify the metabolites presented *in vivo*, the plasma of mouse and rat were collected and pooled, respectively, according to the AUC rule described by Cornelis Hop *et al* [1] The plasma samples were mixed with 4-fold volume of acetonitrile. The mixtures were then centrifuged at 20,879 × *g* for 10 minutes and the supernatants were dried in vacuum. The residue of each sample was reconstituted, and the supernatant was submitted to UPLC-QE-HRMS for metabolite identification and profiling as described above. The housing conditions and in-life procedures were approved by the Institutional Animal Care and Use Committees (IACUC). The experiments were carried out in accordance with the Guiding Principles for the Care and Use of Laboratory Animals and complied with the ARRIVE guidelines.

References:

1. Hop CECA, Wang Z, Chen Q, Kwei G. Plasm-pooling methods to increase throughput for in vivo pharmacokinetic screening. *J Pharm Sci.* 1998;*87*, 901-903. doi: 10.1021/js970486q