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Supplemental information

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Supplementary methods

Surface Plasmon Resonance assay

Binding assay to Kelch domain of KEAP1 was performed by Surface Plasmon resonance (SPR) on a Biacore T200 (GE Healthcare) at 25°C.

Immobilization: HIS-Thromb-KEAP1 (A321-C624) (Novalix, Batch PU-P01; MW:33812.9 Da) is immobilized on the active flow cell of a CM5 chip at a concentration of 40 µg/mL (coupling buffer : 10 mM Na acetate pH 5.5) using the standard wizard method (amine coupling). Amine blank is used as the reference flow cell.

The immobilization buffer is 10 mM Hepes, 150 mM NaCl, 3 mM EDTA pH 7.4, 0.05% P20 (HBS-EP+), 1mM TCEP pH 7.4. Immobilization target is reached at 2500 RU, with a 10 µL/min flowrate and at 25°C.

Response bound (RU) :	FcAct
20170913 N=1	2510 RU
20170913 N=2	2627 RU

Assay: The running buffer is 10 mM Hepes, 150 mM NaCl, 3 mM EDTA pH 7.4, 0.05% P20 (HBSEP) pH 7.4, 1 mM TCEP + 5% DMSO.

Compounds bindings are monitored by Single-Cycle Kinetics (High Performance), with 5 increasing concentrations per cycle. Injection runs for 180 seconds at a 50 µL/min flowrate, dissociation runs for 360 seconds at a 50 µL/min flowrate, both at 25°C. For very slow

dissociation rate, dissociation time was extended to 1h30 to get reliable data. Extra wash after the 5 cycles is a solution of 50% DMSO. Carry-over and solvent correction are applied.

Step	**Solution**	**Time (s)**	**Flowrate (µL/min)**	**Running Buffer**	**# of iteration**
Association Dissociation	S compound Running Buffer	180 360 / 5400	50 50	HBSEP, 1 mM TCEP, 5% DMSO	x5
Extra wash	50% DMSO				

NRF2 translocation assay

PathHunter® U2OS Keap1-NRF2 Nuclear Translocation Cell Line was provided by Eurofins (DiscoverX). PathHunter U2OS cells were grown in Minimum Essential Medium (MEM) (#30-2003, ATCC), 10% FBS (#P30-193306), PanBiotech), 500 µg/ml Geneticine, 250 µg/ml Hygromycine, 0.25 µg/ml Puromycine (10131-027, 10687-010 and A1113802 respectively, Life Technologies). The night before experiment, cells were harvested using Tryple Xpress (Ref.126905, Bibco) for 5min at 37°C, resuspended in Opti-MEM (Ref.31985-047, Gibco), 1% FBS (#P30-193306, Panbiotech) and then plated into 384 well plates (Ref.6007680, Perkin Elmer) at the density of 7,500 cells/well in 20 µl. Cells are stored overnight at 37°C (5% CO2) until used. Compounds in 100% DMSO (0.315 µl/well) were resuspended in 20 µl MEM, 1% FBS, 3.4% DMSO. Compounds and Andrographolide (10 µM final concentration; Ref.365645-500MG, Aldrich) as positive control, were dispensed on cell, 5 µl/well, and then incubated for 3h at 37°C and 5% CO2. After incubation, PathHunter reagents were dispensed on cells (12 µl/well, Ref.93-0001, DiscoverX) and incubated for 60 min at room temperature in the dark.

Then luminescence signal was measured using multimodal reader (Pherastar, BMG Labtech). Data were normalized between 1% DMSO (basal signal) and 10 µM Andrographolide (positive signal) and analyzed using Activity Base software.

HepG2 NRF2 transactivation assay

CellSensor™ ARE-bla Hep G2 Cell Line (#K133) containing a betalactamase reporter gene under control of an ARE was provided by Thermofisher. CellSensor® ARE-bla HepG2 cells were grown to confluence in DMEM GlutaMAXTM (#61965-026, Thermo Fisher), 10% dialyzed FBS (#P30-193306, PanBiotech), 12mM Hepes (#15630-056, Gibco), 0.1mM Non-Essential Amino Acid (NEAA) Cell Culture Supplement (#11140-35, Gibco), 1% Na-pyruvate (#S8636, Sigma), 2.5 µg/ml Blasticidine (#210-01, Invitrogene), 1% Penicilline/streptomycine (15070-063, Gibco) in collagen I (50 µg/ml, #A10483-1, Life technologies) coated flasks, 37°C, 5% CO2. Eighteen hours before the experiment, cells were harvested using TrypleXpress (Ref126905, Bibco) for 10 min at 37°C, resuspended in DMEM GlutaMAXTM (#61965-026, Thermo Fisher), 1% dialyzed FBS (#P30-193306, PanBiotech), 25 mM Hepes (#15630-056, Gibco) 0.1mM NEAA (#11140-35, Gibco), 1% Na-pyruvate (#S8636, Sigma), 2.5 µg/ml Blasticidine (#210-01, Invitrogen), 1% Penicilline/streptomycine (15070-063, Gibco) then plated into 384 wells Cell culture microclear plates (#781091, Greiner) at the density of 30 000 cells/well in 32 µl. Cells are stored at 37°C, 5% CO2 until used. Compounds in 100% DMSO (0.315 µl/well) were resuspended in 20 µl DMEM GlutaMAXTM, 1% dialyzed FBS, 25mM hepes, 0.1mM NEAA, 1% Na-pyruvate (#S8636, Sigma), 2.5 µg/ml Blasticidine, 3.4 % DMSO, 1% Penicilline/streptomycine (15070-063, Gibco). Compounds and Andrographolide (10 µM final concentration, #365645-500MG, Aldrich) as positive control, were dispensed on cell, 8 µl/well, and then incubated for 16h at 37°C and 5% CO2. The day after Live Blazer reagent (Live Blazer FRET B/G (CCF4-M), #K1089, Invitrogen), was dispensed on cells (8 µl/well) and incubated for 2h at room temperature in the dark. Then, Fluorescence Resonance Energy Transfer (FRET)

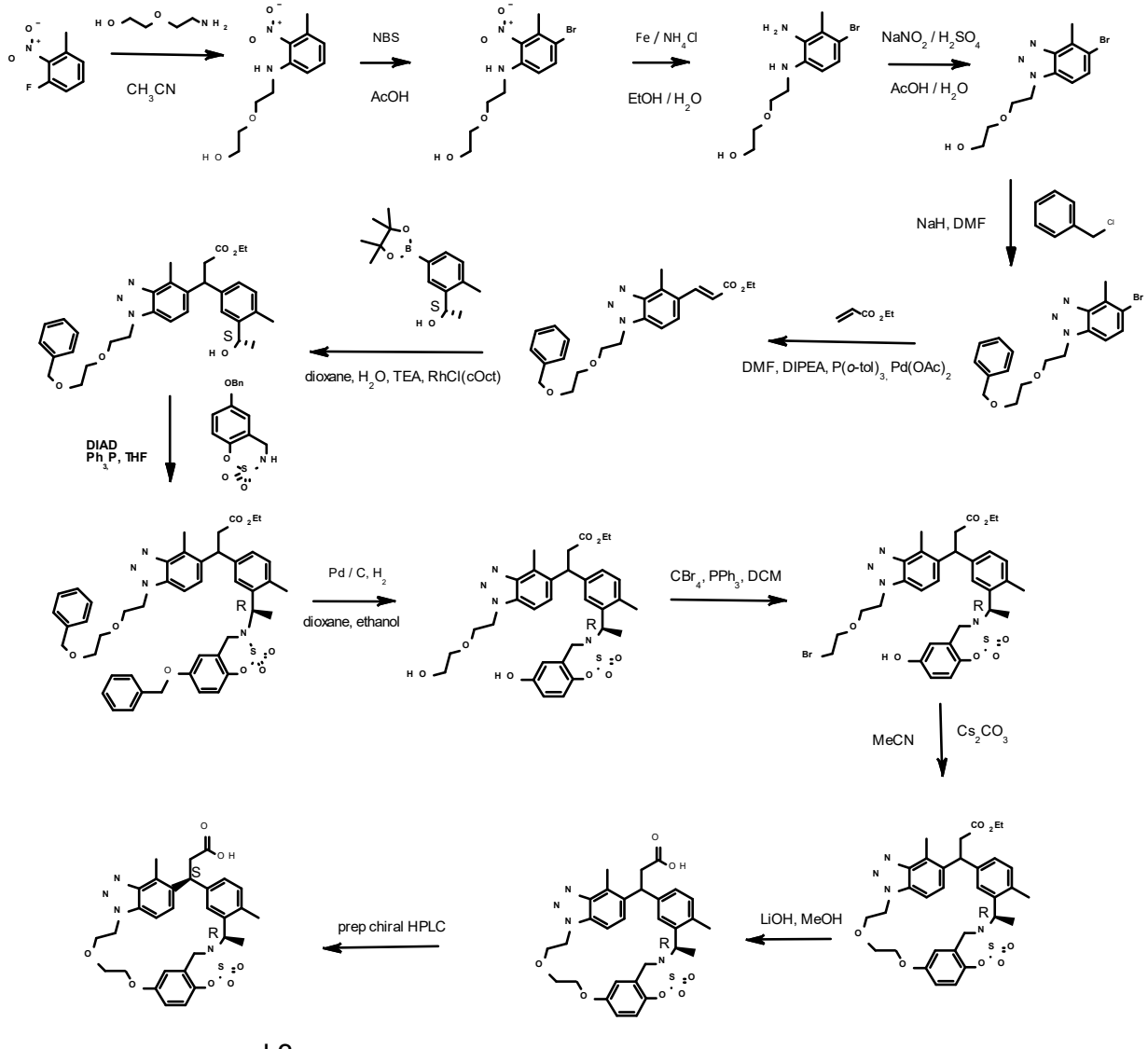
signal was measured using multimodal reader (Ex 409nM/Em and 530 nM; Envision, Perkin Elmer). Data were normalized between 1% DMSO (basal signal) and 10 µM Andrographolide (positive signal) and analyzed using Activity Base software.

ROS quantification in HepG2 cells

HepG2 cells (ATCC, were seeded at 20,000 cells/well in a 96 well-plate (Dutcher #655090) and cultured in complete medium for 24h. Cells were pre incubated with compounds of interest or vehicle (DMSO 0.1%) for 24h. In order to measure ROS generation, cell culture medium is harvested and replaced by Live-cell imaging medium (Invitrogen #A14291DJ). ROS are then triggered with H2O2 (200µM) for 90min in total. The CM-H2DCFDA probe (Invitrogen #C6827) is added 45 min after H2O2 (Sigma H1009) treatment to the wells for an additional 45 min. Cells are then washed with PBS and Live-cell imaging culture medium is added into the wells. Probe's fluorescence is then read on a Biotek Synergy 2 fluorometer (Ex/Em 492-495/517-527 nm) and results were analyzed using Prism (Graphpad).

Chemical synthesis of compound 3.

Synthesis overview:

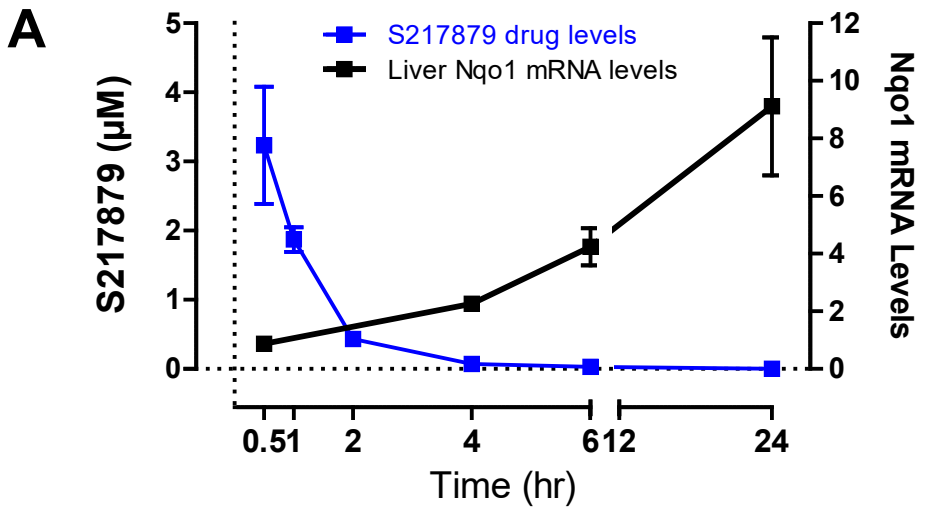


compound 3

Preparation of crystalline form for Xray crystallography: Compound 3 was added at room temperature portion-wise to THF under stirring until suspension was formed. Solid was filtered off, THF was slowly concentrated under decreased pressure. Crystals were filtered off. Absolute configuration of compound 3 was proved with Xray crystallography. Xray crystallography data are uploaded to Cambridge Structural database.

Supplementary figures

Fig. S1: single administration of S217879 (30mg/kg) leads to NRF2 activation *in vivo*. A: Male C57BL/6J mice received a single administration of S217879 (30mg/kg) by gavage in HEC 1% as vehicle. S217879 plasma levels were analyzed by LC-MS/MS. Liver Nqo1 mRNA levels were quantified by RT-qPCR (n=3 per time point). **B**: Male C57BL/6J mice on MCD diet received a single administration of S217879 (30mg/kg) by gavage or vehicle (HEC 1%). 24h post administration, animals were sacrificed by cardiac puncture under isoflurane anesthesia. Liver samples were collected for gene expression studies by RT-qPCR. (*: p<0.05 treated vs. vehicle; n=3 per group).



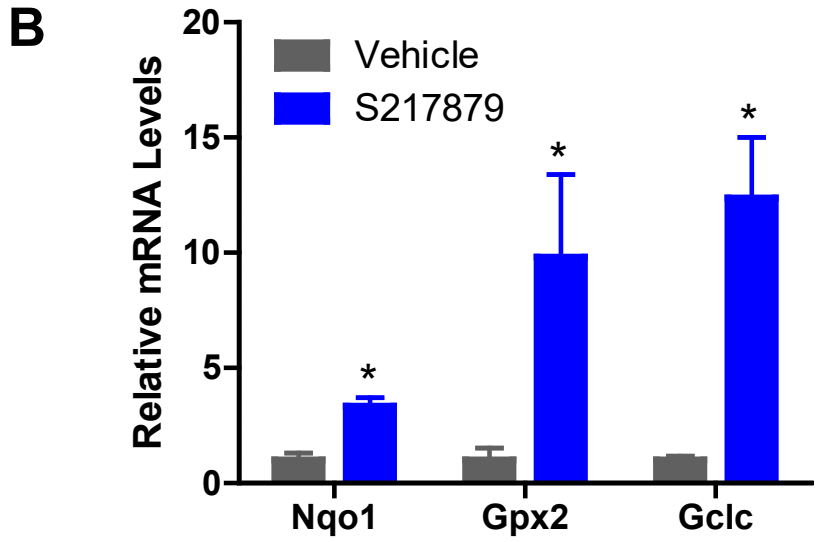


Fig. S2: S217879 treatment leads to reduction in NAS score in MCD-fed mice (A: vehicle; **B:** S218789 3mg/kg; **C:** S217879 30mg/kg). Representative pictures – HE staining.

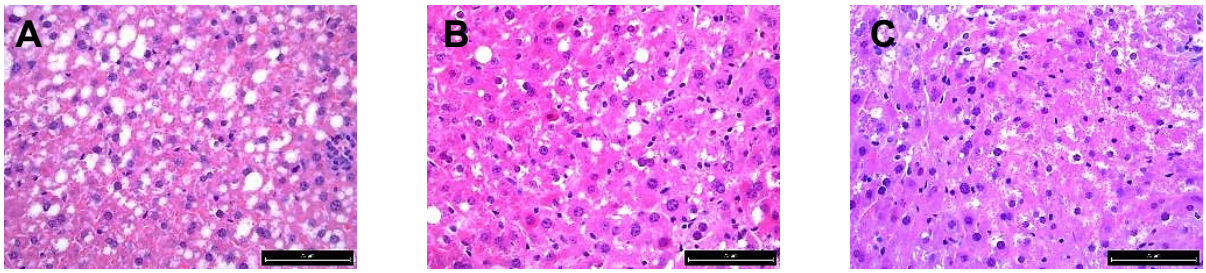


Fig. S3: S217879 treatment leads to reduction in liver triglycerides in MCD-fed mice. Data expressed as mean ± SEM (n=15 per group). Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparison test. (###: p<0.001 A04 vs. MCD vehicle; ***: p<0.001 S217879 vs. MCD vehicle).

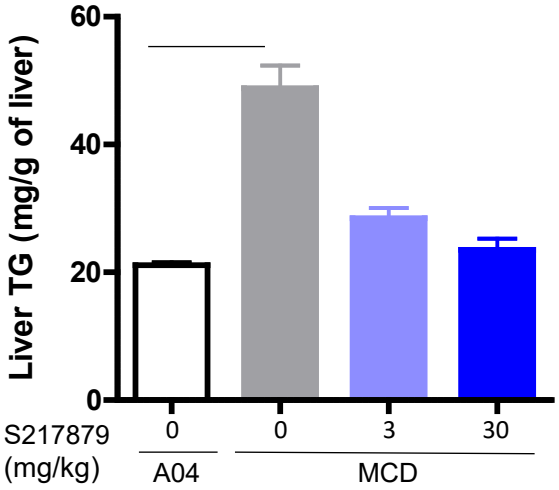


Fig. S4: S217879-mediated NRF2 activation leads to the up-regulation of the antioxidant response and the inhibition of expression of proinflammatory genes in MCD-fed mice. Liver mRNA levels expressed as fold induction over control (A04 fed group) (n=15 per group). Statistical significance was assessed by one-way ANOVA followed by Dunnett's multiple comparison test. ###: p<0.001, ##: p<0.01, #: p<0.05 MCD vs. A04; ***: p<0.001, **: p<0.01 and *: p<0.05 S217879-treated vs. vehicle.

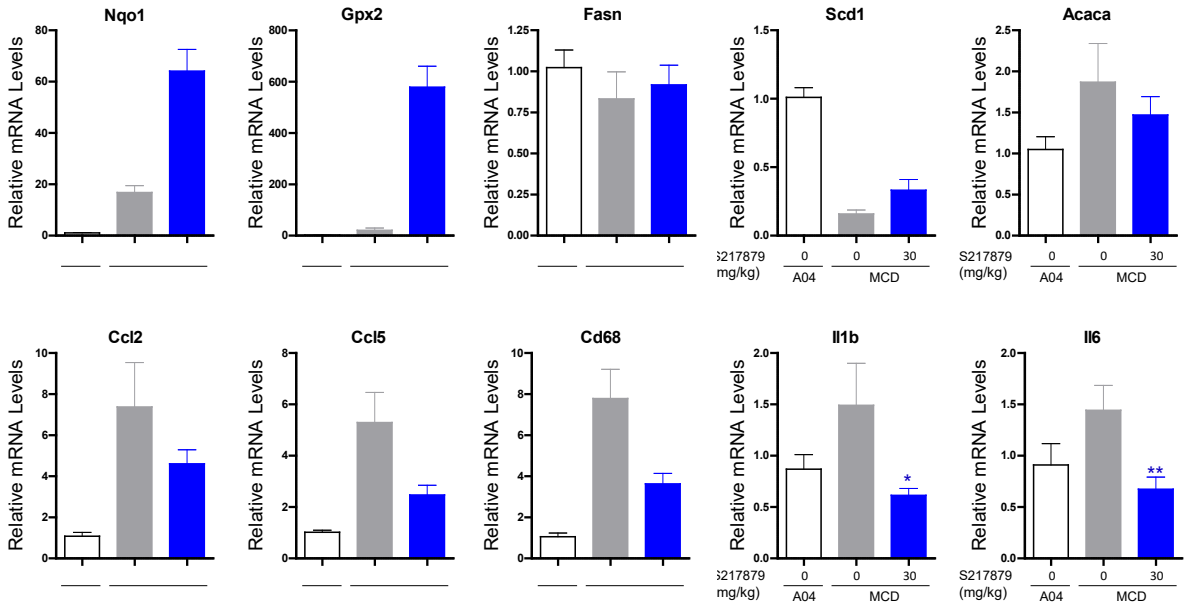


Fig. S5: S217879 treatment leads to reduction in NAS score in MCD-fed mice in a dose-dependent manner. A NAFLD activity scores. **B** Liver Nqo1 mRNA levels expressed as fold induction over control (A04 fed group) (n=15 per group). (***: p<0.001 vs. MCD treated with vehicle).

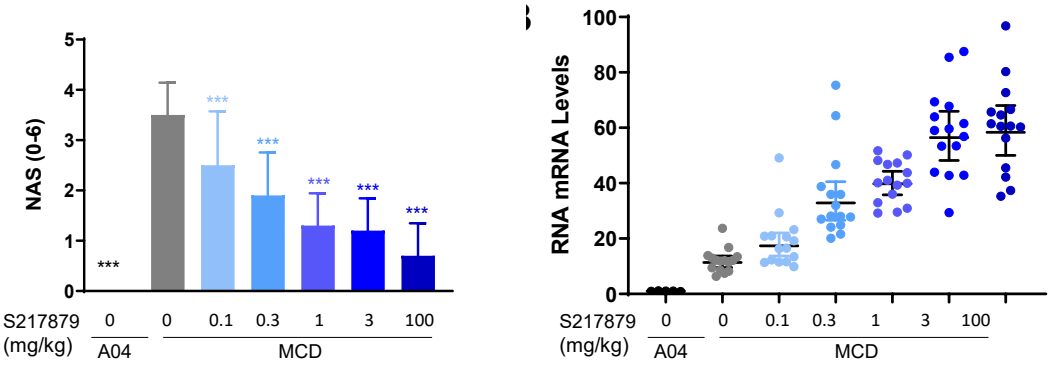


Fig. S6: S217879 treatment did not impact food intake in DIO NASH mice. Food intake expressed as grams per day. Data expressed as Mean ± SEM.

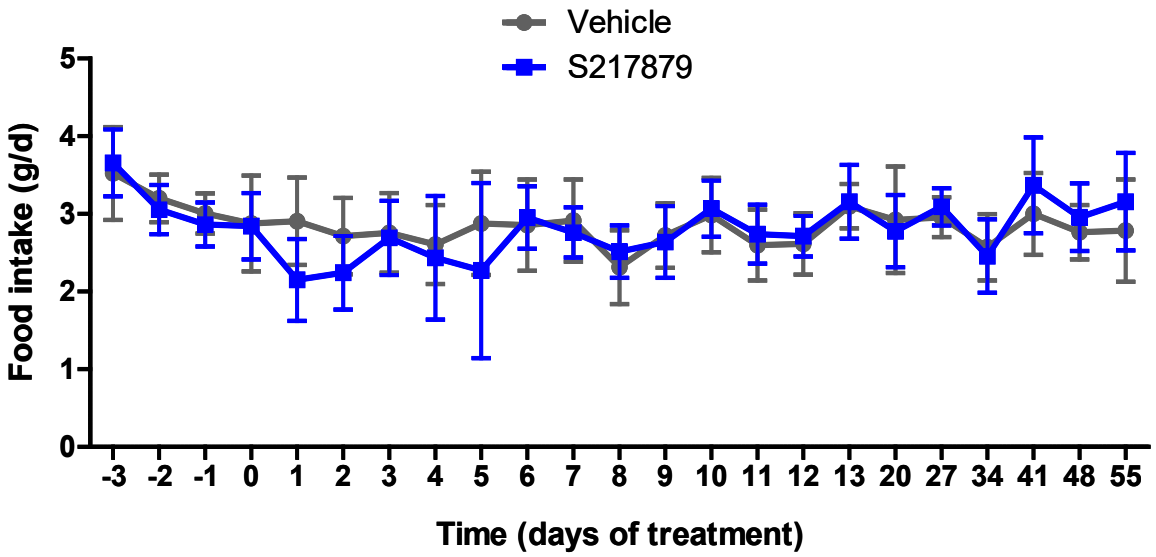


Fig. S7: S217879-mediated NRF2 activation leads to the up-regulation of the antioxidant response and the suppression of a wide spectrum of genes involved in disease progression. Heatmap of core enriched genes derived from GSEA (Differentially Expressed Genes from S217879-treated DIO NASH mice vs. vehicle (Log2 fold change cutoff >1; FDR-adjusted p-value cutoff: 0.05). A: Reactive Oxygen Species, B: Inflammatory response, C: Collagen formation.

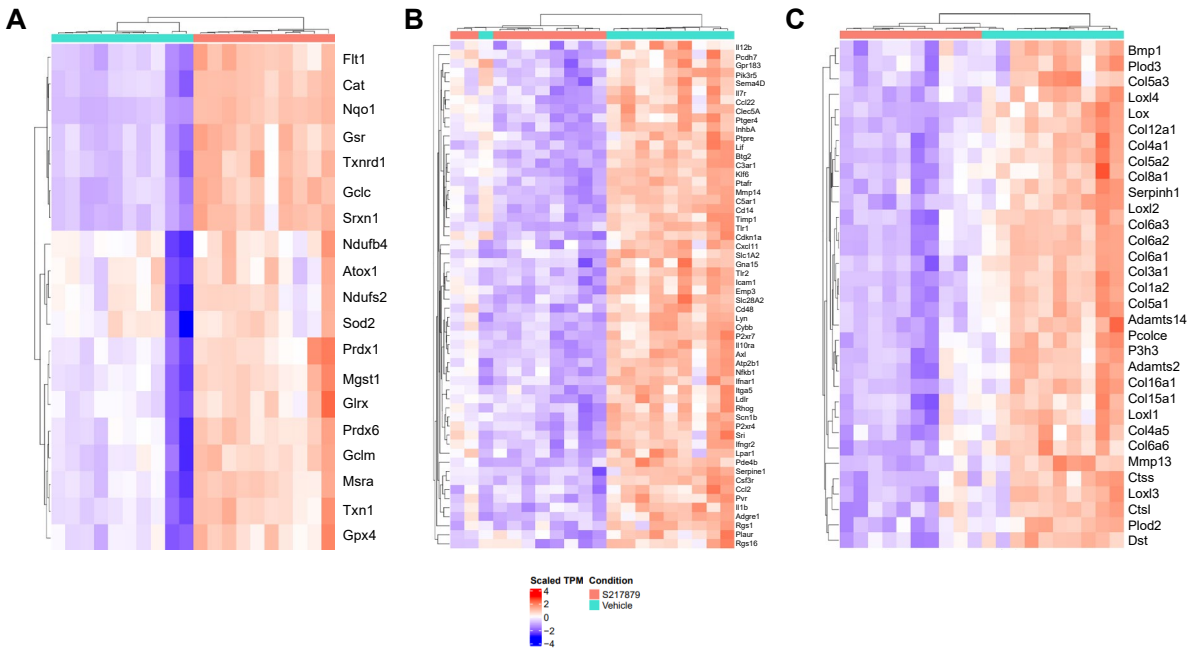
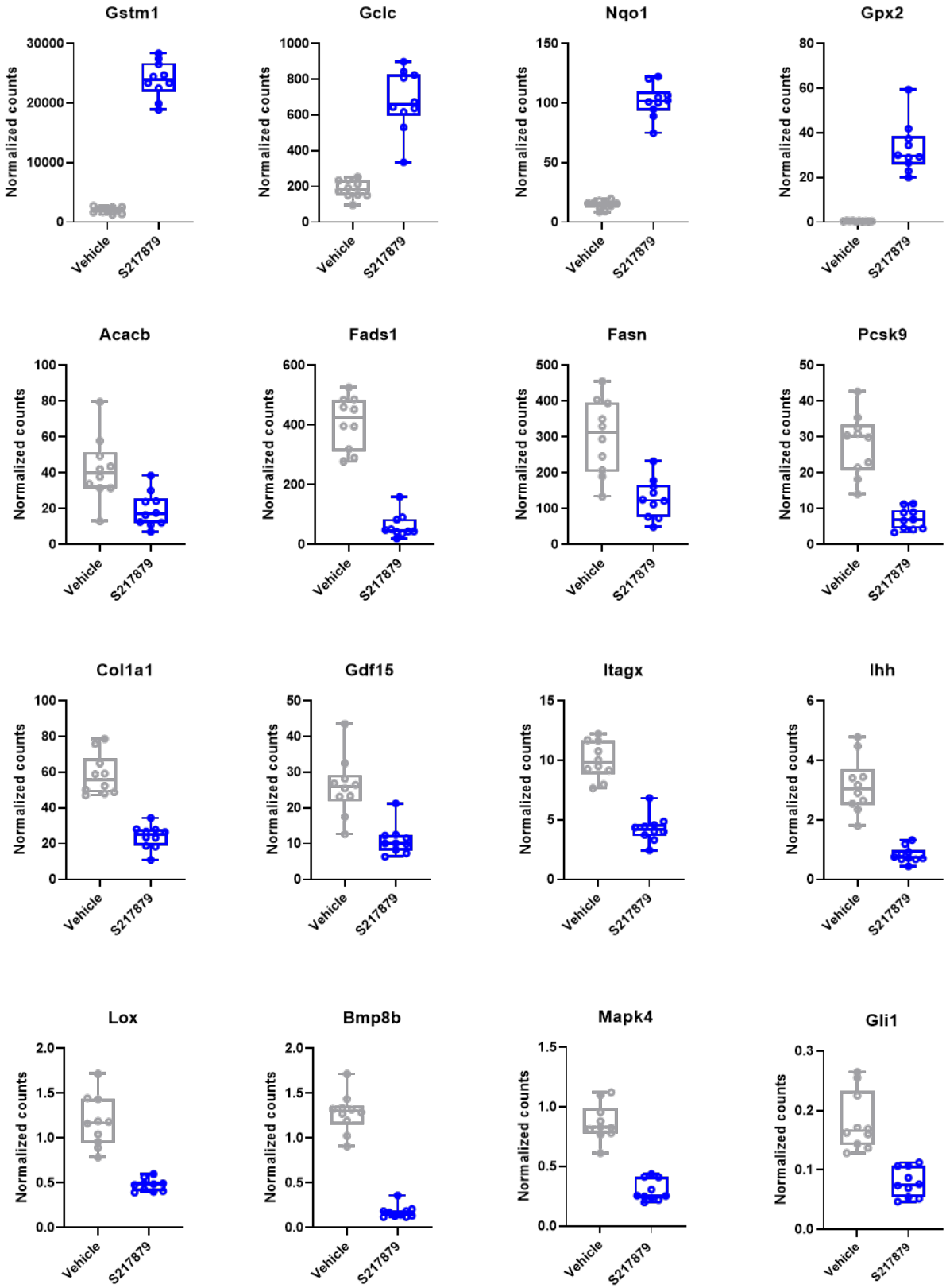


Fig. S8: Expression of differentially expressed genes DIO NASH mice (S217879 30mg/kg vs. vehicle). RNA Seq data are expressed as normalized counts.



Supplementary tables

Table S1: binding characteristics of S217879 to human KEAP1 (Aa 321-624)

	Kd (M)	K_{off} (s-1)	K_{on} (s-1/M)	Residency time (s)
N=1	3.7 e-9	1.3 e-2	3.6 e+6	76
N=2	4.7 e-9	2.5 e-2	5.4 e+6	40

Table S2: Selectivity data in the CEREP safety panel (S217879 tested at 10µM) – Results expressed as % of inhibition.

Assay	% inhibition	Assay	% inhibition
5-HT (h) (antagonist radioligand)	-4,00	M1 (h) (antagonist radioligand)	-2,00
5-HT1A (h) (agonist radioligand)	-9,00	M2 (h) (antagonist radioligand)	-11,00
5-HT1B (h) (antagonist radioligand)	-11,00	M3 (h) (antagonist radioligand)	5,00
5-HT1D (agonist radioligand)	-12,00	M4 (h) (antagonist radioligand)	1,00
5-HT2A (h) (antagonist radioligand)	-2,00	MT1 (ML1A) (h) (agonist radioligand)	6,00
5-HT2B (h) (agonist radioligand)	-14,00	MT2 (ML1B) (h) (agonist radioligand)	-1,00
5-HT2C (h) (antagonist radioligand)	3,00	mu (MOP) (h) (agonist radioligand)	5,00
5-HT3 (h) (antagonist radioligand)	-17,00	N neuronal alpha 4beta 2 (h) (agonist radioligand)	-6,00
5-HT4e (h) (antagonist radioligand)	-8,00	N neuronal alpha 7 (h) (antagonist radioligand)	-11,00
A1 (h) (antagonist radioligand)	-3,00	Na+ channel (site 2) (antagonist radioligand)	25,00
A2A (h) (agonist radioligand)	-3,00	NK1 (h) (agonist radioligand)	23,00
alpha 1A (h) (antagonist radioligand)	-11,00	NK2 (h) (agonist radioligand)	6,00
alpha 1B (h) (antagonist radioligand)	-11,00	NMDA (antagonist radioligand)	5,00
alpha 1D (h) (antagonist radioligand)	4,00	norepinephrine transporter (h) (antagonist radioligand)	1,00
alpha 2A (h) (antagonist radioligand)	17,00	PPARgamma (h) (agonist radioligand)	4,00
alpha 2B (h) (antagonist radioligand)	-18,00	PR (h) (agonist radioligand)	5,00
alpha 2C (h) (antagonist radioligand)	-4,00	sigma 1 (h) (agonist radioligand)	-2,00
AMPA (agonist radioligand)	2,00	sigma 2 (h) (agonist radioligand)	7,00
AR (h) (agonist radioligand)	11,00	SKCa channel (antagonist radioligand)	0,00
AT1 (h) (antagonist radioligand)	8,00	Y (non-selective) (agonist radioligand)	-20,00
B2 (h) (agonist radioligand)	-3,00	15-Lipoxygenase-2 (h) (recombinant)	26,00
beta 1 (h) (agonist radioligand)	2,00	Abl kinase (h)	20,00
beta 2 (h) (antagonist radioligand)	5,00	ACE (h)	17,00
BZD (central) (agonist radioligand)	-14,00	acetylcholinesterase (h)	13,00
Ca2+ channel (L, dihydropyridine site) (antagonist radioligan	-5,00	adenylyl cyclase (inhibitor effect)	11,00
Ca2+ channel (L, diltiazem site) (benzothiazepines) (antago	17,00	Akt1/PKBalpha (h)	10,00
Ca2+ channel (L, verapamil site) (phenylalkylamine) (antago	1,00	ATPase (Na+/K+)	7,00
CB1 (h) (agonist radioligand)	4,00	CaMK2alpha (h)	6,00
CB2 (h) (agonist radioligand)	8,00	caspase-1 (h)	5,00
CCK1 (CCKA) (h) (agonist radioligand)	-14,00	caspase-3 (h)	5,00
CGRP (h) (agonist radioligand)	0,00	caspase-8 (h)	4,00
choline transporter (CHT1) (h) (antagonist radioligand)	-13,00	CDC2/CDK1 (h) (cycB)	4,00
D1 (h) (antagonist radioligand)	15,00	constitutive NOS (h) (endothelial)	3,00
D2S (h) (antagonist radioligand)	4,00	COX1(h)	3,00
D3 (h) (antagonist radioligand)	4,00	COX2(h)	3,00
D4.4 (h) (antagonist radioligand)	4,00	ECE-1 (h)	3,00
D5 (h) (antagonist radioligand)	3,00	EGFR kinase (h)	2,00
delta (DOP) (h) (agonist radioligand)	11,00	FGFR1 kinase (h)	1,00
dopamine transporter (h) (antagonist radioligand)	28,00	guanylyl cyclase (h) (inhibitor effect)	1,00
Estrogen ER alpha (h) (agonist radioligand)	14,00	inducible NOS	0,00
ETA (h) (agonist radioligand)	0,00	IRK (h) (InsR)	0,00
ETB (h) (agonist radioligand)	-8,00	Lipoxygenase 12-LO	0,00
GABA (non-selective) (agonist radioligand)	-10,00	Lyn A kinase (h)	0,00
H1 (h) (antagonist radioligand)	-31,00	MAO-A (h)	-1,00
H2 (h) (antagonist radioligand)	-26,00	MAO-B (h) recombinant enzyme	-1,00
H3 (h) (agonist radioligand)	-6,00	MEK1/MAP2K1 (h)	-1,00
H4 (h) (agonist radioligand)	-10,00	p38alpha kinase (h)	-1,00
I2 (antagonist radioligand)	-7,00	PDE1B (h)	-3,00
kainate (agonist radioligand)	4,00	PDE2A1 (h)	-4,00
kappa (h) (KOP) (agonist radioligand)	37,00	PDE3A (h)	-4,00
KATP channel (antagonist radioligand)	59,00	PDE4D2 (h)	-5,00
KV channel (antagonist radioligand)	-8,00	PDE5 (h) (non-selective)	-5,00
RAF-1/MEK1 kinase (h)	-17,00	PKA (h)	-8,00
sPLA2 (h) (type V)	-21,00	PKCalpha (h)	-9,00
TRKB (h)	-23,00	PLC	-17,00

Table S3: S217879 ADME-T properties

Parameter	'Species'	Parameter value
Fabs (predicted absorbtion)	Caco-2	97%*
Efflux ratio	Caco-2	4.5**
Solubility	Caco-2	>100 µM
Clint (ml/min/g prot) microsomes / hepatocytes / hepatocytes +plasma	mouse	226 / 340 / 87
	rat	22 / 130 / 58
	dog	122 / 244 / 15
	cyno	243/ 940 / 179
	human	35 / 106 / 21
CYP inhibition (IC50)	human	2C8 ~5 µM, 2C9 ~18 µM
CYP phenotyping	human	>95 % 3A4
hERG inhibition	human	10% @10µM
Nav1.5 inhibition	human	17% @10µM
LD_{50} HepG2 cells	Human	>30µM

* Papp(A to B) = 4.5^{e}-6 cm/s (mass recovery 63%) corresponding to the Fabs=97% value of the table
** Papp(B to A) = 20e-6 cm/s (mass recovery=77%) à efflux ratio = 4.5 (=20/4.5)

Table S4: PK parameters of S217879 at steady state (5 days of administration) in MCD mice (HEC 1% as vehicle)

	3 mg/kg	30 mg/kg
Cmax (µM)	0.3	6.1
AUC (µM.h)	0.62	6.85
Tmax (h)	0.5	0.5

Table S5: S217879 treatment leads to reduction in NAS score in MCD-fed mice.

Steatosis Activity Score

			Difference vs. MCD vehicle		
	Mean	**95% CI**	**Mean**	**95% CI**	**p-value**
Vehicle	2.2	[2.0; 2.4]			
S217879 3mg/kg	1.6	[1.0; 2.1]	-0.6	[-1.2; -0.1]	0.029
S217879 30mg/kg	1	[0.6; 1.4]	-1.2	[-1.7; -0.7]	<0.001

p-value obtained with a statistical model using beta distribution with group as fixed effect on NAS parameter, followed by comparisons of the control and treated groups to the MCD vehicle group. Holm adjustment for multiplicity is applied on the comparison of each dose of S217879 with the MCD vehicle group.

Lobular Inflammation Activity Score

			Difference vs. MCD vehicle		
	Mean	**95% CI**	**Mean**	**95% CI**	**p-value**
Vehicle	1.7	[1.3; 2.0]			
S217879 3mg/kg	1.7	[1.3; 2.0]	0.0	[-0.5; 0.5]	1.000
S217879 30mg/kg	1.2	[0.9; 1.5]	-0.5	[-0.9; 0.0]	0.015

p-value obtained with a statistical model using beta distribution with group as fixed effect on NAS parameter, followed by comparisons of the control and treated groups to the MCD vehicle group. Holm adjustment for multiplicity is applied on the comparison of each dose of S217879 with the MCD vehicle group.

Table S6: S217879 treatment leads to reduction in NAS score in DIO NASH mice.

Steatosis Activity Score – treatment effect

			Difference vs. baseline		
	Mean	**95% CI**	**Mean**	**95% CI**	**p-value**
Vehicle	3	[3.0; 3.0]			
S217879 30mg/kg	2.9	[2.65; 3.07]	-0.04	[-0.32;0.25]	1

Lobular inflammation Activity Score – treatment effect

			Difference vs. baseline		
	Mean	**95% CI**	**Mean**	**95% CI**	**p-value**
Vehicle	2.1	[1.92; 2.23]			
S217879 30mg/kg	1.7	[1.44; 1.98]	-1.41	[-2.06;-0.29]	0.0093

Hepatocellular ballooning – treatment effect

			Difference vs. baseline		
	Mean	**95% CI**	**Mean**	**95% CI**	**p-value**
Vehicle	0.4	[0.13; 0.73]			
S217879 30mg/kg	0.1	[-0.07; 0.35]	-0.12	[-0.52;0.27]	1

Fibrosis stage – treatment effect

			Difference vs. baseline		
	Mean	**95% CI**	**Mean**	**95% CI**	**p-value**
Vehicle	2.4	[2.13; 2.73]			
S217879 30mg/kg	1.8	[1.45; 2.12]	-1.27	[-1.88;-0.66]	0.0003

NAFLD Activity Score – treatment effect

			Difference vs. baseline		
	Mean	**95% CI**	**Mean**	**95% CI**	**p-value**
Vehicle	5.5	[5.2;5.8]			
S217879 30mg/kg	4.7	[4.36; 5.07]	-0.82	[-1.27;-0.38]	0.0008