Chem, Volume *6*

Supplemental Information

Discovery of a Small Molecule Drug Candidate for Selective NKCC1 Inhibition in Brain Disorders

Annalisa Savardi, Marco Borgogno, Roberto Narducci, Giuseppina La Sala, Jose Antonio Ortega, Maria Summa, Andrea Armirotti, Rosalia Bertorelli, Andrea Contestabile, Marco De Vivo, and Laura Cancedda

Supplemental Figures

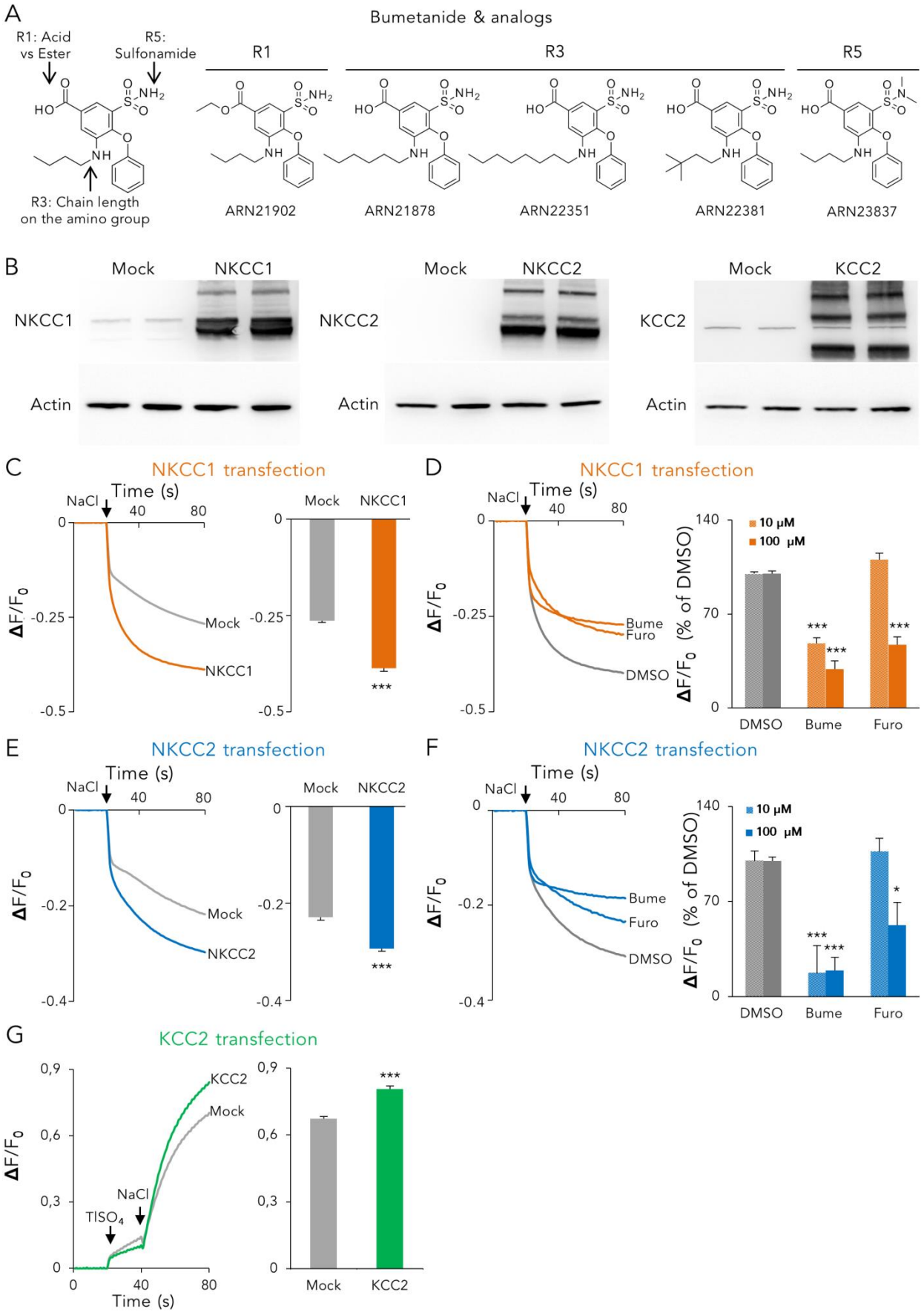


Figure S1. The Cl^- influx assay in HEK293 cells is a reliable tool to test chloride transporter inhibition by newly synthetized compounds. (**A**) Schematic representation of the point of intervention in the bumetanide structure to synthesize novel bumetanide analogs. (**B**) Representative immunoblots for NKCC1 (left), NKCC2 (center) and KCC2 (right) on protein extracts from HEK293 cells transfected with control plasmid (mock), or with NKCC1, NKCC2 or KCC2, respectively. (**C**) Left, example traces obtained in the Cl^- influx assay on mock-transfected or NKCC1-transfected HEK293 cells. The arrow indicates the addition of NaCl (74 mM) to initiate the NKCC1-mediated Cl^- influx. Right, quantification of the fluorescence decrease in experiments as on the left. Data represent mean ± SEM from 20 independent experiments (Mann-Whitney Rank Sum Test, *** $p<0.001$). (**D**) Left, example traces obtained in the Cl^- influx assay on NKCC1-transfected HEK293 cells treated with DMSO as negative control, and the indicated drugs (100 µM). Right, quantification of the NKCC1 inhibitory activity of the indicated drugs (10, 100 µM) in experiments as on the left. Data represent mean ± SEM from 5 independent experiments (10 µM: Kruskal-Wallis One-Way ANOVA on Ranks, H= 79.753, DF=2, $P<0.001$; 100 µM: Kruskal-Wallis One-Way ANOVA on Ranks, H= 63.740, DF=2, $P<0.001$; Dunn's *post hoc* test, *** $p<0.001$). (**E**) Left, example traces obtained in the Cl^- influx assay on mock-transfected or NKCC2-transfected HEK293 cells. The arrow indicates the addition of NaCl (74 mM) to initiate the NKCC2-mediated Cl^- influx. Right, quantification of the fluorescence decrease in experiments as on the left. Data represent mean ± SEM from 15 independent experiments (Mann-Whitney Rank Sum Test, *** $p<0.001$). (**F**) Left, example traces obtained in the Cl^- influx assay on NKCC2-transfected HEK293 cells treated with DMSO as negative control, and the indicated drugs (100 µM). Right, quantification of the NKCC2 inhibitory activity of the indicated drugs (10, 100 µM) in experiments as on the left. Data represent mean ± SEM from 3 independent experiments (10 µM: Kruskal-Wallis One-Way ANOVA on Ranks, H= 15.464, DF=2, $P<0.001$; 100 µM: Kruskal-Wallis One-Way ANOVA on Ranks, H= 29.872, DF=2, $P<0.001$; Dunn's *post hoc* test, * $p<0.05$, *** $p<0.001$). (**G**) Left, example traces obtained in the Tl influx assay on mock-transfected or KCC2-transfected HEK293 cells. The arrows indicate the additions of $TlSO_4$ (2mM) and NaCl (74 mM). Right, quantification of the fluorescence increase in experiments as on the left. Data represent mean ± SEM from 8 independent experiments (Two-tailed t-test, $t = 7.867$, $P<0.001$).

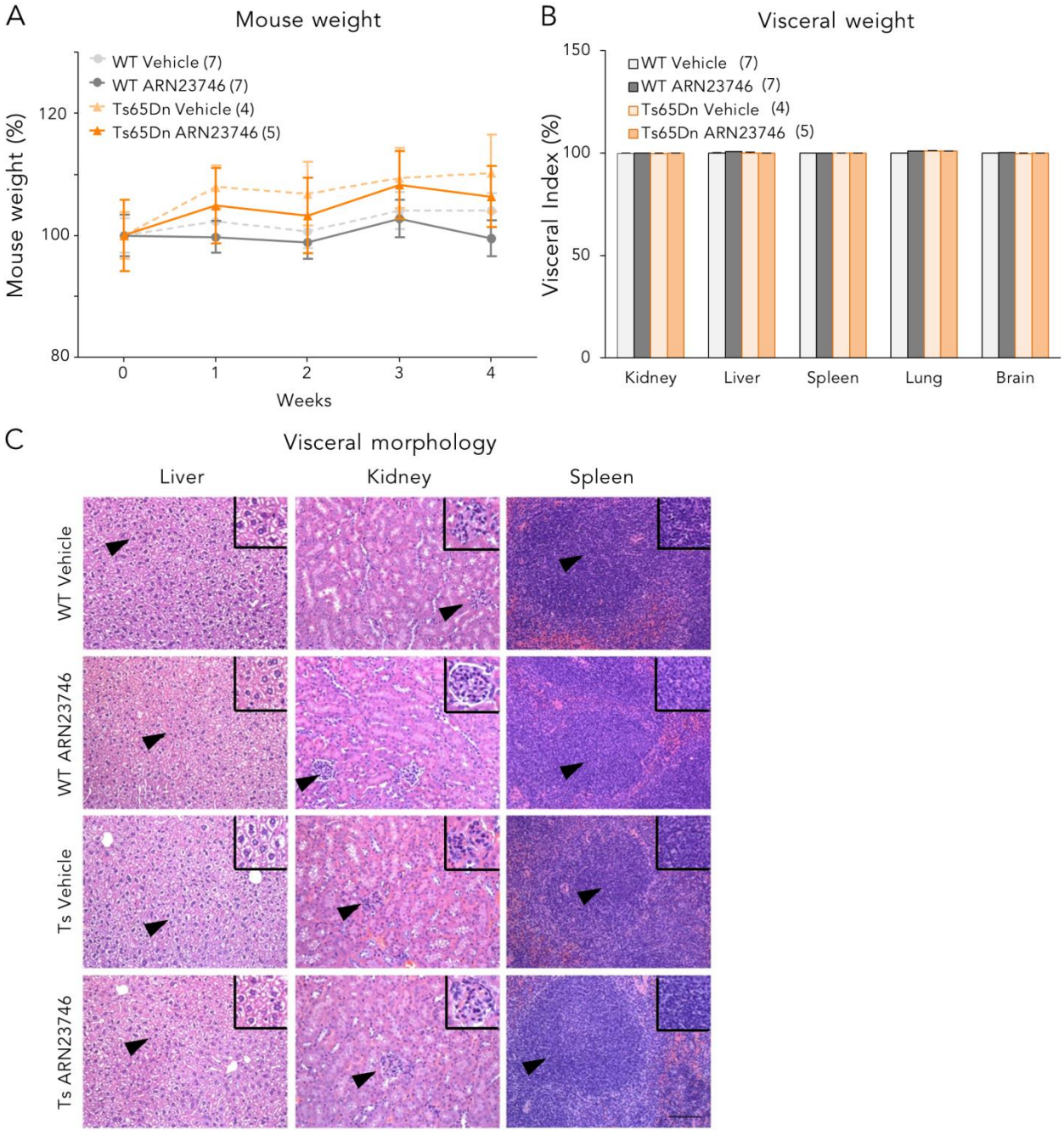

A
Mouse weight
WT Vehicle (7)
WT ARN23746 (7)
Ts65Dn Vehicle (4)
Ts65Dn ARN23746 (5)
Mouse weight (%)
120
100
80
0
1
2
3
4
Weeks
B
Visceral weight
WT Vehicle (7)
WT ARN23746 (7)
Ts65Dn Vehicle (4)
Ts65Dn ARN23746 (5)
Visceral Index (%)
150
100
50
0
Kidney
Liver
Spleen
Lung
Brain
C
Visceral morphology
Liver
Kidney
Spleen
WT Vehicle
WT ARN23746
Ts Vehicle
Ts ARN23746

Figure S2. ARN23746 does not show overt toxic effect after four weeks of chronic treatment in mice. (**A**) Quantification of the body weight of WT and TS65Dn mice across the four weeks of treatment with vehicle (control) or ARN23746. Chronic treatment with ARN23746 does not affect body weight either in WT or in Ts65Dn mice. (**B**) Visceral index expressed as viscera weight/body weight of 5 different organs collected from WT and TS65Dn mice after chronic treatment with vehicle (control) and ARN23746. Chronic treatment with ARN23746 does not affect the visceral index for any of the organs analyzed. In all graphs, the number in parenthesis indicate the number of animals analyzed. (**C**) Representative images of Hematoxylin&Eosin staining to evaluate the morphology of liver, kidney and spleen in WT and Ts65Dn treated with ARN23746 or vehicle. For each panel, the boxed areas correspond to the high-magnification images of the regions indicated with the arrow heads. Scale bar: 100 μm. Chronic treatment with ARN23746 does not affect the morphology of the analyzed organs.

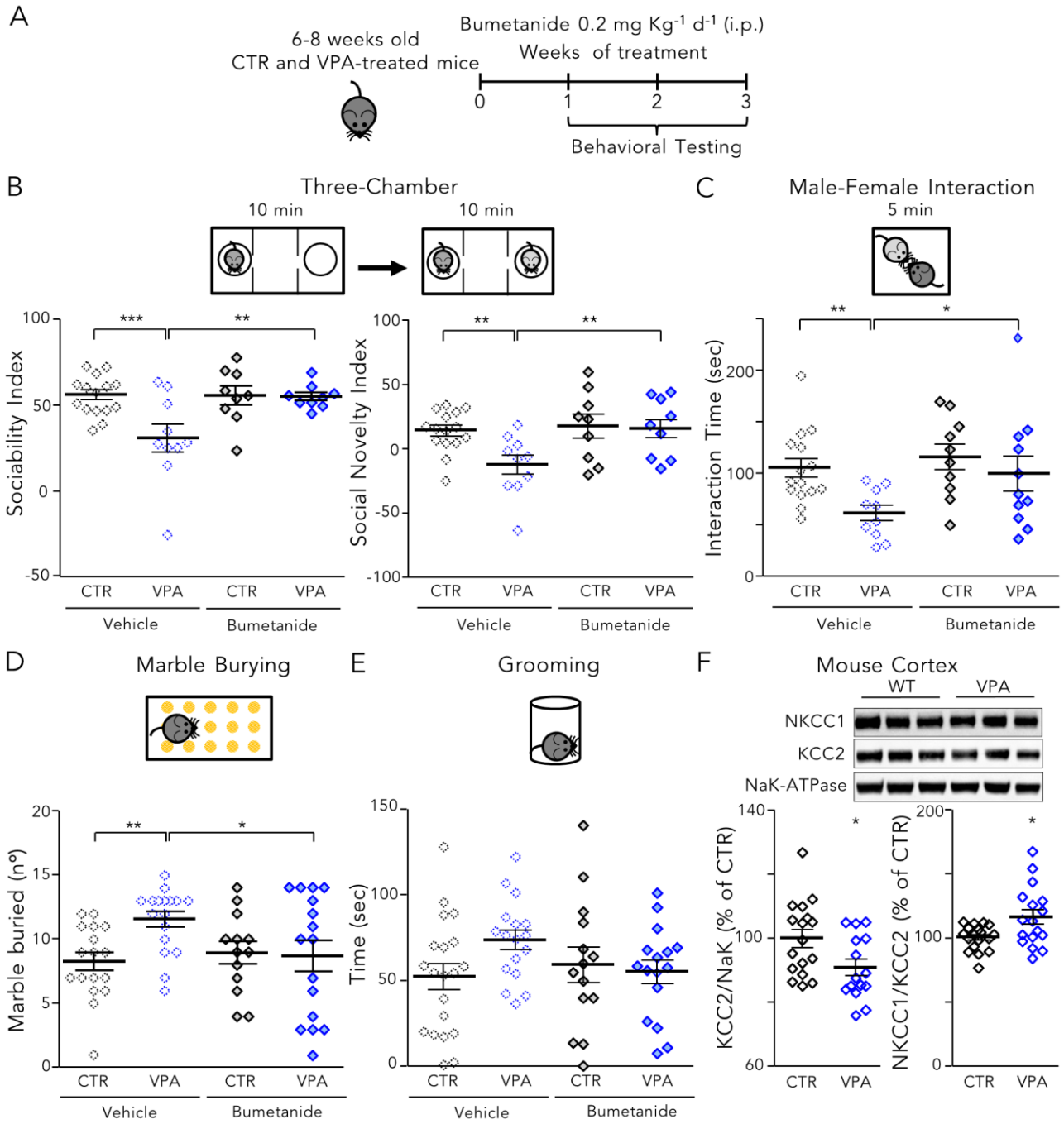

A
6-8 weeks old
CTR and VPA-treated mice
Bumetanide 0.2 mg Kg-1 d-1 (i.p.)
Weeks of treatment
0
1
2
3
Behavioral Testing
B
Three-Chamber
10 min
10 min
Sociability Index
Social Novelty Index
CTR
VPA
CTR
VPA
Vehicle
Bumetanide
C
Male-Female Interaction
5 min
Interaction Time (sec)
D
Marble Burying
Marble buried (nº)
E
Grooming
Time (sec)
F
Mouse Cortex
WT
VPA
NKCC1
KCC2
NaK-ATPase
KCC2/NaK (% of CTR)
NKCC1/KCC2 (% of CTR)

Figure S3. Bumetanide rescues sociability and repetitive behaviour in the valproic acid mouse model of autism (**A**) Schematic cartoon of the experimental protocol for the treatment of young adult control (CTR) and valproic acid (VPA) mice with bumetanide for *in vivo* efficacy assessment to rescue sociability and repetitive behaviors in autism. In all the graphs, the vehicle-treated animal data (dotted symbols) were taken from Fig.4 and shown here for comparison. (**B**) Top, schematic representation of the three-chamber test. Bottom left, quantification of the mean ± SEM and single animal cases of sociability index in mice treated with the indicated drugs (two-way ANOVA, $F_{interaction\ (1,39)}$= 6.058, P = 0.018, Tukey's *post hoc* test, ** P<0.01, ***$P < 0.001$). Bottom right, quantification of the mean ± SEM and single animal cases of social novelty index in mice treated with the indicated drugs (two-way ANOVA, $F_{treatment\ (1,39)}$= 5.528, P = 0.024, Tukey's *post hoc* test, ** P<0.01). (**C**) Top, schematic representation of the male-female interaction test. Bottom, quantification of the mean ± SEM and single animal cases of the interaction time in mice treated with the indicated drugs (two-way ANOVA on Ranks, $F_{condition\ (1,42)}$= 10.490, P = 0.002, Tukey's *post hoc* test, * P<0.05, ** P<0.01). (**D**) Top, schematic representation of the marble burying test. Bottom, quantification of the mean ± SEM and single animal cases of the number of marble buried by mice treated with the indicated drugs (two-way ANOVA, $F_{interaction\ (1,59)}$= 4.134, P = 0.047, Tukey's *post hoc* test, * P<0.05, ** P<0.01). (**E**) Top, schematic representation of the grooming test. Bottom, quantification of the mean ± SEM and single animal cases of the grooming time for mice treated with the indicated drugs. (**F**) Top, representative immunoblots for NKCC1 and KCC2 on extracts of membrane-enriched protein fractions from cortices of VPA mice and CTR mice. Bottom left, quantification of KCC2 in cortex samples from VPA mice in comparison to CTR mice (Two-tailed t-test, t = 2.382, P= 0.0238). Bottom right, quantification of NKCC1/KCC2 expression ratio in samples from cortices of VPA mice in comparison to CTR mice (Mann-Whitney Rank Sum Test, P = 0.042).

Figure 4H

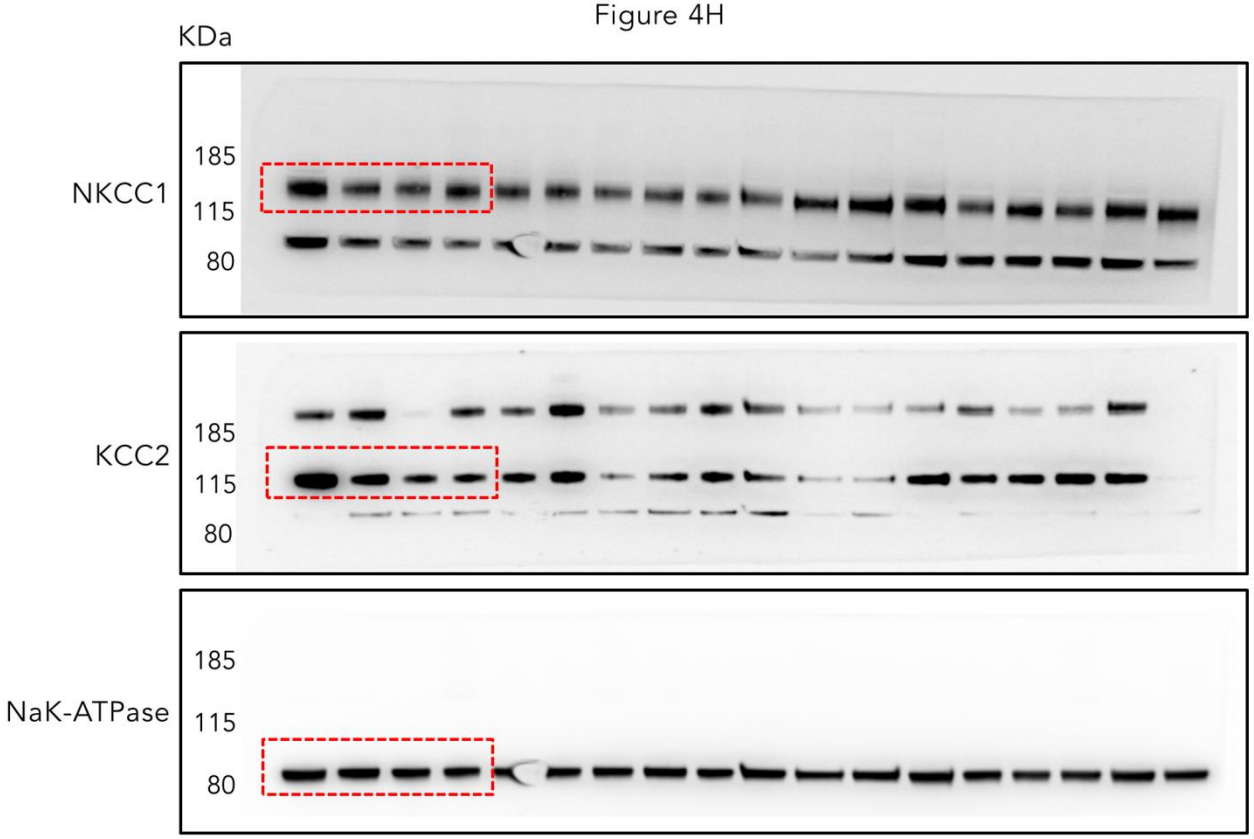


Figure S3F

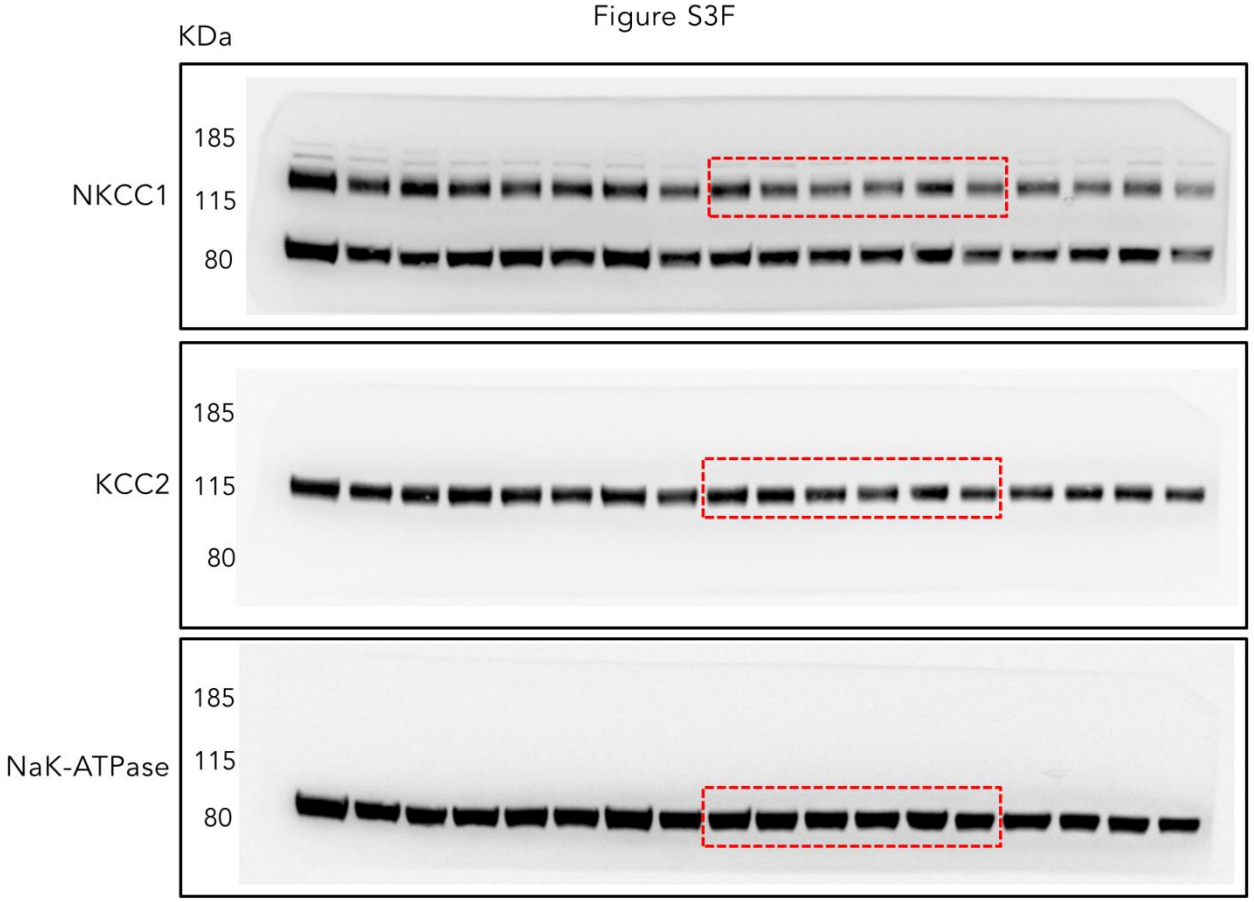
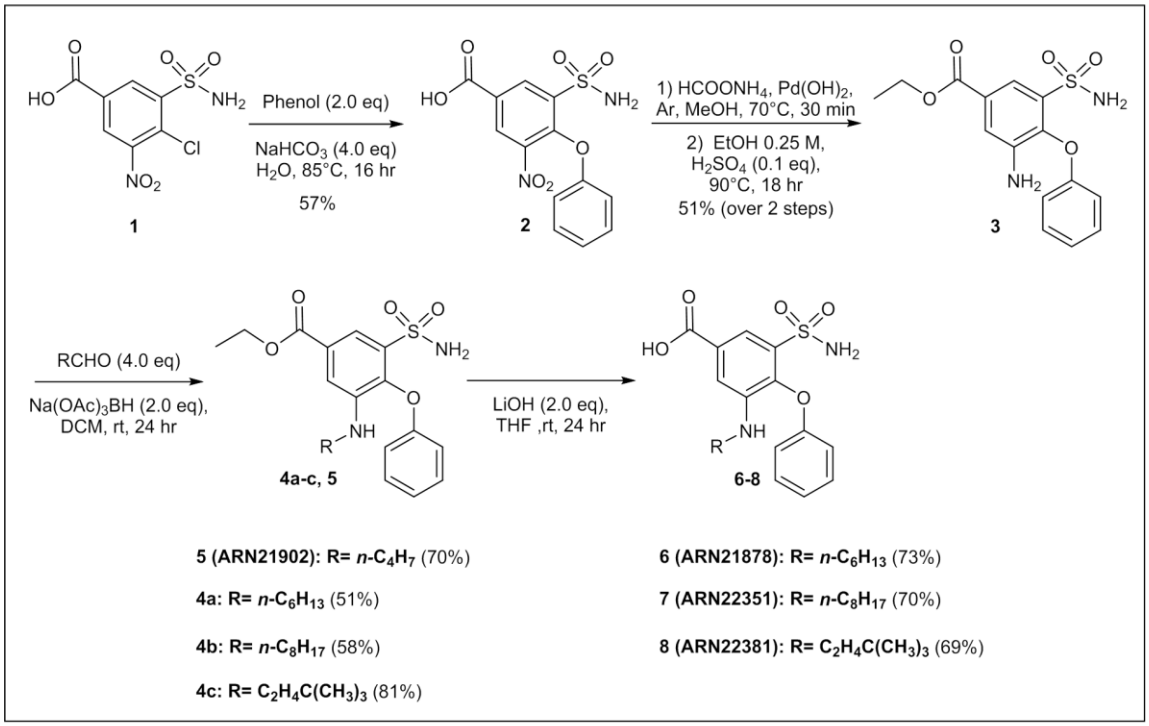
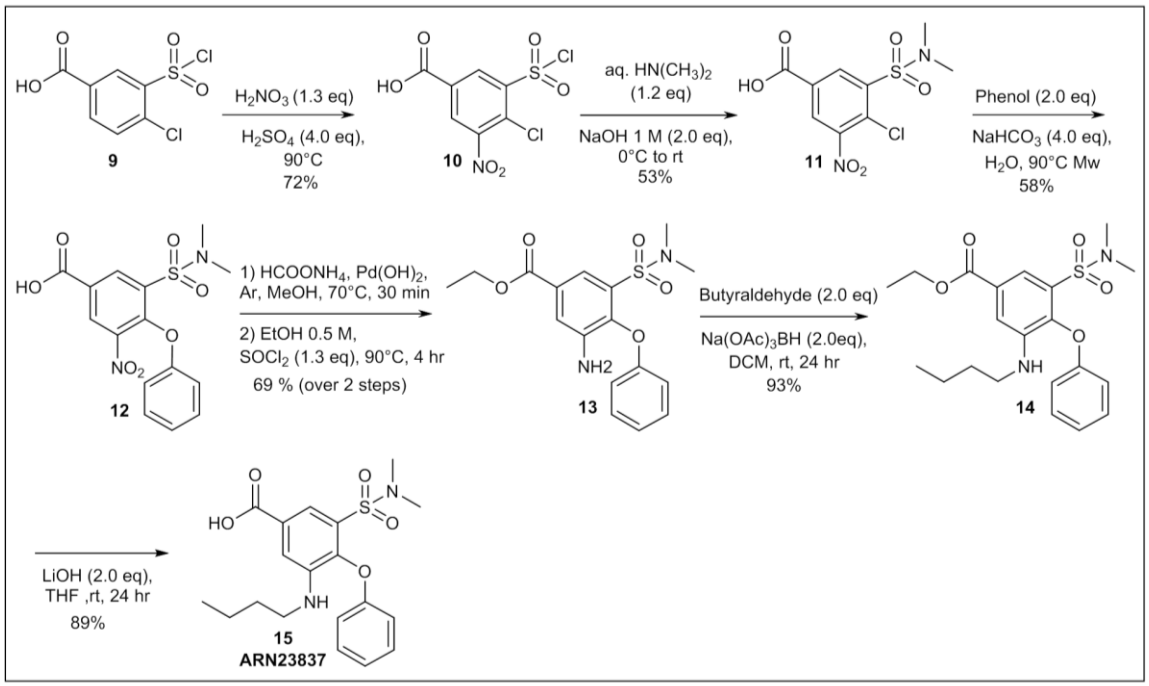


Figure S4. Uncropped blots images. Full-length blot images corresponding to the cropped western blot bands (red boxes) presented in the main figure and supplemental figure of this study.

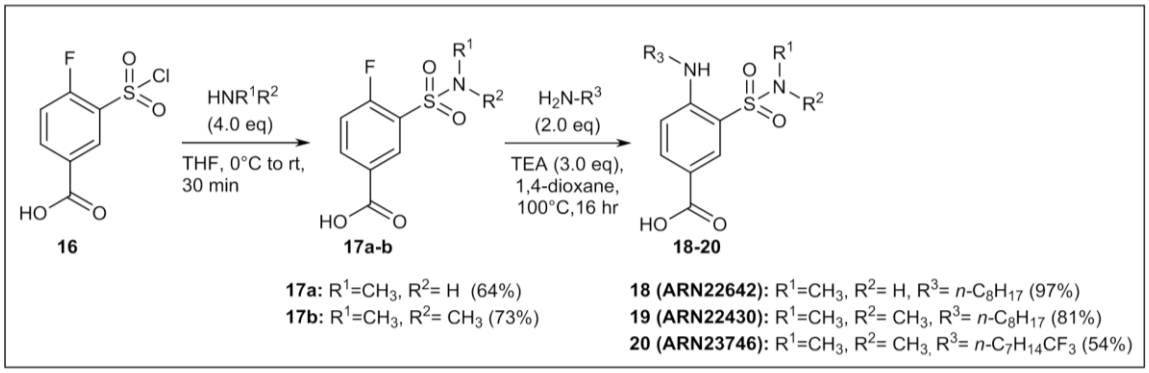
Supplemental Schemes



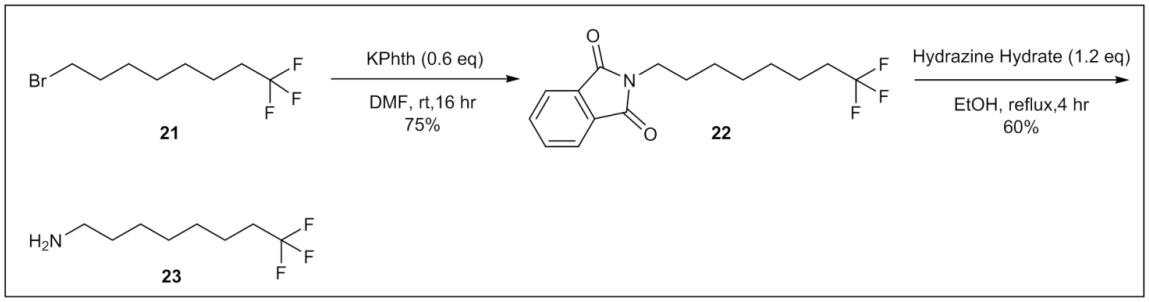
Scheme S1. Synthesis of R3 substituted bumetanide analogs.



Scheme S2. Synthesis of N,N-dimethylsulfamoyl bumetanide.



Scheme S3. Synthesis of 4-amino-3-sulfamoyl-benzoic acid derivatives.



Scheme S4. Synthesis of 8,8,8-trifluorooctan-1-amine.

Supplemental Tables

ARN	Kinetic solubility PBS pH 7.4	$t_{1/2}$ plasma (mouse)	$t_{1/2}$ liver microsomes (mouse)
ARN22642	53 µM	> 60 mins	12 mins
ARN22430	52 µM	> 120 mins	13 mins
ARN23746	> 250 µM	> 120 mins	> 60 mins

Table S1. *In vitro* solubility and metabolic stability.

Analysis	Species	Bumetanide	ARN23746
$S_{kinetic}$ PBS pH 7.4 (µM)	-	>250	>250
$S_{thermodynamic}$ PBS pH 7.4 (µM)	-	n.a	181
$t_{1/2}$ plasma (min)	mouse	>120	>120
$t_{1/2}$ liver microsomal stability (min)	mouse	>60	>60
Residual liver compound	mouse	n.a	65%
$t_{1/2}$ liver microsomal stability (min)	rat	n.a	189
$t_{1/2}$ liver microsomal stability (min)	human	n.a	395

Table S2. *In vitro* ADME properties.

Gene Symbol	Assay Mode	ARN23746 10μM % of Response		
GPCRs		Replicate1	Replicate2	Average
ADORA2A	Agonist	0.0	0.0	0.0
ADORA2A	Antagonist	35.6	42.8	39.2
ADRA1A	Agonist	1.4	0.1	0.7
ADRA1A	Antagonist	2.8	5.4	4.1
ADRA2A	Agonist	13.2	12.3	12.7
ADRA2A	Antagonist	0.0	0.0	0.0
ADRB1	Agonist	0.0	0.0	0.0
ADRB1	Antagonist	67.2	83.7	75.4
ADRB2	Agonist	0.0	0.0	0.0
ADRB2	Antagonist	27.0	39.3	33.2
AVPR1A	Agonist	4.4	3.2	3.8
AVPR1A	Antagonist	4.4	0.0	2.2
CCKAR	Agonist	2.5	1.9	2.2
CCKAR	Antagonist	0.0	1.5	0.7
CHRM1	Agonist	0.0	0.0	0.0
CHRM1	Antagonist	37.9	23.1	30.5
CHRM2	Agonist	15.0	12.2	13.6
CHRM2	Antagonist	0.0	0.0	0.0
CHRM3	Agonist	0.0	0.0	0.0
CHRM3	Antagonist	11.8	14.6	13.2
CNR1	Agonist	66.7	68.9	67.8
CNR1	Antagonist	0.0	0.0	0.0
CNR2	Agonist	6.2	6.4	6.3
CNR2	Antagonist	7.8	9.4	8.6
DRD1	Agonist	0.1	0.0	0.1
DRD1	Antagonist	44.8	48.7	46.8
DRD2S	Agonist	24.6	15.0	19.8
DRD2S	Antagonist	0.0	0.0	0.0
EDNRA	Agonist	4.4	2.0	3.2
EDNRA	Antagonist	0.0	0.0	0.0
HRH1	Agonist	1.3	1.6	1.5
HRH1	Antagonist	7.0	11.3	9.2
HRH2	Agonist	0.0	0.0	0.0
HRH2	Antagonist	82.0	83.2	82.6

HTR1A	Agonist	22.3	18.2	20.2
HTR1A	Antagonist	0.0	0.0	0.0
HTR1B	Agonist	21.7	16.8	19.2
HTR1B	Antagonist	0.0	0.0	0.0
HTR2A	Agonist	5.1	3.6	4.3
HTR2A	Antagonist	49.1	52.9	51.0
HTR2B	Agonist	2.8	0.9	1.8
HTR2B	Antagonist	37.3	47.2	42.2
OPRD1	Agonist	20.6	24.5	22.6
OPRD1	Antagonist	0.0	0.0	0.0
OPRK1	Agonist	48.9	49.9	49.4
OPRK1	Antagonist	0.0	0.7	0.4
OPRM1	Agonist	37.3	47.2	42.3
OPRM1	Antagonist	0.0	0.0	0.0
Nuclear Hormone Receptors		Replicate1	Replicate2	Average
AR	Agonist	0.0	0.0	0.0
AR	Antagonist	16.1	12.0	14.1
GR	Agonist	0.0	0.0	0.0
GR	Antagonist	30.0	16.1	23.1
Transporters		Replicate1	Replicate2	Average
DAT	Blocker	7.4	0.0	3.7
NET	Blocker	0.0	1.8	0.9
SERT	Blocker	0.0	0.0	0.0
Ion Channels		Replicate1	Replicate2	Average
CAV1.2	Blocker	0.0	0.0	0.0
GABAA	Opener	0.6	1.4	1.0
GABAA	Blocker	10.2	20.0	15.1
hERG	Blocker	0.0	0.0	0.0
HTR3A	Opener	0.6	0.0	0.3
HTR3A	Blocker	10.2	17.1	13.6
KvLQT1/minK	Opener	9.5	12.9	11.2
KvLQT1/minK	Blocker	0.0	0.0	0.0
nAChR(a4/b2)	Opener	0.0	0.0	0.0
nAChR(a4/b2)	Blocker	0.0	0.0	0.0
NAV1.5	Blocker	36.1	34.6	35.4
NMDAR (1A/2B)	Opener	0.0	0.0	0.0
NMDAR (1A/2B)	Blocker	0.0	0.0	0.0

Non-Kinase Enzymes		Replicate1	Replicate2	Average
AChE	Inhibitor	2.2	1.1	1.7
COX1	Inhibitor	3.8	2.7	3.2
COX2	Inhibitor	3.5	2.7	3.1
MAOA	Inhibitor	0.0	0.0	0.0
PDE3A	Inhibitor	0.0	0.0	0.0
PDE4D2	Inhibitor	10.0	14.0	12.0
Kinases		Replicate1	Replicate2	Average
INSR	Inhibitor	9.4	7.2	8.3
LCK	Inhibitor	0.9	0.0	0.5
ROCK1	Inhibitor	14.3	0.0	7.2
VEGFR2	Inhibitor	4.9	5.4	5.2

Table S3. Off target profile of ARN23746 in Safety47™ Panel (Eurofins).

% Object preference OL

Objects	WT Vehicle	WT ARN23746	Ts65Dn Vehicle	Ts65Dn ARN23746	F and P
A	51.44 ± 2.94	52.81 ± 2.24	56.46 ± 4.55	51.61 ± 1.87	Two-way ANOVA on Ranks $F_{interaction\ (1,45)} = 2.278$, P=0.138
B	48.56 ± 2.94	47.19 ± 2.24	43.54 ± 4.55	48.39 ± 1.87	Two-way ANOVA on Ranks $F_{interaction\ (1,45)} = 2.278$, P=0.138

% Object preference NOR

Objects	WT Vehicle	WT ARN23746	Ts65Dn Vehicle	Ts65Dn ARN23746	F and P
A	28.46 ± 2.12	29.25 ± 2.06	30.23 ± 2.88	34.81 ± 2.39	Two-way ANOVA $F_{interaction\ (1,46)} = 0.649$, P=0.425
B	40.55 ± 1.90	41.22 ± 2.43	44.19 ± 3.18	40.51 ± 2.59	Two-way ANOVA $F_{interaction\ (1,46)} = 0.724$, P=0.399
C	30.99 ± 1.91	29.53 ± 2.54	25.58 ± 2.78	24.68 ± 1.96	Two-way ANOVA $F_{genotype\ (1,46)} = 4.741$, P=0.035

Total Exploration Time (sec) OL

	WT Vehicle	WT ARN23746	Ts65Dn Vehicle	Ts65Dn ARN23746	F and P
Acquisition	39.32 ± 3.73	33.49 ± 3.28	46.23 ± 8.37	50.37 ± 5.93	Two-way ANOVA on Ranks $F_{interaction\ (1,45)} = 2.585$, P=0.115
Trial	51.29 ± 4.02	48.85 ± 3.34	54.24 ± 6.54	71.45 ± 7.78	Two-way ANOVA $F_{genotype\ (1,45)} = 5.793$, P=0.020 Tukey *post hoc* test Within Ts65Dn Veh vs ARN P=0.039 Within ARN23746 WT vs Ts P=0.005

Total Exploration Time (sec) NOR

	WT Vehicle	WT ARN23746	Ts65Dn Vehicle	Ts65Dn ARN23746	F and P
Acquisition	79.95 ± 10.08	61.73 ± 2.85	84.52 ± 10.49	92.63 ± 11.12	Two-way ANOVA on Ranks $F_{genotype\ (1,46)} = 4.768$, P=0.034 Tukey *post hoc* test Within ARN23746 WT vs Ts P=0.006
Trial	65.68 ± 6.44	76.76 ± 5.74	90.21 ± 10.22	87.24 ± 6.47	Two-way ANOVA on Ranks $F_{genotype\ (1,46)} = 6.459$, P=0.014 Tukey *post hoc* test Within Vehicle WT vs Ts P=0.027

Freezing Time (%) CFC

	WT Vehicle	WT ARN23746	Ts65Dn Vehicle	Ts65Dn ARN23746	F and P
Pre Shock	8.11 ± 0.89	8.59 ± 0.97	10.27 ± 1.90	9.69 ± 1.02	Two-way ANOVA on Ranks $F_{interaction\ (1,46)} = 0{,}0000166$, P=0.997
Post Shock	59.09 ± 3.56	53.38 ± 4.25	60.75 ± 7.14	50.61 ± 3.64	Two-way ANOVA $F_{interaction\ (1,41)} = 0.213$, P=0.647
New Context	4.32 ± 0.58	5.05 ± 1	5.30 ± 1.16	5.37 ± 1.14	Two-way ANOVA on Ranks $F_{interaction\ (1,46)} = 0.0110$, P=0.917

Locomotor activity

	WT Vehicle	WT ARN23746	Ts65Dn Vehicle	Ts65Dn ARN23746	F and P
Distance travelled	36.41 ± 3.27	40.67 ± 3.82	42.24 ± 5.64	55.74 ± 5.98	Two-way ANOVA on Ranks $F_{genotype\ (1,47)} = 4.854$, P=0.033 Tukey *post hoc* test Within ARN23746 WT vs Ts P=0.022

Table S4. Control parameters in the NOL, NOR and CFC tasks of WT and Ts65Dn mice.

Case Number	Disorder	Age (years)	Gender
1668	CTR	19	M
5889	CTR	27	M
5235	CTR	28	M
5563	CTR	29	M
5825	CTR	41	M
5614	CTR	31	M
6096	CTR	28	M
5643	CTR	17	M
5875	CTR	45	M
6104	ASD	20	M
6146	ASD	22	M
6087	ASD	22	M
5888	ASD	23	M
5406	ASD	34	M
5443	ASD	25	M
5619	ASD	22	M
6167	ASD	19	M
5969	ASD	45	M

Table S5. Human sample information.

Cohort #	Test 1	Test 2	Test 3	Test 4
1	Grooming	Marble burying	Three-chamber	Male-Female
2	Male-Female	Marble burying	Three-chamber	Grooming
3	Three-chamber	Male-Female	Grooming	Marble burying
4	Marble burying	Grooming	Three-chamber	Male-Female
5	Marble burying	Grooming		

Table S6. Mice cohorts subjected to behavioral testing.

Supplemental experimental procedures

***In vitro* mouse plasma stability.** Compounds were diluted in preheated (37 °C) mouse plasma (Rockland Immunochemicals Inc.) with 5% DMSO to favor solubilization. The final compound concentration was 2.0 µM. At time points (0, 5, 15, 30, 60, 120 min), a 30 µL aliquot of the incubation solution was diluted in 200 µL of cold acetonitrile spiked with 200 nM Warfarin, as internal standard. After vortexing for 30 s, the solution was centrifuged at 3500g for 15 min at 4 °C, and the supernatant transferred for LC-MS/MS analysis on a Waters Acquity UPLC/MS TQD system. Compound stability was evaluated on the basis of the corresponding peak areas plotted *vs* time. The compounds' half-lives were calculated using a one-phase fitting decay of the peak area *vs* time profiles.

***In vitro* mouse liver microsomes stability.** 10mM DMSO stock solution of test compound was pre-incubated at 37 ˚C for 15 min with liver microsomes (Sekisui Xenotech, LCC), 0.1M Tris-HCl buffer (pH 7.4), and 10% DMSO. The final concentration was 4.6 µM. After pre-incubation, the cofactors (NADPH, G6P, G6PDH, $MgCl_2$ pre-dissolved in 0.1M Tris-HCl) were added to the incubation mixture and the incubation was continued at 37 ˚C for 1h. At each time point (0, 5, 15, 30, 60 min), 30µL of incubation mixture was diluted with 200 µL cold acetonitile spiked with 200 nM of warfarin as internal standard, followed by centrifugation at 3500 g for 30 min. The supernatant was further diluted with H2O (1:1) for analysis. An aliquot of 200 µl of the supernatant was removed, and the concentration of the test compound was quantified by LC-MS/MS. The percentage of the test compound remaining at each time point relative to t=0 was calculated. The half-lives (t½) were determined by a one-phase decay equation using a non-linear regression of compound concentration *vs* time.

***In vitro* rat and human liver microsomes stability.** Rat (Gibco) or human (BD Gentest) hepatocytes were thawed in Hepatocyte Plating Supplement pack (Life Technologies) and placed in 37 ± 1 °C shaking water. Hepatocytes were re-suspended in Williams E medium containing Cell Maintenance Supplement Pack (Life Technologies), and counted using

Trypan blue solution to a final concentration of 0.25 x 106 cells/mL. Samples of the test compound at 10 µM were incubated for 0, 10, 30, 60, 120, and 240 min at 37 °C. Blank samples were prepared by incubating 250 µl of cell solution without any compound for 240 min. The incubations were quenched 1:1 with ice-cold acetonitrile spiked with 600 nM labetalol as internal standard. Samples were then centrifuged at 12000 rpm for 5 min at 4 °C. Aliquots of 200 µl of the supernatant were removed and the concentration of the test compound was quantified by LC-MS/MS. The percentage of the test compound remaining at each time point relative to t=0 was calculated. The half-lives (t½) were determined by a one-phase decay equation using a non-linear regression of compound concentration *vs* time.

In vitro off-target activity profiling. An external contractor performed the study. The activity of 10 µM ARN23746 as agonist or antagonist for several receptors, ion channels, enzymes, and transporters was assessed using validated assays under conditions defined by the contractor (https://www.discoverx.com/services/drug-discovery-development-services/safetyscan-profiling). A complete list of the off-targets evaluated is provided in Supplementary Table 3.

Viscera Index and Histological Analysis. Ts65Dn animals were analyzed after 28 days of treatment with vehicle or ARN23746 at 0.2 mg*kg^{-1} concentration. Liver, kidney, spleen, lung, and brain samples were excised and weighed to calculate the viscera index, which we used to evaluate hyperplasia, swelling, or atrophy of different organs potentially induced by ARN23746 treatment. Viscera Index = (Visceral weight (g) / Mouse weight (g)) *100. Then, liver, kidney, and spleen samples were fixed in 10% formalin solution and embedded in paraffin. Serial sections of 5-µm thickness were obtained and stained with Hematoxylin&Eosin (H&E) to evaluate morphology, and analyzed with a Leica DM5500 optical microscope (n=5 each group). The results were examined blind.

Synthetic procedures

All chemicals were purchased from Acros, Aldrich, Merck, Fluorochem, TCI or Alfa Aesar and used as such unless stated otherwise. All the commercial available reagents and solvents were used as purchased from vendors without further purification. Dry solvents were purchased from Sigma-Aldrich. Automated column chromatography purifications were done using a Teledyne ISCO apparatus (CombiFlash® Rf) with pre-packed silica gel or basic alumina columns of different sizes (from 4 g up to 120 g) and mixtures of increasing polarity of cyclohexane and ethyl acetate (EtOAc), cyclohexane or dicloromethane (DCM) and methanol (MeOH). NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for ^{1}H, and 100.62 MHz for ^{13}C), equipped with a BBI probe and Z-gradients. Spectra were acquired at 300 K, using deuterated dimethylsulfoxide (DMSO–d_6) or deuterated chloroform ($CDCl_3$) as solvents. For ^{1}H-NMR, data are reported as follows: chemical shift, multiplicity (s= singlet, d= doublet, dd= double of doublets, t= triplet, q= quartet, h= sextet, m= multiplet), coupling constants (Hz) and integration. UPLC/MS analyses were run on a Waters ACQUITY UPLC/MS system consisting of a SQD (single quadrupole detector) mass spectrometer equipped with an electrospray ionization interface and a photodiode array detector. The PDA range was 210–400 nm. Analyses were performed on an ACQUITY UPLC BEH C18 column (100x2.1mmID, particle size 1.7 µm) with a VanGuard BEH C18 pre-column (5x2.1 mmID, particle size 1.7 µm). Mobile phase was 10 mM NH_4OAc in H2O at pH 5 adjusted with CH_3COOH (A) and 10 mM NH_4OAc in CH_3CN–H_2O (95:5) at pH 5.0. Two types of gradients were applied depending on the analysis, gradient 1 (5 % to 95 % mobile phase B in 3 min) or gradient 2 (50 % to 100 % mobile phase B in 3 min). Electrospray ionization in positive and negative mode was applied. Electrospray ionization in positive and negative mode was applied. ESI was applied in positive and negative mode. All tested compounds showed ≥ 95% purity by NMR and UPLC/MS analysis.

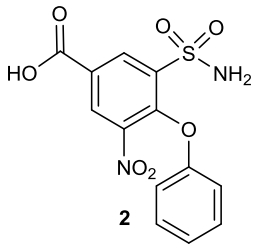
General reductive amination procedure A for the synthesis of compounds 4a-c, 5, 14 (Scheme S1, S2). To a suspension of ethyl benzoate **3** or **13** (1.0 mmol) in dry dichloromethane (15 mL) was added the proper aldehyde (4.0 mmol). After 30 min of stirring sodium triacetoxyborohydride was added (2.0 mmol) and the mixture was stirred at room temperature for 18 hr. At reaction completion, the crude was portioned between

CH_2Cl_2 (25 ml) and $NaHCO_3$ saturated solution (40 ml) and the layers separated. The organic layer was dried over Na_2SO_4 and concentrated to dryness at low pressure. Purification by silica gel flash chromatography with cyclohexane/EtOAc finally afforded the pure titled compounds.

General ester hydrolysis procedure B for the synthesis of compounds 6-8, 15 (Scheme S1, S2). To a suspension of the proper ester (1.0 mmol) in tetrahydrofuran (10 mL) was added a 0.5 LiOH aqueous solution (2.0 mmol) and the mixture was stirred at room temperature for 16 hr. At reaction completion, the crude was portioned between EtOAc (25 ml) and a NH_4Cl saturated solution (25 ml) and the layers separated. The organic layer was dried over Na_2SO_4 and concentrated to dryness at low pressure. Finally, trituration with cyclohexane afforded the pure titled compounds.

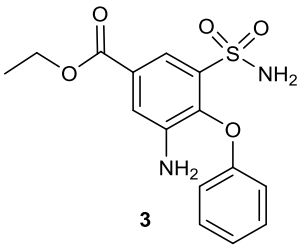
General procedure C for the synthesis of sulfonamides 17a-b (scheme S3). 4-Fluoro-3-chlorosulfonyl-benzoic acid **16** (1 mmol) solved in 1,5 mL of THF was added dropwise to 8 mL of an ice cold solution of the proper amine (2 mmol) in THF and stirred for 1 hr at rt. At reaction completion the reaction mixture was evaporated to dryness. The dry residue was dissolved in water and treated with 2N HCl until it reached pH3. The precipitated product was filtered and rinsed with water to afford the pure titled compounds.

General nucleophilic aromatic substitution procedure D for the synthesis of compounds 18-20 (scheme S3). A suspension of intermediates **17a-b** (1 mmol) and the appropriate amine (2 mmol) in dry 1,4-dioxane (3 ml) was stirred under Argon atmosphere at 100°C for 6 hours. After reaction completion the mixture was evaporated to dryness at low pressure and the residue was treated with a saturated NH_4Cl aqueous solution (15 ml) and extracted twice with EtOAc (2x15 ml). The combined organic layers were dried over Na_2SO_4 and concentrated to dryness at low pressure. Purification by silica gel flash chromatography with CH_2Cl_2/MeOH followed by trituration with a suitable solvent (cyclohexane or diethyl ether) afforded finally the pure title compounds.



2

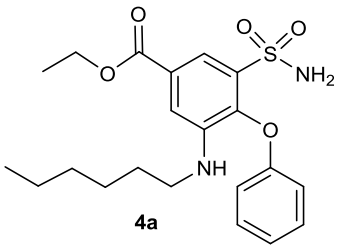
3-nitro-4-phenoxy-5-sulfamoyl-benzoic acid (2, Scheme S1). To a suspension of commercial 4-Chloro-3-nitro-5-sulfamoylbenzoic acid (4 g, 14.11 mmol) and $NaHCO_3$ (4.79 g, 56.45 mmol) in water (31.4 mL) was added phenol (2.82 g, 29.64 mmol) and the mixture was stirred at 85°C for 24 hours. At reaction completion, the reaction mixture was cooled in an ice bath and acidified with concentrated HCl until pH 3. The precipitated solid was filtrated and washed twice with cold water (2 x 8 mL) to afford pure benzoic acid **2** (2.71 g, 57 % yield) as a yellow solid. UPLC/MS: Rt = 1.31 min (gradient 1); MS (ESI) m/z: 337.1 $[M-H]^-$, $[M-H]^-$ calculated: 337.02. ^1H NMR (400 MHz, DMSO-d_6) **δ** 8.70 (d, *J* = 2.1 Hz, 1H), 8.63 (d, *J* = 2.2 Hz, 1H), 7.87 (s, 2H), 7.42 – 7.24 (m, 2H), 7.14 – 7.03 (m, 1H), 6.92 (d, *J* = 7.7 Hz, 2 H).



3

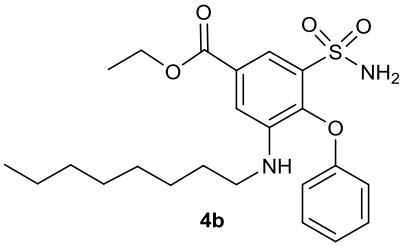
Ethyl 3-amino-4-phenoxy-5-sulfamoyl-benzoate (3, Scheme S1) Under argon atmosphere, to a suspension of benzoic acid **2** (2.71 g, 8.00 mmol) and $Pd(OH)_2/C$ (541 mg) in anhydrous methanol (159.9 mL) was added ammonium formate (2.61 g, 39.98 mmol) and the reaction crude was stirred at reflux temperature for 30 minutes. At completion, the reaction crude was filtered through a celite coarse patch and the filtrate concentrated to dryness at low pressure. This crude material (3.1 g) was solved in absolute ethanol (40 mL) and the solution degassed with nitrogen. Then, concentrated sulphuric acid was added (54 µL, 1.0 mmol) and the reaction mixture was stirred at reflux temperature for 16 hours. At completion, the reaction crude was evaporated to dryness at low pressure. The dry residue was treated with 30 mL of a saturated $NaHCO_3$ solution and extracted twice with CH_2Cl_2 (2 x 30 mL). The combined organic layers were dried over

anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford pure benzoate **3** (1.86 g, 69 % yield over two steps) as a white solid. UPLCS/MS: Rt = 1.84 min (gradient 1); MS (ESI) m/z: 335.1 $[M-H]^-$, $[M-H]^-$ calculated: 335.1. ^1H NMR (400 MHz, DMSO-d_6) δ 7.69 – 7.61 (m, 2H), 7.32 – 7.24 (m, 4H), 7.01 (t, *J* = 7.4 Hz, 1H), 6.88 – 6.80 (m, 2H), 5.34 (s, 2H), 4.33 (q, *J* = 7.1 Hz, 2H), 1.33 (t, *J* = 7.1 Hz, 3H).



4a

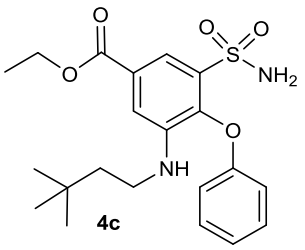
Ethyl 3-(hexylamino)-4-phenoxy-5-sulfamoyl-benzoate (4a, Scheme S1). Compound **4a** was synthesized following the general procedure previously described using ethyl benzoate **3** (100 mg, 0.29 mmol) and hexanal (145 µL, 0.42 mmol). Purification by typical silica gel flash chromatography (cyclohexane/EtOAc from 100:0 to 80:20) finally afforded the pure **4a** (62.6 mg, 51% yield) as a white solid. UPLCS/MS: Rt = 1.84 min (gradient 2); MS (ESI) m/z: 421.2 $[M+H]^+$, $[M+H]^+$calculated: 420.2. ^1H NMR (400 MHz, DMSO-d_6) δ 7.66 (d, *J* = 2.0 Hz, 2H), 7.40 (d, *J* = 2.0 Hz, 1H), 7.27 – 7.21 (m, 2H), 7.00 (t, *J* = 7.3 Hz, 1H), 6.81 (d, *J* = 8.1 Hz, 2H), 4.32 (q, *J* = 7.1 Hz, 2H), 3.02 (t, *J* = 6.7 Hz, 2H), 1.38 – 1.27 (m, 5H), 1.17 – 0.97 (m, 6H), 0.76 (t, *J* = 6.8 Hz, 3H).



4b

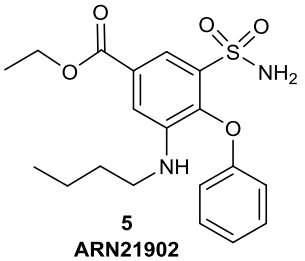
Ethyl 3-(octylamino)-4-phenoxy-5-sulfamoyl-benzoate (4b, Scheme S1). Compound **4b** was synthesized following the general procedure previously described using ethyl benzoate **3** (80 mg, 0.24 mmol) and octanal (147 µL, 0.94 mmol). Purification by typical silica gel flash chromatography (cyclohexane/EtOAc from 100:0 to 85:15) finally afforded the pure **4b** (62.9 mg, 58% yield) as a white solid. UPLCS/MS: Rt = 2.20 min (gradient 2); MS (ESI) m/z: 449.5 $[M+H]^+$, $[M+H]^+$ calculated: 448.2. ^1H NMR (400 MHz, DMSO-d_6) δ

7.69 (d, *J* = 2.0 Hz, 1H), 7.41 (d, *J* = 2.0 Hz, 1H), 7.34 (s, 2H), 7.30 – 7.21 (m, 2H), 7.05 – 6.97 (m, 1H), 6.87 – 6.81 (m, 2H), 5.08 (t, *J* = 5.7 Hz, 1H), 4.34 (q, *J* = 7.1 Hz, 2H), 3.05 (q, *J* = 6.5 Hz, 2H), 1.35 (m, 5H), 1.27 – 1.01 (m, 10H), 0.84 (t, *J* = 7.1 Hz, 3H).



4c

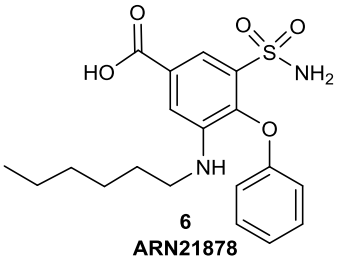
Ethyl 3-((3,3-dimethylbutyl)amino)-4-phenoxy-5-sulfamoylbenzoate (4c, Scheme S1). Compound **4c** was synthesized following the general procedure previously described using ethyl benzoate **3** (80 mg, 0.24 mmol) and 3,3-dimethylbutyraldehyde (118 µL, 0.94 mmol). Purification by typical silica gel flash chromatography (cyclohexane/EtOAc from 100:0 to 80:20) finally afforded the pure **4c** (82.2 mg, 81% yield) as a white solid. UPLCS/MS: Rt = 1.70 min (gradient 2); MS (ESI) m/z: 421.5 $[M+H]^+$, $[M+H]^+$ calculated: 421.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.69 (d, *J* = 2.0 Hz, 1H), 7.44 (d, *J* = 2.1 Hz, 1H), 7.33 (s, 2H), 7.28 – 7.23 (m, 2H), 7.05 – 6.96 (m, 1H), 6.86 – 6.78 (m, 2H), 5.16 (t, *J* = 5.8 Hz, 1H), 4.34 (q, *J* = 7.1 Hz, 2H), 3.13 – 3.05 (m, 2H), 1.33 (t, *J* = 7.1 Hz, 3H), 1.31 – 1.25 (m, 2H), 0.86 (s, 9H).



5
ARN21902

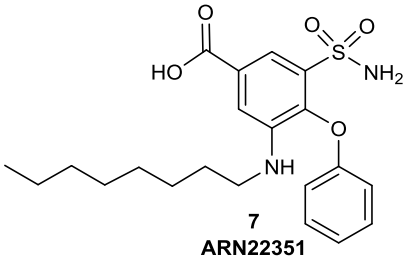
Ethyl 3-(butylamino)-4-phenoxy-5-sulfamoylbenzoate (5, Scheme S1), ARN21902. Compound **5** was synthesized following the general procedure previously described using ethyl benzoate **3** (100 mg, 0.29 mmol) and butyraldehyde (106 µL, 1.18 mmol). Purification by typical silica gel flash chromatography (cyclohexane/EtOAc from 100:0 to 75:25) finally afforded the pure **5** (79.6 mg, 70% yield) as a white solid. UPLCS/MS: Rt = 1.36 min (gradient 2); MS (ESI) m/z: 393.2 $[M+H]^+$, $[M+H]^+$ calculated: 393.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.70 (d, *J* = 1.9 Hz, 1H), 7.42 (d, *J* = 2.0 Hz, 1H), 7.32 (s, 2H), 7.31 –

7.21 (m, 2H), 7.06 – 6.97 (m, 1H), 6.85 (d, *J* = 6.5 Hz, 2H), 5.06 (t, *J* = 5.7 Hz, 1H), 4.35 (q, *J* = 7.1 Hz, 2H), 3.07 (q, *J* = 6.6 Hz, 2H), 1.41 – 1.31 (m, 5H), 1.11 (h, *J* = 7.4 Hz, 2H), 0.78 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 164.96 (CO), 156.22 (C), 142.49 (C), 139.83 (C), 137.75 (C), 129.07 (C), 127.15 (C), 122.25 (CH), 115.53 (CH), 114.83 (CH), 114.48 (CH), 61.04 (CH_2), 41.95 (CH_2), 30.07 (CH_2), 19.23 (CH_2), 14.15 (CH_3), 13.52 (CH_3). qNMR: 96.2%.



6
ARN21878

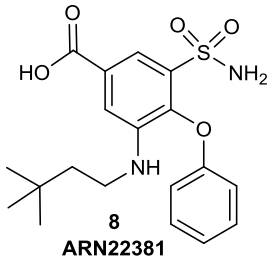
3-(hexylamino)-4-phenoxy-5-sulfamoylbenzoic acid (6, Scheme S1), ARN21878. Compound **6** was synthesized following the general procedure B previously described using ethyl ester **4a** (61 mg, 0.14 mmol). Final trituration in cyclohexane twice (2 × 1 mL) afforded the pure **6** (40.4 mg, 73% yield) as a white solid. UPLCS/MS: Rt = 0.50 min (gradient 2); MS (ESI) m/z: 391.2 $[M-H]^-$, $[M-H]^-$ calculated: 392.1. ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 7.69 (d, *J* = 1.9 Hz, 1H), 7.41 (d, *J* = 2.0 Hz, 1H), 7.32 (s, 2H), 7.29 – 7.24 (m, 2H), 7.01 (t, *J* = 7.3 Hz, 2H), 6.84 (d, *J* = 7.8 Hz, 2H), 3.05 (t, *J* = 6.8 Hz, 2H), 1.42 – 1.32 (m, 2H), 1.25 – 1.02 (m, 6H), 0.80 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 166.55 (CO), 156.30 (C), 142.35 (C), 139.57 (C), 137.65 (C), 129.10 (CH), 128.06 (C), 122.24 (CH), 115.56 (CH), 115.14 (CH), 114.76 (CH), 42.29 (CH_2), 30.86 (CH_2), 27.96 (CH_2), 25.78 (CH_2), 22.00 (CH_2), 13.85 (CH_3). qNMR: 97.3%.



7
ARN22351

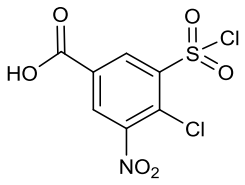
3-(octylamino)-4-phenoxy-5-sulfamoylbenzoic acid (7, Scheme S1), ARN22351. Compound **7** was synthesized following the general procedure B previously described

using ethyl ester **4b** (63 mg, 0.14 mmol). Final trituration in cyclohexane twice (2 × 1 mL) afforded the pure **7** (42.9 mg, 73% yield) as a white solid. UPLCS/MS: Rt = 0.87 min (gradient 2); MS (ESI) m/z: 419.5 $[M-H]^-$, $[M-H]^-$ calculated: 420.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.69 (d, *J* = 1.9 Hz, 1H), 7.41 (d, *J* = 1.9 Hz, 1H), 7.34 (s, 2H), 7.26 (t, *J* = 7.8 Hz, 2H), 7.00 (t, *J* = 7.3 Hz, 1H), 6.84 (d, *J* = 8.1 Hz, 2H), 5.04 (t, *J* = 5.7 Hz, 1H), 3.05 (q, *J* = 6.5 Hz, 2H), 1.37 (p, *J* = 7.0 Hz, 2H), 1.27 – 1.02 (m, 10H), 0.84 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.53 (CO), 156.28 (C), 142.31 (C), 139.52 (C), 137.63 (C), 129.06 (CH), 128.10 (C), 122.19 (CH), 115.54 (CH), 115.12 (CH), 114.73 (CH) , 42.25 (CH_2), 31.14 (CH_2), 28.58 (CH_2), 27.98 (CH_2), 26.15 (CH_2), 26.08 (CH_2), 22.05 (CH_2), 13.92 (CH_3). qNMR: 94.6%



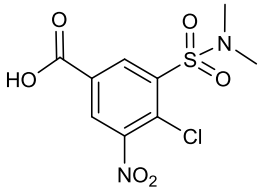
8
ARN22381

3-((3,3-dimethylbutyl)amino)-4-phenoxy-5-sulfamoylbenzoic acid (8, Scheme S1), ARN22381. Compound **8** was synthesized following the general procedure **B** previously described using ethyl ester **4c** (82.2 mg, 0.2 mmol). Final trituration in cyclohexane twice (2 × 1 mL) afforded the pure **8** (54.9 mg, 69% yield) as a white solid. Characterization: UPLCS/MS: Rt = 1.84 min (gradient 1); MS (ESI) m/z: 391.5 $[M-H]^-$, $[M-H]^-$ calculated: 392.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.69 (d, *J* = 1.9 Hz, 1H), 7.43 (d, *J* = 2.0 Hz, 1H), 7.33 (s, 2H), 7.26 (t, *J* = 8.0 Hz, 2H), 7.00 (t, *J* = 7.3 Hz, 1H), 6.82 (d, *J* = 8.0 Hz, 2H), 5.06 (t, *J* = 5.8 Hz, 1H), 3.12 – 3.04 (m, 2H), 1.32 – 1.25 (m, 2H), 0.85 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.54 (CO), 156.33 (C), 142.46 (C), 139.52 (C), 137.64 (C), 129.07 (C), 128.16 (CH), 122.15 (CH), 115.50 (CH), 115.18 (CH), 114.78 (CH), 41.64 (CH_2), 39.02 (CH_2), 29.56 (C), 29.17 (CH_3). qNMR: 91.2%



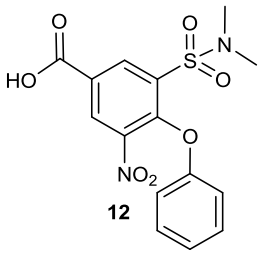
10

4-chloro-3-(chlorosulfonyl)-5-nitrobenzoic acid (10, Scheme S2). Under Ar atmosphere, to an ice cold solution of Nitric Acid (591 µL 15.52 mmol) and Sulfuric acid (2.53 mL, 46.57 mmol) was added 4-chloro-3-chlorosulfonyl-benzoic acid **9** (1.0 g, 3.88 mmol) and the reaction mixture was stirred at 90° for 4 hours. At completion, the reaction mixture was quenched dropwise into 50 mL of ice cold water and extracted twice with EtOAc (2 x 50 ml). The combined organic layers were dried over Na2SO4 filtered, and concentrated to dryness at low pressure to afford pure nitrobenzoic acid **10** (839.8 mg, 72% yield) as a yellow solid. UPLCS/MS: Rt = 1.51 min (gradient 1); MS (ESI) m/z: 298.2 $[M-H]^-$, $[M-H]^-$ calculated: 298.9. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.62 (d, *J* = 2.1 Hz, 1H), 8.37 (d, *J* = 2.1 Hz, 1H).



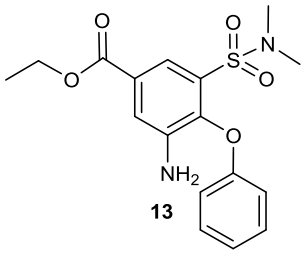
11

4-chloro-3-(N,N-dimethylsulfamoyl)-5-nitrobenzoic acid (11, Scheme S2). To a an ice cold mixture of 1N sodium hydroxide (5.54 mL) and aqueous 40% methylamine solution (0.5 mL, 3.32 mmol) nitro-benzoic acid **10** (839.8 g, 2.77 mmol) was added in portions, while stirring. Then the resulting solution was left standing until it had reached room temperature and stirred for additional 5 minutes. Then the reaction mixture was slowly acidified with 2N hydrochloric acid until pH 3. The resulting precipitate was collected by suction and recrystallized from aqueous ethanol to afford pure **11** (427.5 mg, 53% yield) as a brownish solid. UPLCS/MS: Rt = 1.27 min (gradient 1); MS (ESI) m/z: 307.3 [M-H]-, [M-H]- calculated: 307.9. 1H NMR (400 MHz, DMSO-d6) δ 8.71 (d, J = 2.0 Hz, 1H), 8.57 (d, J = 2.0 Hz, 1H), 2.88 (s, 6H).



12

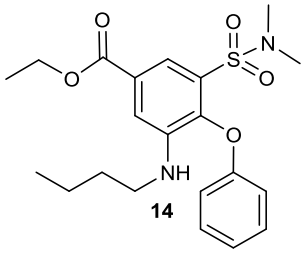
3-(N,N-dimethylsulfamoyl)-5-nitro-4-phenoxybenzoic acid (12, Scheme S2). To a suspension (427.5 mg, 1.37 mmol) and $NaHCO_3$ (465.3 mg, 5.48 mmol) in water (7 mL) was added phenol (237.7 mg, 2.88 mmol) and the mixture was stirred at 90°C for 8 hours under microwave irradiation (CEM Explorer 48 SP apparatus, power 200 W). At reaction completion, the reaction mixture was cooled in an ice bath and acidified with concentrated HCl until pH 3. The resulting precipitate was filtrated and recrystallized from aqueous ethanol to afford pure intermediate **12** (291.1 mg, 58% yield) as a brownish solid. UPLCS/MS: Rt = 1.51 min (gradient 1); MS (ESI) m/z: 365.4 $[M-H]^-$, $[M-H]^-$ calculated: 366.05. ^1H NMR (400 MHz, DMSO-d_6) **δ** 8.69 (d, *J* = 2.2 Hz, 1H), 8.65 (d, *J* = 2.2 Hz, 1H), 7.37 – 7.30 (m, 2H), 7.14 – 7.08 (m, 1H), 6.96 – 6.90 (m, 2H), 2.79 (s, 6H).



13

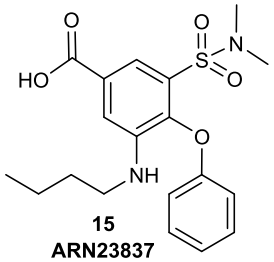
ethyl 3-amino-5-(N,N-dimethylsulfamoyl)-4-phenoxybenzoate (13, Scheme S2). Under argon atmosphere, to a suspension of benzoic acid **12** (291.1 mg, 0.79 mmol) and $Pd(OH)_2/C$ (58.0 mg) in anhydrous methanol (15.9 mL) was added ammonium formate (258.0 mg, 3.97 mmol) and the reaction crude was stirred at reflux temperature for 30 minutes. At completion, the reaction crude was filtered through a celite coarse patch and the filtrate concentrated to dryness at low pressure. This crude material (380 mg) was dissolved in absolute ethanol (2.2 mL) and the solution was cooled at 0°C and degassed with nitrogen. Then, thionyl chloride was added (110 µL, 1.45 mmol) and the reaction mixture was stirred at reflux temperature for 4 hours. At completion, the reaction crude was evaporated to dryness at low pressure. The dry residue was treated with 20 mL of a saturated $NaHCO_3$ solution and extracted twice with CH_2Cl_2 (2 x 20 mL). The combined

organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Purification by typical silica gel flash chromatography (cyclohexane/EtOAc from 85:15) finally afforded pure benzoate **13** (198.6 mg, 69% yield over two steps) as a brownish solid. UPLCS/MS: Rt = 2.13 min (gradient 1); MS (ESI) m/z: 365.4 $[M+H]^+$, $[M+H]^+$ calculated: 364.1. ^1H NMR (400 MHz, DMSO-d_6) **δ** 7.71 (d, *J* = 2.1 Hz, 1H), 7.61 (d, *J* = 2.1 Hz, 1H), 7.29 (t, *J* = 7.9 Hz, 2H), 7.03 (t, *J* = 7.3 Hz, 1H), 6.77 (d, *J* = 8.1 Hz, 2H), 5.45 (s, 2H), 4.34 (q, *J* = 7.1 Hz, 2H), 2.69 (s, 6H), 1.33 (t, *J* = 7.1 Hz, 3H).



14

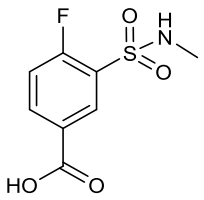
Ethyl 3-(butylamino)-5-(N,N-dimethylsulfamoyl)-4-phenoxybenzoate (14, Scheme S2). Compound **5** was synthesized following the general procedure **A** previously described using ethyl benzoate **13** (75 mg, 0.20 mmol) and butyraldehyde (73 µL, 0.81 mmol). Purification by typical silica gel flash chromatography (cyclohexane/EtOAc from 100:0 to 90:10) finally afforded the pure **15** (78.1 mg, 93% yield) as a white solid. UPLCS/MS: Rt = 1.86 min (gradient 2); MS (ESI) m/z: 419.5 $[M-H]^-$, $[M-H]^-$ calculated: 420.2. ^1H NMR (400 MHz, DMSO-d_6) **δ** 7.62 (d, *J* = 2.0 Hz, 1H), 7.48 (d, *J* = 2.0 Hz, 1H), 7.31 – 7.24 (m, 2H), 7.06 – 7.00 (m, 1H), 6.79 – 6.74 (m, 2H), 5.21 (t, *J* = 5.7 Hz, 1H), 4.35 (q, *J* = 7.1 Hz, 2H), 3.07 (q, *J* = 6.6 Hz, 2H), 2.69 (s, 6H), 1.42 – 1.31 (m, 5H), 1.14 – 1.05 (m, 2H), 0.77 (t, *J* = 7.3 Hz, 3H).



15
ARN23837

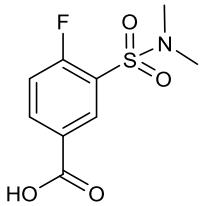
3-(butylamino)-5-(N,N-dimethylsulfamoyl)-4-phenoxybenzoic acid (15, Scheme S2), ARN23837. Compound **15** was synthesized following the general procedure **B** previously described using ethyl ester **14** (78.9 mg, 0.14 mmol). Final trituration in cyclohexane twice

(2 × 1 mL) afforded the pure **15** (48.9 mg, 89% yield) as a white solid. UPLCS/MS: Rt = 1.87 min (gradient 1); MS (ESI) m/z: 391.5 $[M-H]^-$, $[M-H]^-$ calculated: 392.1. ^1H NMR (400 MHz, DMSO-d_6) **δ** 7.63 (d, *J* = 1.9 Hz, 1H), 7.49 (d, *J* = 2.0 Hz, 1H), 7.33 – 7.26 (m, 2H), 7.04 (t, *J* = 7.4 Hz, 1H), 6.80 – 6.75 (m, 2H), 5.15 (t, *J* = 5.7 Hz, 1H), 3.07 (q, *J* = 6.6 Hz, 2H), 2.70 (s, 6H), 1.42 – 1.33 (m, 2H), 1.17 – 1.06 (m, 2H), 0.78 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) **δ** 166.41 (CO), 156.14 (C), 142.80 (C), 139.60 (C), 130.92 (C), 129.34 (CH), 122.29 (CH), 117.48 (CH), 115.66 (CH), 115.07 (CH), 42.01 (CH_2), 37.12 (CH_3), 30.13 (CH_2), 19.31 (CH_2), 13.58 (CH_3). qNMR: 96.2%.



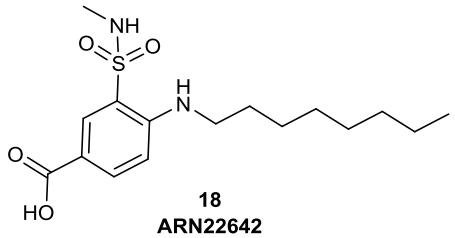
17a

4-fluoro-3-(methylsulfamoyl)benzoic acid (17a, Scheme S3). Compound **17a** was synthesized following the general procedure **C** previously described using intermediate **16** (500 mg, 2.07 mmol) and a 2M methylamine solution in THF (2.07 ml, 4.15 mmol). The described workup afforded pure **17a** (313.8 mg, 64% yield) as a white solid. UPLC/MS: Rt = 1.26 min (gradient 1); MS (ESI) m/z: 232.3 $[M-H]^-$. $[M-H]^-$ calculated: 232.02 ^1H NMR (400 MHz, DMSO-d_6) **δ** 8.30 (dd, *J* = 7.0, 2.2 Hz, 1H), 8.25 – 8.19 (m, 1H), 7.89 (q, *J* = 4.8 Hz, 1H), 7.62 – 7.54 (m, 1H), 2.52 (d, *J* = 4.8 Hz, 3H).



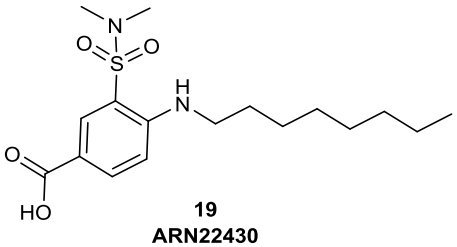
17b

3-(dimethylsulfamoyl)-4-fluoro-benzoic acid (17b, Scheme S3). Compound **17b** was synthesized following the general procedure **C** previously described using intermediate **16** (1 g, 4.15 mmol) and a 2M dimethylamine solution in THF (4.15 ml, 8.30 mmol). The described workup afforded pure **17b** (749 mg, 73% yield) as a white solid. UPLC/MS: Rt = 1.11 min (gradient 1); MS (ESI) m/z: 246.3 $[M-H]^-$. $[M-H]^-$ calculated: 246.03. ^1H NMR (400 MHz, DMSO-d_6) **δ** 8.29 – 8.24 (m, 2H), 7.67 – 7.58 (m, 1H), 2.75 (d, *J* = 1.9 Hz, 6H).



18
ARN22642

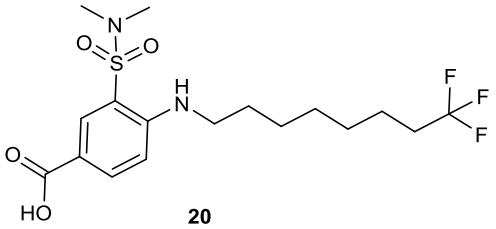
3-(methylsulfamoyl)-4-(octylamino)benzoic acid (18, Scheme S3), ARN22642. Compound **18** was synthesized following the general procedure **D** previously described using intermediate **17a** (50 mg, 0.21 mmol) and octylamine (71 µl, 0.42 mmol) in dry 1,4-Dioxane (0.7 ml). The compound was obtained pure without silica gel purification. Trituration with cyclohexane (1 ml) afforded the pure **18** (69.5 mg, 97% yield) as a white solid. UPLC/MS: Rt = 2.28 min (gradient 1); MS (ESI) m/z: 341.4 $[M-H]^-$. [M-H] calculated: 341.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.15 (d, *J* = 2.1 Hz, 1H), 7.89 (dd, *J* = 8.8, 2.1 Hz, 1H), 6.86 (d, *J* = 8.9 Hz, 1H), 6.44 (t, *J* = 5.4 Hz, 1H), 3.23 (q, *J* = 6.6 Hz, 2H), 2.38 (s, 3H), 1.59 (p, *J* = 7.1 Hz, 2H), 1.40 – 1.20 (m, 10H), 0.89 – 0.82 (m, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.49 (CO), 148.53 (C), 134.88 (CH), 131.99 (CH), 118.64 (C), 116.42 (C), 111.44 (CH), 42.47 (CH_2), 31.19 (CH_2), 28.66 (CH_2), 28.63 (CH_2), 28.16 (CH_3), 28.11 (CH_2), 26.32 (CH_2), 22.06 (CH_2), 13.93 (CH_3). qNMR: 97.2%.



19
ARN22430

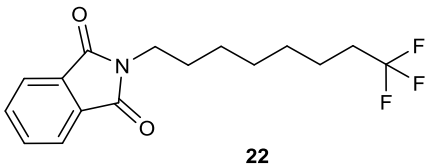
3-(dimethylsulfamoyl)-4-(octylamino)benzoic acid (19, Scheme S3), ARN22430. Compound **19** was synthesized following the general procedure **D** previously described using intermediate **17b** (50 mg, 0.20 mmol) and octylamine (67 µl, 0.40 mmol) in dry 1,4-Dioxane (0.7 ml). The compound was obtained pure without silica gel purification. Trituration with cyclohexane (1 ml) afforded the pure **19** (59.9 mg, 84% yield). UPLC/MS: Rt = 2.44 min (gradient 1); MS (ESI) m/z: 355.4 $[M-H]^-$. $[M-H]^-$ calculated: 355.2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.62 (s, 1H), 8.04 (d, *J* = 2.1 Hz, 1H), 7.93 (dd, *J* = 8.9, 2.1 Hz, 1H), 6.91 (d, *J* = 9.0 Hz, 1H), 6.75 (t, *J* = 5.4 Hz, 1H), 3.23 (q, *J* = 6.6 Hz, 2H), 2.65 (s, 6H), 1.57 (p, *J* = 6.9 Hz, 2H), 1.39 – 1.19 (m, 10H), 0.90 – 0.80 (m, 3H). ^{13}C NMR (101 MHz,

DMSO-d_6) δ 166.79 (CO), 149.99 (C), 135.87 (CH), 132.90 (CH), 117.08 (C), 115.63 (C), 112.49 (CH), 42.76 (CH_2), 37.76 (CH_3, 2C), 31.64 (CH_2), 29.09 (CH_2), 29.06 (CH_2), 28.55(CH_2), 26.80 (CH_2), 22.52 (CH_2), 14.40 (CH_3). qNMR: 93.3%.



20
ARN23746

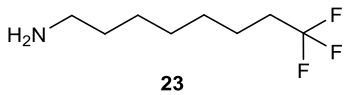
3-(dimethylsulfamoyl)-4-(8,8,8-trifluorooctylamino)benzoic acid (20, Scheme S3), ARN23746. Titled compound was synthesized following the general procedure **D** previously described using intermediate **17b** (50 mg, 0.20 mmol) and amine **23** (89 mg, 0.40 mmol) in dry 1,4-Dioxane (0.7 ml). Purification by silica gel flash chromatography (CH_2Cl_2/MeOH from 100:0 to 98:02). Trituration with cyclohexane (1 ml) afforded then the pure compound **20** (44.3 mg, 54% yield). UPLC/MS: Rt = 2.28 min (gradient 1); MS (ESI) m/z: 409.4 $[M-H]^-$. [M-H] calculated: 409.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.62 (s, 1H), 8.05 (d, *J* = 2.1 Hz, 1H), 7.93 (dd, *J* = 8.8, 2.1 Hz, 1H), 6.91 (d, *J* = 9.0 Hz, 1H), 6.75 (t, *J* = 5.4 Hz, 1H), 3.24 (q, *J* = 6.6 Hz, 2H), 2.66 (s, 6H), 2.29 – 2.14 (m, 2H), 1.64 – 1.52 (m, 2H), 1.52 – 1.39 (m, 2H), 1.40 – 1.25 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.31 (CO), 149.50 (C), 135.38 (CH), 132.42 (CH), 127.68 (CF_3, q, $^1J_{CF}$ = 276.6 Hz), 116.62 (C), 115.18 (C), 112.01 (CH), 42.22 (CH_2), 37.27 (CH_3, 2C), 32.34 (CH_2, q, $^2J_{CF}$ = 26.9 Hz), 28.11 (CH_2), 27.97 (CH_2), 27.79 (CH_2), 26.06 (CH_2), 21.31(CH_2).



22

2-(8,8,8-trifluorooctyl)isoindoline-1,3-dione (22, Scheme S4). A suspension of potassium phthalimide (300 mg, 1.60 mmol) and 8-Bromo-1,1,1-trifluorooctane **21** (400 µL, 2.08 mmol) in dry N,N-dimethylformamide (5.5 mL) was stirred at room temperature for 16 hours. After reaction completion the mixture portioned between water (35 ml) and

EtOAc (35 ml) and the layers separated. The organic layer was dried over Na_2SO_4 and concentrated to dryness at low pressure. Purification by silica gel flash chromatography (cyclohexane/EtOAc from 100:0 to 85:15) afforded the pure compound **22** (392.63 mg, 75% yield) as a colourless oil. UPLC/MS: Rt = 1.76 min (gradient 2); MS (ESI) m/z: 314.4 $[M+H]^+$. $[M+H]^+$ calculated: 314.1. ^1H NMR (400 MHz, Chloroform-*d*) δ 7.86 – 7.81 (m, 2H), 7.73 – 7.67 (m, 2H), 3.70 – 3.65 (m, 2H), 2.11 – 1.97 (m, 2H), 1.68 (p, *J* = 7.2 Hz, 2H), 1.58 – 1.47 (m, 2H), 1.39 – 1.30 (m, 6H).



23

8,8,8-trifluorooctan-1-amine (23, Scheme S4). Intermediate **22** (393 mg, 1.24 mmol) was refluxed in absolute ethanol (5.5 mL) with hydrazine hydrate (140 µL, 1.86 mmol) for 4 hours. At reaction completion the reaction mixture was cooled at room temperature and the resulting precipitated solid was filtered. The solid was rinsed twice with ethanol (2 x 10 mL) and the filtrate concentrated to dryness at low pressure. Purification by basic alumina flash chromatography (CH_2Cl_2/MeOH from 100:0 to 95:05) afforded pure amine **23** (136.3 mg, 60% yield) as a yellow oil. UPLC/MS: Rt = 1.59 min (gradient 1); MS (ESI) m/z: 184.4 $[M+H]^+$. $[M+H]^+$ calculated: 184.1. ^1H NMR (400 MHz, DMSO-d_6) δ 2.78 – 2.68 (m, 2H), 2.30 – 2.15 (m, 2H), 1.61 – 1.41 (m, 4H), 1.38 – 1.21 (m, **6H**).