SUPPORTING INFORMATION

Preclinical development of the Na-K-2Cl cotransporter-1 (NKCC1) inhibitor ARN23746 for the treatment of neurodevelopmental disorders

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***In vitro* permeability determination and P-gp interaction in MADCKII MDR1 cells.** An external contractor performed the study. Additional information on the study conditions can be obtained from the contractor's website (https://www.evotec.com/en/execute/drug-discovery-services). MDCKII-MDR1 cells (SOLVO Biotechnology) were preincubated (37°C, 15 to 30 minutes), on apical (A) and basolateral (B) sides filling both wells with receiver working solutions containing transport buffer (HBSSH). Following pre-incubation, test items (ARN23746, 10 µM) and control items (Digoxin, 10 µM) transport was measured in two directions (apical to basolateral [A→B]) and basolateral to apical [B→A]) and these directions were performed in triplicate sets of wells, in absence and in presence of the P-gp inhibitor 10 µM Elaclidar. For [A→B] directional transport, 75 µL of donor working solution were then added to the A (apical) compartment and 235 µL of receiver working solution to the B (basolateral) compartment. For [B→A] directional transport, 235 µL donor working solution with test items or reference controls were be added to the B compartment and 75 µL receiver working solution to the A compartment. Transport of permeability reference controls, Atenolol and Metoprolol (10 µM) were measured in one direction (apical to basolateral [A→B]) only. The cells were incubated (37°C, with shaking) for 60 minutes. Samples were removed from donor solutions and transport buffer (blank samples) for t=0 samples (C0) and at the end of the incubation period, from the receptor site (basolateral compartment for A→B direction and apical compartment for B→A direction) and from donor side (C0 fin). Samples were transferred into 96 well plate. Samples were then extracted by protein precipitation with acetonitrile containing Rolipram (for positive ion mode) or Diclofenac (for negative ion mode) as generic internal standard compounds and centrifuged for 10 min at 3000 rpm. Samples were analysed via LC MS/MS system to monitor the test item or positive control to internal standard peak area ratios as representative of the test or control items concentrations.

Papp (apparent permeability expressed in nm/sec) values were then calculated according to the following equation:

$$Papp(nm/\sec) = \left(\frac{dQ}{dt}\right) x \left(\frac{1}{C0}\right) x \left(\frac{1}{A}\right)$$

Where:

- dQ/dt is the permeability rate
- C_0 is the initial concentration in the donor solution (expressed as IS ratio)
- A is the surface area of the filter (the surface area of the cell monolayer).

Monolayer efflux ratios (ER) in the presence and absence of the P-gp inhibitor, Elaclidar, are derived using the following equation:

$$EffluxRatio = \left(\frac{B - APapp(nm/\sec)}{A - BPapp(nm/\sec)}\right)$$

Comparing the efflux ratios generated in the presence and absence of Elaclidar indicates whether test items are P-gp substrates. A test item was considered a P-gp substrate when the efflux ratio in the absence of inhibitor is >2 and is significantly reduced in the presence of inhibitor.

***In vitro* permeability determination and BCRP interaction in Caco-2 cells.** An external contractor performed the study. Additional information on the study conditions can be obtained from the contractor's website (https://www.evotec.com/en/execute/drug-discovery-services). Caco-2 cells were seeded on to Millipore Multiscreen Transwell plates at 1 x 10^5 cells/cm^2. Assay

buffer was composed of supplemented HBSS pH 7.4. For assessment of A→B permeability, HBSS was removed from the apical compartment and replaced with ARN23746 810 µM. The apical compartment insert was then placed into a companion plate containing fresh buffer (containing ≤1 % v/v DMSO or 10 µM of the BCRP inhibitor fumitremorgin C, maintaining a ≤1 % v/v DMSO concentration). For assessment of B→A permeability, HBSS was removed from the companion plate and replaced with ARN23746 dosing solution. Fresh buffer (containing ≤1 % v/v DMSO or 10 µM of the BCRP inhibitor fumitremorgin C, maintaining a ≤1 % v/v DMSO concentration) was added to the apical compartment insert, which was then placed into the companion plate. At 120 min the apical compartment inserts and the companion plates were separated and apical and basolateral samples diluted for analysis. Four control compounds were screened alongside ARN23746, atenolol (human absorption 50 %), antipyrine (human absorption 97 %), and estrone 3-sulphate (a BCRP substrate). The BCRP substrate estrone 3-sulphate was screened in the absence and presence of the BCRP inhibitor.

Samples were analysed via LC MS/MS system to monitor the test item or positive control to internal standard peak area ratios as representative of the test or control items concentrations. The starting concentration (C0) was determined from the dosing solution and the experimental recovery calculated from C0 and both apical and basolateral compartment concentrations.

Papp (apparent permeability expressed in nm/sec) values were then calculated according to the following equation:

$$Papp(nm/\sec) = \left(\frac{dQ}{dt}\right) x \left(\frac{1}{C0}\right) x \left(\frac{1}{A}\right)$$

Where:

- dQ/dt is the permeability rate
- C_0 is the initial concentration in the donor solution (expressed as IS ratio)
- A is the surface area of the filter (the surface area of the cell monolayer).

Monolayer efflux ratios (ER) in the presence and absence of the BCRP inhibitor fumitremorgin C, were derived using the following equation:

$$EffluxRatio = \left(\frac{B - APapp(nm/\sec)}{A - BPapp(nm/\sec)} \right)$$

Comparing the efflux ratios generated in the presence and absence of fumitremorgin C indicates whether test items are BCRP substrates. A test item was considered a BCRP substrate when the efflux ratio in the absence of inhibitor is >2 and is significantly reduced in the presence of inhibitor.

***In vitro* mouse 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* mouse rat and human liver hepatocytes stability and metabolites identification.** Mouse (Gibco), 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 Wereiams 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. The generated metabolites were analyzed via LC-HRMS and assigned upon detection of the masses of likely metabolic reactions.

Figure S1

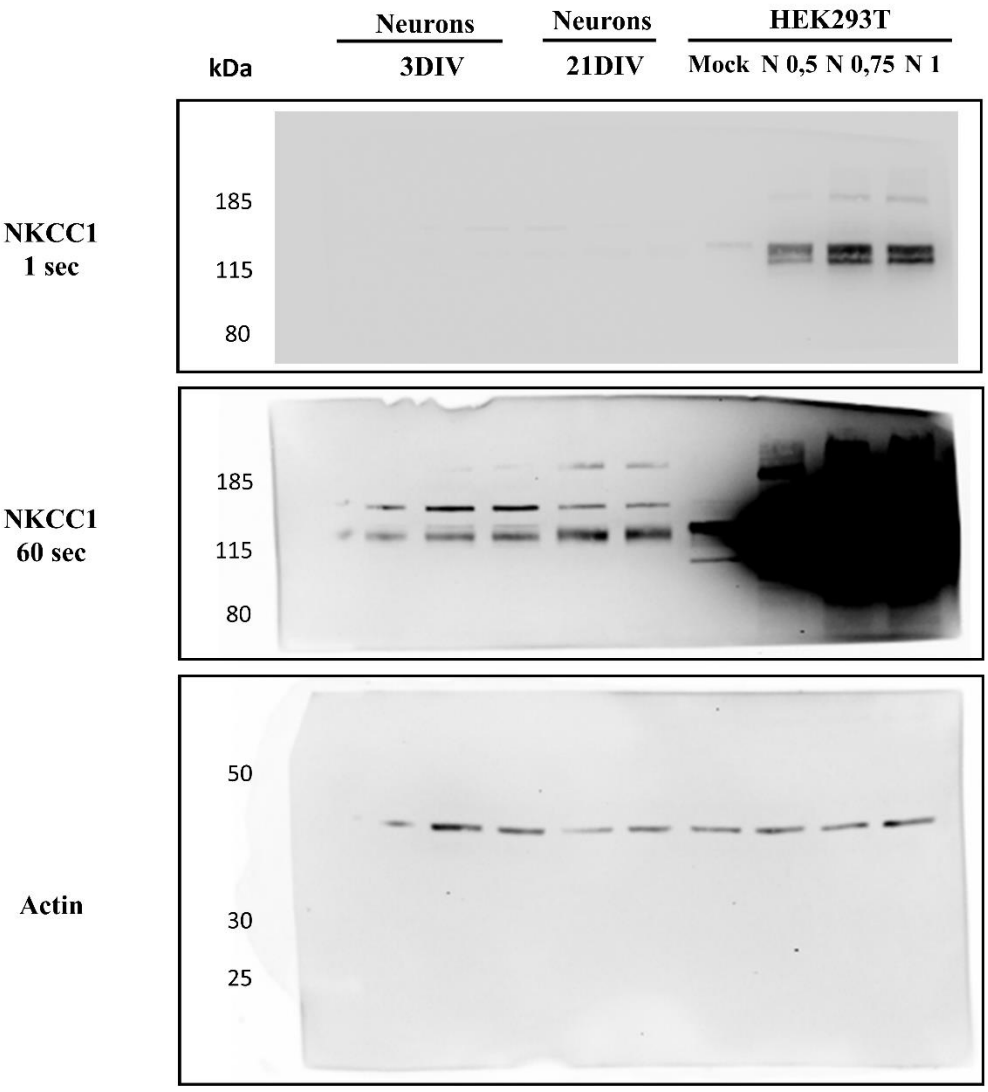


Figure S1: Uncropped blot images. Full-length blot images corresponding to the cropped western blot presented in the main figure (Figure 1A) of this study.

Table S1

A

MDCKII-MDR1 ER (Papp B>A/A>B) wo and w INH[a]	P-gp Eff.[b]	MDCKII-MDR1 Mean Perm.[c]	Caco-2 ER (Papp B>A/A>B) wo and w INH[d]	BCRP Eff.[e]	Caco-2 Mean Perm.[f]
1.3-1.4	NO	150 nm/sec	0.938	NO	25.6 nm/sec

B

$T_{1/2}$ mouse micros.[a]	$T_{1/2}$ mouse hep.[b]	$T_{1/2}$ rat hep.[b]	$T_{1/2}$ human hep.[b]	Cl_{int} mouse[c]	Cl_{int} rat[c]	Cl_{int} human[c]
> 60 min	172 min	69 min	72 min	4.8 $\mu L*min^{-1}$ *million $cells^{-1}$	5.0 $\mu L*min^{-1}$ *million $cells^{-1}$	4.8 $\mu L*min^{-1}$ *million $cells^{-1}$

C

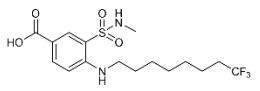
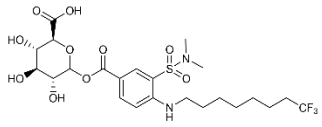
Main observed Metabolites. (proposed structure)	Observed Mass	Rat metabolite formation rate	Human metabolite formation rate
	[M – 14]	$1.61*10^3$	BLQ
	[M + 176]	$1.21*10^3$	$1.11*10^3$

Table S1: *In vitro* permeability assessment and metabolism analysis of ARN23746. (A) *In vitro* permeability determination of ARN23746. ***a***) Efflux ratio between compartments A and B of ARN23746 in MDCK-II cells transfected with MDR1 gene in absence of presence of a P-gp inhibitor (verapamil, 100 µM). Compound was incubated at 10 µM (final DMSO < 1%). ***b***) Estimated presence of P-gp efflux based on efflux ratio. ***c***) Mean permeability in MDCK-II cells transfected with MDR1 gene. ***d***) Efflux ratio between compartments A and B of ARN23746 in

Caco-2 cells transfected with BCRP gene in absence of presence of a BCRP inhibitor (fumitremorgin C, 10 µM). Compound was incubated at 10 µM (final DMSO < 1%). ***e***) Estimated presence of BCRP efflux based on efflux ratio. ***f***) Mean permeability in Caco-2 cells transfected with BCRP gene. (**B**) *In vitro* metabolic half-life and intrinsic clearance determination of ARN23746. ***a***) Metabolic stability in mouse liver microsomes. Compounds were incubated at 5 µM (final DMSO 0.1%). ***b)*** Metabolic stability in mouse, rat, and human hepatocytes. Compounds were incubated at 0.5 µM (final DMSO 0.1%). ***c***) intrinsic clearance determination in mouse, rat, and human hepatocytes. Compounds were incubated at 0.5 µM (final DMSO 0.1%). (**C**) Cross-species analysis of most abundant metabolites of ARN23746 and rate of formation determination. Compound was incubated in rat and human hepatocytes at 10 µM (final DMSO 0.1%). The generated metabolites were analysed via LC-HRMS and assigned upon detection of the masses of likely metabolic reactions. BLQ = below limit of quantification.

Table S2

% Object preference NOR

Objects	WT Vehicle	WT ARN23746	Ts65Dn Vehicle	Ts65Dn ARN23746	F and P
A	30.29 ± 1.88	34.70 ± 2.21	33.37 ± 1.97	32.89 ± 2.12	Two-way ANOVA $F_{interaction\ (1,48)} = 1.439$, P=0.236
B	31.25 ± 1.57	32.74 ± 2.22	34.49 ± 2.04	36.93 ± 2.31	Two-way ANOVA $F_{interaction\ (1,48)} = 0.053$, P=0.818
C	38.46 ± 1.93	33.89 ± 2.70	30.81 ± 1.45	30.18 ± 1.80	Two-way ANOVA $F_{genotype\ (1,48)} = 7.366$, P=0.009 Tukey *post hoc* test Within Vehicle WT vs Ts P=0.013

Total exploration time (sec) NOR

	WT Vehicle	WT ARN23746	Ts65Dn Vehicle	Ts65Dn ARN23746	F and P
Acquisition	75.60 ± 7.07	78.45 ± 5.22	85.71 ± 7.53	73.59 ± 7.27	Two-way ANOVA $F_{interaction\ (1,48)} = 1.218$, P=0.275
Trial	60.23 ± 4.52	66.47 ± 5.75	71.28 ± 7.72	61.98 ± 7.63	Two-way ANOVA $F_{interaction\ (1,48)} = 1.482$, P=0.229

Supplementary table 2. Control parameters in the NOR tasks of WT and Ts65Dn mice.