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Supplemental information

Direct activation of KCC2 arrests benzodiazepine refractory status epilepticus and limits the subsequent neuronal injury in mice

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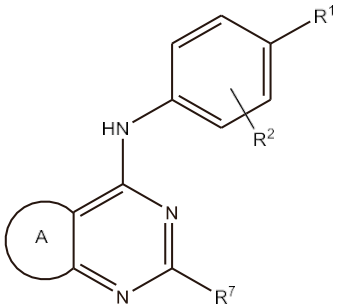
Supplementary Figure 1. KCC2 activators; related to Figure 1.

The subseries described here is captured by the following formula:

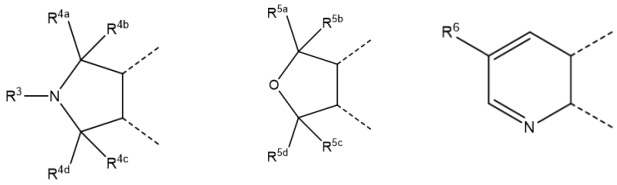


R1 is selected from C2-6alkyl; C2-6alkenyl; C2-6alkynyl; C2-6alkoxy; C2-6alkenyloxy; C2-6alkynyloxy; C3-7cycloalkyl; -O-C3-7cycloalkyl; C6-10aryl; -O-(CH2)m-C6-10aryl; 6 membered heteroaryl; and thiophenyl; wherein alkyl, alkenyl, alkynyl, alkoxy, alkenyloxy, alkynyloxy and cycloalkyl are optionally substituted with 1, 2 or 3 5 substituents selected from -F and -CF3; and wherein aryl and heteroaryl are optionally substituted with 1 or 2 substituents selected from -halo, -C1-3alkyl, -C1-8alkoxy and -C2-8alkynyloxy, wherein -C1-3alkyl, -C1-8alkoxy and -C2-8alkynyloxy are optionally substituted with 1, 2, or 3 substituents selected from -F, -CF3 and -NHC(O)O-C1-6alkyl or two substituents together with the carbon to which they are attached form diazirinyl.

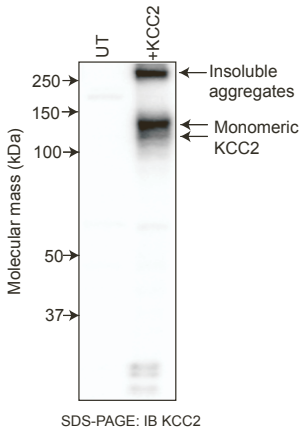
R2 is selected from -H; -halo; and -C1-3alkyl optionally substituted with 1, 2 or 3 substituents selected 10 from -F and -CF3.

R7 is selected from -NR10R11; a 5 to 7 membered monocyclic heterocycloalkyl; and a 5 or 6 membered monocyclic heteroaryl; wherein the heterocycloalkyl and heteroaryl are optionally substituted with 1, 2 or 3 groups selected from -CN; -C1-6alkyl optionally substituted with 1, 2 or 3 substituents selected from -F, -CF3 and 30 6 -OH; -C1-3alkoxy optionally substituted with 1, 2 or 3 substituents selected from -F and -CF3; -C(O)OH; -C1-3alkylene-NHC(O)C1-6alkyl; -C1-3alkylene-NHC(O)OC1-6alkyl; and C3-5cycloalkyl; or the heterocycloalkyl is optionally substituted with two substituents on the same ring carbon which together with the carbon atom to which they are attached form a 5 to 7 membered monocyclic heterocycloalkyl; and wherein when R7 is morpholinyl and R1 is unsubstituted phenyl, R2 is not -H;

A. is selected from:

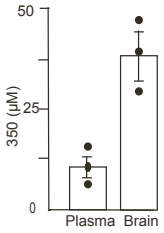


Supplementary Figure 2. Analyzing the molecular mass of KCC2 expressed in HEK-293 cells; related to Figure 1.



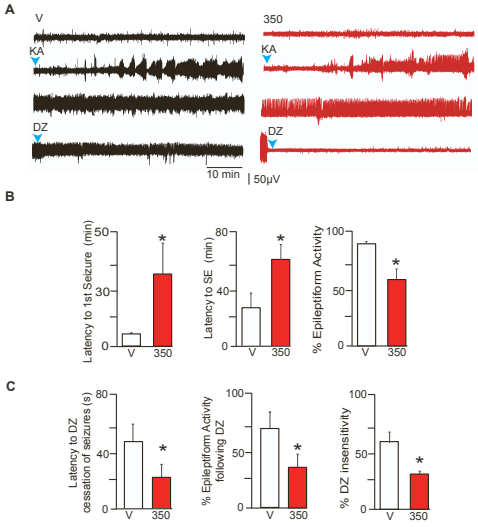
Untransfected HEK-293 (UT) and those transiently expressing KCC2 were subject to SDS-PAGE followed by immunoblotting with KCC2 antibodies. The migration of individual molecular mass markers is indicated by the lefthand arrows. The migration of monomeric KCC2 and denatured aggregates are also indicated.

Supplementary Figure 3. Analyzing the accumulation of 350 in the brain and plasma of mice following IV dosing, *related to Figure 4.*



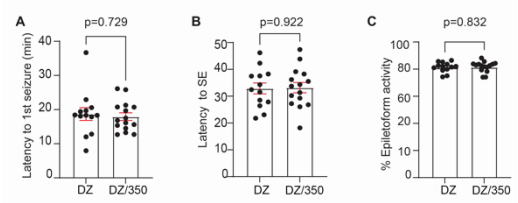
A. Mice were injected IV with 25 mg/kg 350and 30 min later drug accumulation in plasma and brain were measured using LC-MS/MS, n=3 mice.

Supplementary Figure 4. Injection of 350 into the hippocampus prevents the development of RSE, *related to Figure 5.*



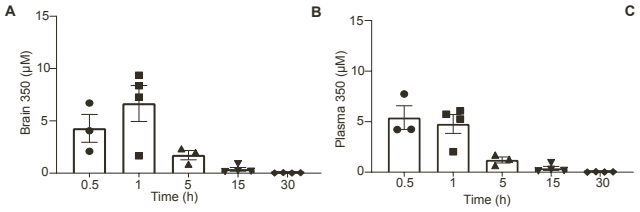
A. Exemplar EEG traces are shown for mice injected with 350 (20 µM) or vehicle into the hippocampus prior to dosing with IP with 20 mg/kg KA. 2h following KA injection mice were dosed with IP with 10 mg/kg DZ (and EEG recordings were extended for a further 1 h. **B**. The time to the 1st seizure (*p = 0.039), the onset of SE (*p = 0.005) and the % total time in epileptiform activity (*p = 0.002) were compared between treatment groups, n=9 mice. **C**. The latency to seizure reduction by DZ, (*p = 0.040), % Epileptiform activity (*p = 0.018), and % DZ insensitivity were then compared between experimental groups. p values were determined using unpaired t-tests, n = 8 mice.

Supplementary Figure 5. Comparing the development of SE in mice treated at 2 h with DZ or DZ/350; related to Figure 6



A. The latency to the first seizure following KA injection was compared for mice subsequently treated at 2h with DZ or DZ/350 (DZ: 13 mice, DZ/350: 15 mice). **B.** The latency to SE following KA injection was compared for mice subsequently treated at 2 h with DZ or DZ/350. % Time mice spent in epileptiform activity was compared for mice subsequently treated at 2 h with DZ or DZ/350 (DZ: 13 mice, DZ/350: 15 mice).

Supplementary Figure 6. Analyzing the accumulation of 350 in the brain and plasma following IP dosing; Related to Figure 6.



Time (h)	Brain (µM)	Plasma (µM)
0.5	4.29 ± 1.33	5.40 ± 1.17
1	6.66 ± 1.72	4.77 ± 0.93
5	1.73 ± 0.44	1.22 ± 0.29
15	0.372 ± 0.177	0.37 ± 0.19
30	0.033 ± 0.014	0.02 ± 0.008

Mice were injected with IP with 50 mg/kg 350 and maintained for up to 30 h. Brain (**A**) and plasma (**B**) levels of 350 were then examined 0.5, 1, 5, 15, and 30 h after injection using LC-MS/MS. This data was used to determined drug levels over time as shown in **C**, n=3-4 mice.