Supplementary Information

Inhibition of drug-induced seizure development in both zebrafish and mouse models by a synthetic nanoreceptor

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Experimental section

Materials and instrument

CB[7] was synthesized according to the reported method[1]. PTZ was purchased from Alfa Aesar (Hong Kong) and used as received. Ultrapure water was obtained from a Millipore water system (Millipore, Bedford, Massachusetts). Fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). The E3 medium was composed of 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$ (pH 7.2 – 7.3) in ultrapure water. ^{1}H NMR spectra was obtained using Bruker 600 MHz NMR spectrometer and isothermal titration calorimetry analysis was performed using A Malvern MicroCal PEAQ ITC instrument. The liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS) analysis was performed using an UltiMate 3000 HPLC (Thermo Scientific, USA) instrument, and an LTQ Orbitrap XL™ hybrid iontrap-orbitrap mass spectrometer (Thermo Scientific, USA). Chromatographic separation was performed using an eclipse XDB-C18 analytical column (4.6 × 250 mm, 5 μm particle size, Agilent, USA).

Animals

Zebrafish fish

Wild type zebrafish were used for locomotion assays, Briefly, adult zebrafish were raised in an aquaculture system with 14 h light/ 10 h dark cycles, and fed twice daily with freshly hatched brine shrimp. Mature male and female zebrafish (at a ratio of 1 male to 2 females) were transferred into a breeding tank and separated by a mesh screen the night before breeding. All fertilized embryos were examined under a microscope and only those that developed normally were selected for experiments. The collection of embryos, and exposure experiments were all performed in E3 medium and were maintained at 27 ± 1°C.

Mice

Thirty-six adult male C57BL/6 mice (Animal Facility, University of Macau, Macau)

were used as experimental subjects. They were maintained on a 12:12 light-dark cycle and at an ambient temperature of 20 ± 2°C with free access to food and water. All animal experiments were conducted in accord with the ethical guidelines of the Institute of Chinese Medical Sciences, University of Macau, and the protocol was approved by the Animal Ethics Committee, University of Macau.

^{1}H NMR titration

Briefly, PTZ (1 mM) was dissolved in D_2O, it is then used to dissolve different equivalent of CB[7] (0.5 eq. and 1.6 eq.), these samples were then sent for NMR analysis after sonication.

Isothermal Titration calorimetry (ITC) analysis

CB[7] (2 mM) and PTZ (0.15 mM) were prepared with ultrapure water. CB[7] (2 mM) and PTZ (0.2 mM) were prepared in E3 medium, and CB[7] (2 mM) and PTZ (20 mM) were prepared in fetal bovine serum (FBS), respectively. All these solutions were subsequently sonicated for degassing before ITC experiments. ITC analysis was conducted in these three different media at 25°C, and data was analyzed with the built- in software of MicroCal PEAQ ITC Analysis instrument.

Zebrafish locomotion

CB[7] (2 mM) and PTZ (4 mM) were dissolved in E3 medium. Zebrafish larvae (7 dpf) were placed into the wells of a 96-well plate (1 larvae per well). The zebrafish was acclimatized for 30 min and their basal swimming behavior was monitored for 20 min using a Viewpoint Zebrabox system (http://www.viewpoint.fr/en/p/equipment/zebrabox). Various concentrations of CB[7] (1 and 2 mM), PTZ (2 and 4 mM) and PTZ@CB[7] were added to each well (10-12 fish in each group and leaving 100 µL solution per well). With an acclimation for 30 min, the swimming behavior of the larvae was then recorded for 20 min. The quantification of seizure-type locomotion was achieved using the Zebrabox system and software to analyze data to give numbers of movements, distances travelled and 3 predefined 'speed' categories. These 3 speed categories were: low speed movements of below 5 mm/s; medium speed movements of between 5 and 20 mm/s; and high-speed movements of greater than 20 mm/s. The judgment of seizure-like was the distance travelled in the high speed category (≥ 20 mm/s), and analyzed the high speed category only.[2] Because seizure-like zebrafish were presence of increased firing activity, small-amplitude "interictal"-like activity at the beginning, followed by high-frequency firing and a period of clonic activity.[3]

Mice seizure scoring

Mice were divided into four groups (n = 9 in each group) randomly and treated 1) saline, 2) CB[7] 500 mg/kg, 3) PTZ 60 mg/kg, 4) CB[7]-PTZ complex with *i.p*. injection, then each mice was individually placed in a transparent experimental cage with beddings for a 30 min observation, seizure scores were given by three independent observers according to the Racine Seizure Scoring System[4] (summarized in Table. S1)

Determination of PTZ level in Zebrafish

Wild-type zebrafish at 7 days post fertilization were used for this study. The zebrafish (N = 50 in each group) were incubated with E3 medium (control), PTZ (4 mM) in E3 medium, and CB[7]-PTZ complex (CB[7]: 2 mM, PTZ: 4 mM) in E3 medium for 30 min, respectively. After incubation, the zebrafish were washed and collected in the 1.5 mL polypropylene tubes. All the samples were homogenized in 300 µL water/ acetonitrile (1:1 v/v). After centrifugation at 13000 rpm for 20 min at 4 °C, the supernatants were collected and dried with nitrogen at room temperature. The dried samples were reconstituted with 150 µL of the mobile phase and after centrifugation at 13000 rpm for 20 min, 50 µL of supernatant was injected into LC-MS/MS (UltiMate 3000 HPLC instrument, equipped with an LTQ Orbitrap XL™ hybrid iontrap-orbitrap mass spectrometer). Chromatographic separations were performed using an eclipse XDB-C18 analytical column (4.6 × 250 mm, 5 µm particle size) and maintained at a temperature 30 °C. The mobile phase was a mixture of 0.1 % formic acid and 10 mM ammonium formate in ultrapure water and acetonitrile (7:3, v/v), and the diode array detector (DAD) was set at 202 nm and the total run time was 15 minutes. The injection volume and flow rate were set at 50 µL and 1.0 mL/ min, respectively. ESI interface parameters were as follows: spray voltage 4.5 kV, sheath gas (N2) 20 psi, aux gas (N2) 15 scales, and capillary temperature 350 °C, capillary voltage 35 V. The LC-MS system was tuned by using Xcalibur system software version 1.2. The standard curve was measured and established (0.1 – 10 µg/L), as shown in Fig. S2. The quantity of PTZ in each sample was calculated by comparing the sample PTZ integration against the standard curve. The experiment was performed for 4 times. Statistical significance of the results was analyzed using either a two-tailed independent samples t-test. Values of $*p < 0.05$ were considered be statistically significant

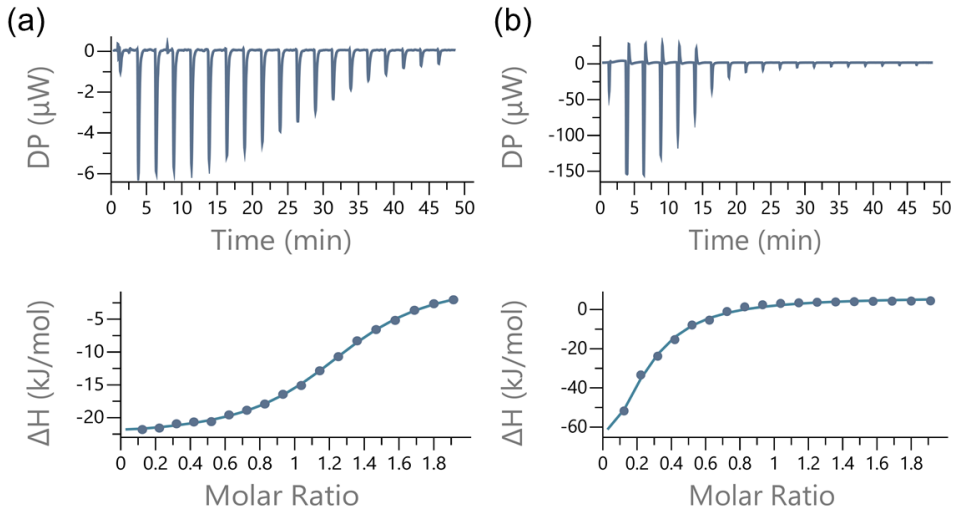

Fig. S1 ITC profile of CB[7]-PTZ complexation in E3 medium (a) and FBS (b). Top: (a) Thermogram of 19 drops (2 µL per drop) of CB[7] (2.00 mM, 0.04 mL) injected into PTZ solution (0.20 mM, 0.20 mL); (b) PTZ (20.00 mM, 0.04 mL) was injected into CB[7] solution (2.00 mM, 0.20 mL). Bottom: the dependence of ΔH against the molar ratio between CB[7] and PTZ during the titration, and the solid line represents the best fit plot by using "one set of binding sites" binding model, affording binding constants of (a) K_a = (8.40 ± 0.51) x 10^4 M^{-1}, (b) K_a = (3.16 ± 0.62) x 10^3 M^{-1}, respectively.

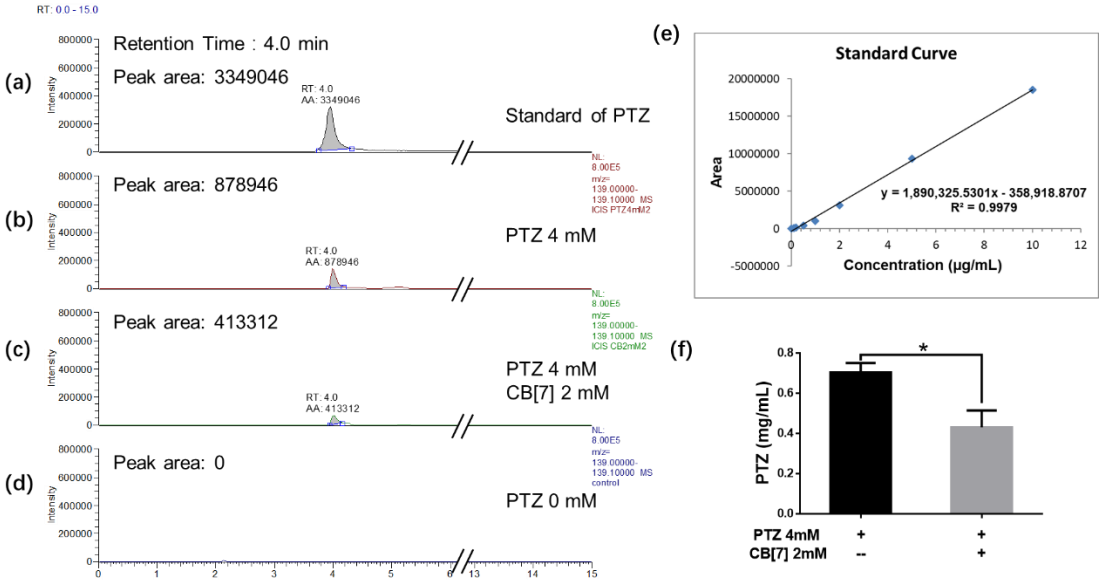

Fig. S2 Quantification of the PTZ levels in zebrafish. (a-d) HPLC-MS chromatograms of PTZ standard, PTZ extracted from zebrafish treated with 4 mM PTZ in the absence and in the presence of 2 mM CB[7], respectively, and the control group. (e) Standard curve (0.1 – 10 µg/L) of PTZ. (f) The concentration of PTZ in the zebrafish (N = 50) was determined when the fish were incubated with 4 mM PTZ in the absence or in the presence of 2 mM CB[7] for 30 min, to be 0.706 ± 0.081 and 0.432 ± 0.146, respectively. Data are expressed as mean ± S.E.M (n = 4), *p < 0.05, indicates significant difference between both groups.

Table. S1 Rodent seizure scoring table.

Racine score	**Behavioral signs**
1	Reduced motion with facial movement and shivering
2	Head nodding
3	Forelimb clonus
4	Rearing with forelimb clonus
5	Rearing and falling with forelimb clonus

1 A. Day, A. P. Arnold, R. J. Blanch and B. Snushall, *J. Org. Chem.*, 2001, **66**, 8094-8100.

2 M. J. Winter, W. S. Redfern, A. J. Hayfield, S. F. Owen, J.-P. Valentin and T. H. Hutchinson, *J. Pharmacol. Toxicol. Methods*, 2008, **57**, 176-187.

3 G. A. Hortopan, M. T. Dinday and S. C. Baraban, *Dis. Model Mech.*, 2010, **3**, 144-148.

4 R. J. Racine, *Electroencephalogr. Clin. Neurophysiol.*, 1972, **32**, 281-294.