Supporting Information

Computer-aided selective optimization of side activities of talinolol

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Experimental Procedures

Chemistry materials and general procedures
Dry solvents were purchased from the supplier Acros, the starting materials from Apollo and TCI. Retention factors were determined from silica coated TLC plates (particle size 60 µm) from Merck. ^{1}H-NMR spectra were recorded on a Bruker DPX250, Avance-300, Avance-400 spectrometers. Chemical shifts were given in ppm from tetramethylsilane with the solvent resonance as internal standard ($CDCl_3$ 7.26 ppm, DMSO d_6 2.50 ppm). Deuterated solvents were purchased from Euriso-top. Liquid chromatography was performed on the device IntelliFlash 310 from AnaLogix using prepacked silica gel columns from Biotage. Reaction which were heated via microwave irradiation were performed on an Initiator 2.0 from Biotage. The according microwave-vials were purchased from Biotage. Mass detection (ESI) occurred on a LCMS-2020 from shimadzu. High resolution mass spectra were measured by a MALDI LTQ Orbitrap XL spectrometer from Thermo Scientific. The purity of the morpholino-talinolol was determined with a column Luna® (10 µm CN 100 Å) from Phenomenex via the device LCMS-2020 from Shimadzu. Elution started with 30% acetonitrile and 70% 0.1% aqueous formic acid till minute 5. After 10 minutes 50% acetonitrile were used (linear gradient) and after 15 minutes a linear gradient led to 80% acetonitrile. The flow rate was adjusted to 1 mL/min.

Synthesis of 1-(*tert*-butyl(2-hydroxyethyl)amino)-3-(4-nitrophenoxy)propan-2-ol (5):
In a 10–20 microwave vial 2-[(4-nitrophenoxy)methyl]oxirane (**3**, 1.00 g, 0.32 mmol) and 2-(*tert*-butylamino)ethanol (**4**, 0.72 g, 6.14 mmol, 1.2eq) were dissolved in 5 mL ethanol und heated up to 150 °C via microwave irradiation. The solution was diluted with water and the aqueous phase was extracted with dichloromethane. After drying over $MgSO_4$ and filtration the solvent was removed under reduced pressure. A yellow oil was obtained (0.32 g, 1.01 mmol, 20%). $C_{15}H_{24}N_2O_5$, MW: 312,36 g/mol; R_f (DCM/MeOH 9/1)= 0.44; LRMS: (ESI (m/z)) required: 312.2, found: 312.9 [M+H$^+$], ^{1}H-NMR ^{1}H-NMR (250 MHz, CDCl3): δ 8.22 - 8.15 (m, 2H), 7.01 - 6.93 (m, 2H), 4.12 - 3.94 (m, 3H), 3.69 - 3.57 (m, 2H), 3.29 - 2.20 (m, 5H), 2.16 (s, 1H), 1.11 (s, 9H); ^{13}C-NMR (75 MHz, CDCl3): δ ppm 207.3, 163.8, 141.8, 126.04, 116.0, 71.0, 68.2, 62.4, 56.6, 54.0, 53.6, 31.1, 27.1.

Synthesis of 4-(*tert*-butyl)-2-((4-nitrophenoxy)methyl)morpholine (6):
1-(*tert*-butyl(2-hydroxyethyl)amino)-3-(4-nitrophenoxy)propan-2-ol (**5**, 99 mg, 0.32 mmol) was dissolved in 5 mL dry THF and cooled with an ice bath. A suspension of NaH (31 mg, 1.28 mmol, 4 eq) in THF was added dropwise. After stirring the reaction 15 min at 0 °C the ice bath was removed and it was stirred for further 60 minutes. *p*-toluolsulfonyl chloride (61 mg, 0.32 mmol) was added at 0 °C and the reaction stirred for additional 72 h while allowing to come to room temperature. After quenching with NH_4Cl-solution at 0 °C the product was extracted with ethyl acetate. The organic layer was washed with a solution of brine and $NaHCO_3$-solution (1:1 v/v) and dried over $MgSO_4$. Filtration and evaporation in vacuo gave a brown residue. This was purified via flash chromatography (dichloromethane / methanol 95:5). An orange solid was obtained (57 mg, 0.19 mmol, 61%). $C_{15}H_{22}N_2O_4$, MW: 294,35 g/mol; R_f (DCM/MeOH 95/5)= 0.46; LRMS: (ESI (m/z)) required: 294.2, found: 294.9 [M+H$^+$]; ^{1}H-NMR (400 MHz, CDCl3): δ 8.22 - 8.16 (m, 2H), 7.02 - 6.95 (m, 2H), 4.16 - 3.78 (m, 4H), 3.74 - 3.66 (m, 1H), 2.96 (td, J=2.2, 11.2 Hz, 1H), 2.82 - 2.76 (m, 1H), 2.36 (ddd, J=11.3, 11.3, 3.2 Hz, 1H), 2.20 (t, J=10.5 Hz, 1H), 1.07 (s, 9H); ^{13}C-NMR (75 MHz, CDCl3): δ ppm 163.9. 141.9, 126.0, 114.8, 74.5. 70.3, 67.8, 54.1, 48.2, 45.8, 25.8.

Synthesis of 4-((4-(*tert*-butyl)morpholin-2-yl)methoxy)aniline (7):
4-(*tert*-butyl)-2-((4-nitrophenoxy)methyl)morpholine (**6**, 53 mg, 0.18 mmol) was dissolved in 10 mL ethanol and 9 mg Pd/C were added. The suspension was exposed to a hydrogen atmosphere (via a balloon) and it was stirred for 6 h at room temperature. After filtration via celite the organic solvent was evaporated and an orange oil was obtained (46 mg, 0.17 mmol, 97%) $C_{15}H_{24}N_2O_2$, MW: 264,36 g/mol; R_f (DCM/MeOH 9/1)= 0.60; LRMS: (ESI (m/z)) required: 264.2, found: 265.0 [M+H$^+$]; ^{1}H-NMR (250 MHz, DMSO): δ 6.69 - 6.61 (m, 2H), 6.53 - 6.45 (m, 2H), 4.59 (s, 2H), 4.03 - 3.55 (m, 4H), 3.47 (dt, J=2.7, 10.9 Hz, 1H), 2.90 (td, J=2.2, 11.1 Hz, 1H), 2.75 - 2.65 (m, 1H), 2.19 (dt, J=3.2, 11.3 Hz, 1H), 2.01 (dd, J=10.1, 10.8 Hz, 1H), 1.00 (s, 9H); ^{13}C-NMR (75 MHz, DMSO): δ ppm 149.7, 142.5, 115.4, 114.8, 74.4, 69.8, 66.5, 53.2, 47.9, 45.4, 25.4.

Synthesis of 1-(4-((4-(tert-butyl)morpholin-2-yl)methoxy)phenyl)-3-cyclohexylurea (2, morpholino-talinolol):
Under an inert atmosphere 4-((4-(*tert*-butyl)morpholin-2-yl)methoxy)aniline (**7**, 167 mg, 0.63 mmol) was dissolved in 8 mL dry dichloromethane and DIPEA (0.3 mL, 1.89 mmol, 3 eq). Via an Eppendorf pipette cyclohexyl isocyanate **8** (805 µL, 0.63 mmol) was added and the approach stirred

for 18 h at room temperature. After removing the solvent the residue was suspended in *n*-hexane and filtered hot. The solid was triturated with ethanol and *n*-hexane. Filtration gave a white solid (80 mg, 0.21 mmol, 33%). $C_{22}H_{35}N_3O_3$, MW: 389,53 g/mol, r_t = 6,75, purity 99%; LRMS: (ESI (m/z)) required: 389.2, found: 390.3 [M+H$^+$]; HRMS (MALDI): found 390.27495, simulated 390.27512, Δ ppm = 0.44; ^{1}H-NMR (300 MHz, DMSO): δ 8.16 (d, J=59.4 Hz, 1H), 7.34 - 7.22 (m, 2H), 6.94 - 6.77 (m, 2H), 6.04 - 5.89 (m, 1H), 4.22 - 3.76 (m, 3H), 3.73 - 3.61 (m, 1H), 3.53 - 3.40 (m, 2H), 2.91 (d, J=11.1 Hz, 1H), 2.72 (d, J=10.5 Hz, 1H), 2.27 - 2.14 (m, 1H), 2.08 - 1.98 (m, 1H), 1.89 - 1.43 (m, 7H), 1.38 - 0.69 (m, 12H); ^{13}C-NMR (75 MHz, DMSO): δ ppm 154.6, 152.9, 133.9, 121.8, 119.1, 118.1, 114.6, 74.3, 69.3, 66.5, 53.2, 47.7, 47.5, 45.4, 33.0, 32.5, 25.4, 25.2, 24.5, 24.3.

Protein expression and purification

The cloning of the full-length sEH and the C terminal hydrolase domain (sEH-H; aa222–aa555)) as well as the expression and purification were described previously.[1] In short the expression of the sEH-H was performed in E.coli BL21-(DE3) cells with ZYP5052 autoinduction media at 16 °C for 36 h. The expressed protein was purified by nickel affinity chromatography followed by a size exclusion chromatography. As buffer for the size exclusion chromatography (50 mM NaCl, 50 mM sodium phosphate, 10 % (v/v) glycerol (98%), 2 mM DTT, pH 7.4) was used.

sEH hydrolase assay

Fluorescence-based assay of sEH hydrolase activity was performed in 96-well format as described previously[1]. Non-fluorescent PHOME[2] (3-phenyl-cyano-(6-methoxy-2-naphthalenyl)methyl ester-2-oxirane-acetic acid) was used as the substrate, which can be hydrolyzed by the sEH to the fluorescent 6-methoxynaphtaldehyde. The formation of the product was measured (λem= 330 nm, λex= 465 nm) by a Tecan Infinite F200 Pro plate reader. Recombinant human sEH (2 µg/well) was dissolved in pH 7 bis-Tris buffer with 0.1 mg/ml BSA containing a final concentration of 0.01% Triton-X 100. 90 µL aliquots of protein were incubated with different concentrations of compounds (final DMSO concentration 1%) for 30 min at room temperature. 10 µl aliquots of substrate were added (to the final concentration 50 µM). The hydrolyzed substrate was measured for 30 min (one point every minute). All samples where measured in triplicates on the plate as well as in 3 separate experiments. The percent inhibition was calculated in comparison to activities of blank (without protein) and positive control wells (without inhibitor). To calculate IC_{50} vales data obtained from measurements with at least six different inhibitor concentrations were fitted with a sigmoidal dose-response function using GraphPad Prism software (version 5.03; GraphPad Software, Inc.).

Differential scanning fluorimetry (DSF)

The differential scanning fluorimetry experiments of the sEH-H domain were performed according to Lukin et.al.[1] with some minor modifications. In brief, 200 µM of the inhibitor in DMSO (or pure DMSO in case of the control) were mixed with 5 µM sEH-H in buffer (25 mM Tris, pH 7), 0.01% (w/v) Triton X-100 and 5x SYPRO™ Orange protein gel stain (Invitrogen) in transparent 96-well PCR plates (MicroAmp, Applied Biosystems) at a final volume of 40 µL. To exclude inhibitor/SYPRO™ interactions background fluorescence was measured without enzyme. The melting curves were measured in a Icycler IQ single color real time PCR (BioRad) at an excitation wavelength of 490 nm and emission wavelength of 570 nm covering a temperature range starting at 20 °C up to 89 °C. The heating rate was set to 1 °C/min with a sampling speed of one data point per minute. Each condition was measured 12x on a single plate. The first derivatives of the measured melting curves were automatically calculated by MyIQ 1.0 software and the maxima of the curves, which were considered as the melting point, were determined in Microsoft Excel. The graphic representation of presentative curves were created in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Membrane permeability assay (MPA):

The assay was performed according to the manufactures protocol in MultiScreen Permeability PCF .45u clear con-sterile plates (Merck, MPC4NTR10). In brief, artificial membranes were prepared by applying 15 µl of a 5% (w/v) hexadecane in hexane solution to the centre of each well in the donor plate before drying it for 1 h under a fume hood. Then 125 µL of a 100 µM compound solution (or pure DMSO as negative control) in PBS with 5% (v/v) DMSO final concentration was added to the donor plate wells as well as 200 µL of pure 5% (v/v) DMSO in PBS solution in each acceptor plate well. The donor plate was placed in the acceptor plate and incubated for 16 h at RT (covered in wet paper and placed in a sealed plastic bag).

After the incubation inhibitor concentrations in the acceptor and donor wells were analysed by UPLS MS on an ACQUITY UPLC with TUV detector (Waters) coupled to a single quadrupole mass detector (QDa Waters). Therefore, acceptor as well as donor plate solution were first transferred in 1.5 mL Eppendorf tubes, before a 1:1 dilution with 5% DMSO PBS was performed and tubes were centrifuged at max speed (21130 g) for 10 mins in an Eppendorf centrifuge 5424R. Supernatant was further diluted 1:10 in acetonitrile (UPLC grade). For a measurement, 2 µL or 10 µL of the sample was injected. Separation was achieved on a ACQUITY UPLC® HSS T3 (1.8µm, 2.1 x 100mm) reverse phase column (Waters) with a column temperature of 45 °C. Acetonitrile and H2O with 0.1% formic acid were used as eluents following the gradient range from 5 to 95% in 5 min at a flow rate of 0.5 mL/min. The following compounds were analysed in positive single ion mode [M+H+]+ using Selected Ion Monitoring (SIM) approach: Carbamazepine m/z 237.2, Propranolol m/z 260.2, Talinolol m/z 364.2, and Morpholino-talinolol m/z 390.2. The obtained ion chromatograms were integrated with Epower 3 software. The peak areas determined for the compounds in the acceptor and donor wells were used to calculate the log(P_e) value for each well according to the manufactures protocol. The following formula was used:

$$logP_c(cm/s) = \log\left[\frac{-\ln\left(1-\frac{C_A}{C_{equilibrium}}\right)}{S\left(\frac{1}{V_D}+\frac{1}{V_A}\right)t}\right],$$

where C_A = final drug concentration in the acceptor well (µM), $C_{equilibrium}$ = theoretical equilibrium concentration = $(C_0 \cdot V_0 + C_A \cdot V_A)/(V_D+V_A)$, where C_D = final drug concentration in the donor well (µM), V_D = volume in the donor well (cm3), C_A = final drug concentration in the acceptor well (µM), V_A = volume in the acceptor well (cm^3), S = surface area (cm^2), typically 0.268 cm^2, V_D = volume in the donor well (cm^3) = 0.125 cm^3, V_A = volume in the acceptor well (cm^3) = 0.2 cm^{3}and the t = incubation time (s) = 57600 s. Mean values and the corresponding standard deviations were calculated for the obtained log(P_e) values.

In vitro drug metabolism in rat liver microsomes

The assay was performed according to the previously described procedure[3]. Solutions of the test compounds (1 mM) were prepared in 100% DMSO. 432 µL of phosphate buffer (0.1 M, pH 7.4) and 50 µL of NADPH-regenerating system (30 mM glucose 6-phosphate, 4 U/mL glucose 6-phosphate dehydrogenase, 10 mM NADP, 30 mM $MgCl_2$) and 5 µL of the corresponding test compound were preincubated at 37 °C. The final concentration of the investigated compound is 10 µM. After 5 min the reaction was started by the addition of 13 µL of microsome mix from the liver of Sprague-Dawley rats (Gibco, Darmstadt, Germany; 20 mg of protein/mL in 0.1 M phosphate buffer). The incubation was performed in a shaking water bath at 37 °C. The reaction was stopped by the addition of 500 µL of ice-cold methanol at 0, 15, 30, and 60 min. The samples were centrifuged at 10 000g for 5 min at 4 °C. The supernatants were analyzed and quantified by HPLC. Control samples were always performed to check the stability of the compounds in the reaction mixture. First control was without NADPH, which is needed for the enzymatic activity of the microsomes. Second control

was with inactivated microsomes (microsomes that were incubated for 20 min at 90 °C). Third control was without test compounds (to determine the baseline). As positive control, a solution of 7-ethoxycoumarin (1 mM) was used. The final concentration of the control compound, under assay conditions, was again 10 μM. The amounts of the test compounds were quantified by an external calibration curve.

sEH activity in HEP-G2 Cell lysates

Quantification of cellular sEH metabolic activity was performed as initially published by Zha et al.[4] and previously described[5]. For this, HEP-G2 cells were harvested, washed twice with PBS and sonicated in PBS for disruption of cell integrity. Then, 1 μg of total cell homogenate diluted in 100 μl of PBS containing 0.1 mg/ml BSA was incubated with the compounds or vehicle for 15 min at 37°C. After this, 25 ng (±)14(15)-EET-d_{11} (Cayman Chemical, Ann Arbor, MI, United States) were added per sample and the incubation was continued for additional 10 min at 37°C. A blank was performed using PBS (containing 0,1 mg/mL BSA). The reactions were stopped by adding 100 μL of ice-cold methanol. After centrifugation (2000 rpm, 4°C, 5 min), supernatants were analyzed by LC-MS/MS and the amounts of (±)14(15)-EET-d_{11} and the corresponding (±)14(15)-DHET-d_{11} were determined.

Determination of (±)14(15)-EET-d_{11}/(±)14(15)-DHETd_{11} by LC/MS-MS

14(15)-EET-d_{11} and 14(15)-DHET-d_{11} content of the extracted samples were analyzed employing liquid chromatography tandem mass spectroscopy (LC-MS/MS). The LC/MS-MS system comprised an API 5500 QTrap (Sciex, Darmstadt, Germany), equipped with a Turbo-V-source operating in negative ESI mode, an Agilent 1200 binary HPLC pump and degasser (Agilent, Waldbronn, Germany) and an HTC Pal autosampler (Chromtech, Idstein, Germany) fitted with a 25 μL LEAP syringe (Axel Semrau GmbH, Sprockhövel, Germany). High purity nitrogen for the mass spectrometer was produced by a NGM 22-LC/MS nitrogen generator (cmc Instruments, Eschborn, Germany). All substances were obtained from Cayman Chemical, Ann Arbor, MI, United States. Stock solutions with 2,500 ng/mL of both analytes were prepared in methanol. Working standards were obtained by further dilution with a concentration range of 0.1–250 ng/mL for 14(15)-EET-d_{11} and 14(15)-DHET-d_{11}. Sample extraction was performed with liquid–liquid-extraction. Therefore, 150 μL of matrix homogenates were gently mixed with 20 μL of internal standard [14(15)-EET and 14(15)-DHET all with a concentration of 100 ng/ml in methanol], and were extracted twice with 600 μL of ethyl acetate. Samples for standard curve and quality control were prepared similarly, instead of 150 μL of matrix homogenates, 150 μL PBS were added. Further 20 μL methanol, 20 μL working standard and 20 μL internal standard were added. The organic phase was removed at a temperature of 45 °C under a gentle stream of nitrogen. The residues were reconstituted with 50 μL of methanol/water/(50:50, v/v), centrifuged for 2 min at 10,000 *g* and then transferred to glass vials (Macherey-Nagel, Düren, Germany) prior to injection into the LC-MS/MS system. For the chromatographic separation a Gemini NX C18 column and pre-column were used (150 mm × 2 mm i.d., 5 μm particle size and 110 Å pore size from Phenomenex, Aschaffenburg, Germany). A linear gradient was employed at a flow rate of 0.5 mL/min mobile phase with a total run time of 17.5 min. Mobile phase was A water/ammonia (100:0.05, v/v) and B acetonitrile/ammonia (100:0.05, v/v). The gradient started from 85% A to 10% within 12 min. This was held for 1 min at 10% A. Within 0.5 min the mobile phase shifted back to 85% A and was held for 3.5 min to equilibrate the column for the next sample. The injection volume of samples was 20 μL. Quantification was performed with Analyst Software V 1.5.1 (Sciex, Darmstadt, Germany) employing the internal standard method (isotope- dilution mass spectrometry). Ratios of analyte peak area and internal standard area (*y*-axis) were plotted against concentration (*x*-axis) and calibration curves were calculated by least square regression with 1/concentration^2 weighting.

Crystallization and structure determination

The crystallization experiments of the sEH-H were performed as described previously[1]. In short apo sEH-H crystals were grown at 277 K by sitting-drop vapor diffusion, mixing 1 μL of the protein solution (5-10 mg/mL, 50 mM NaCl, 50 mM sodium phosphate, 10% glycerol (98%), 2 mM DTT at pH 7.4 1:1 with precipitant mixture (23 %-28 % (w/v) polyethylenglycol (PEG) 6000, 70 mM ammonium acetate, 200 mM magnesium acetate, 100 mM sodium cacodylate at pH 6.1-6.5). Formed apo crystals were soaked with inhibitor/cryoprotectant solution for 24 h.

Soaked and flash frozen crystals were measured at beamline station ID29 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. All diffraction data was obtained from a single crystal. Datasets of the two crystals were processed in the XDS[6] software package. Initial structures were obtained by the PHASER[7] program using PDB record 6FR2 where coordinates for heteroatoms (water and ligands) were excluded from the starting model. Final models were obtained after several iterative rounds of model building with Coot[8] and model refinement using the PHENIX[9] software package. The binding of both inhibitors was validated using polder maps[10] around the ligand with a solvent exclusion radius of 3 Å and a resolution factor of 0.3. For talinolol only two of the possible ligand conformations were modeled in the structure covering both main orientations. The sEH-H structure with talinolol reached a value of R_{work} and R_{free}-factors of 0.1744 and 0.2035, while the morpholino-talinolol reached a value of 0.2023 and 0.2536. The coordinates and structure-factor amplitudes of the sEH-H/ talinolol structure (PDB record 6HGV) as well as the sEH-H/morpholino-talinolol structure (PDB record 6HGX) were deposited in the Protein Data Bank (PDB) using the PDB Extract tool for data preparation. For both crystals statistics of data collection and structural refinement are summarized in Table S1, using the PHENIX table one tool. The graphical representations were made using MOE.

Table S1. Data collection and refinement statistics.

	Talinolol	**Morpholino-talinolol**
Wavelength	1.072	1.072
Resolution range	45.94 - 2.0 (2.072 - 2.0)	52.85 - 2.16 (2.237 - 2.16)
Space group	I 2 2 2	I 2 2 2
Unit cell	79.73 91.88 106.05 90 90 90	79.8 92 105.7 90 90 90
Total reflections	350882 (35155)	135440 (14095)
Unique reflections	26671 (2623)	21231 (2100)
Multiplicity	13.2 (13.4)	6.4 (6.7)
Completeness (%)	99.90 (99.81)	99.78 (99.57)
Mean I/sigma(I)	20.75 (2.73)	10.09 (1.55)
Wilson B-factor	38.33	41.22
R-merge	0.07425 (0.828)	0.131 (0.9442)
R-meas	0.0773 (0.8612)	0.1429 (1.025)
R-pim	0.0213 (0.2352)	0.05643 (0.3959)
CC1/2	0.999 (0.897)	0.996 (0.758)
CC*	1 (0.972)	0.999 (0.929)
Reflections used in refinement	26655 (2620)	21198 (2093)
Reflections used for R-free	1698 (166)	2043 (201)
R-work	0.1744 (0.2525)	0.2023 (0.2933)
R-free	0.2035 (0.2842)	0.2536 (0.3333)
CC(work)	0.965 (0.899)	0.960 (0.811)
CC(free)	0.948 (0.862)	0.928 (0.723)
Number of non-hydrogen atoms	2825	2715
macromolecules	2623	2574
ligands	53	29
solvent	149	112
Protein residues	319	319
RMS(bonds)	0.007	0.008
RMS(angles)	0.81	1.01
Ramachandran favored (%)	98.11	97.79
Ramachandran allowed (%)	1.58	1.89
Ramachandran outliers (%)	0.32	0.32
Rotamer outliers (%)	0.00	0.71
Clashscore	3.63	5.49
Average B-factor	51.71	55.38
macromolecules	50.96	55.03
ligands	78.93	95.05
solvent	55.19	53.13
Number of TLS groups	8	4

Statistics for the highest-resolution shell are shown in parentheses.

Diabetic neuropathic pain model

All procedures and animal care adhered the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications 8th Edition, 2011) and were performed in accordance with the protocols approved by the Animal Use and Care Committee (IACUC) of the University of California, Davis. Great care was taken to minimize suffering of the animals and to reduce the number of animals used. Male Sprague-Dawley rats (250-300 g, purchased from Charles River) were housed under standard conditions (25°C) in a fixed 12-h light/dark cycle with ad libitum food and water. For all the behavioral tests, the rats were randomly assigned to treatment groups and tested intermingled for the studies.

Pain was modeled using diabetic neuropathy induced by streptozocin which targets and kills the pancreatic beta islet cells rendering the rats with type I diabetes and neuropathic pain. The decrease in nociceptive thresholds develops within five days and persists the lifetime of the animal. Prior to model induction rats were acclimated for one hour and tested for naive baseline mechanical withdrawal thresholds (MWTs) assessed with the von Frey assay using an electronic aesthesiometer (IITC, Woodland Hills, CA). Subsequently, streptozocin in saline (55 mg/kg) was injected via tail vein. After five days the allodynia of diabetic rats was confirmed and rats that scored 65% or lower of the original pain free baseline were considered allodynic and included in the study.

For the von Frey assay rats were placed in clear acrylic chambers on a steel mesh floor. The hind paw of the rat was probed through the mesh with a rigid tip probe on the aesthesiometer connected to an electronic readout pressure meter set to the maximum hold setting. The withdrawal thresholds per rat were measured 3–5 times at 1 minute intervals for each time point. For the diabetic neuropathy model, rats were oral gavaged with single administration of the test compounds (all at 10 mg/kg) in PEG300 and assessed over a 4 hours time course. Diabetic baseline scores were normalized to 0 to reflect the response to treatments which are reported as % of post diabetic neuropathic baseline[11].

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