Supporting Information

Inhibition of inflammatory pain and cough by a novel charged sodium channel blocker

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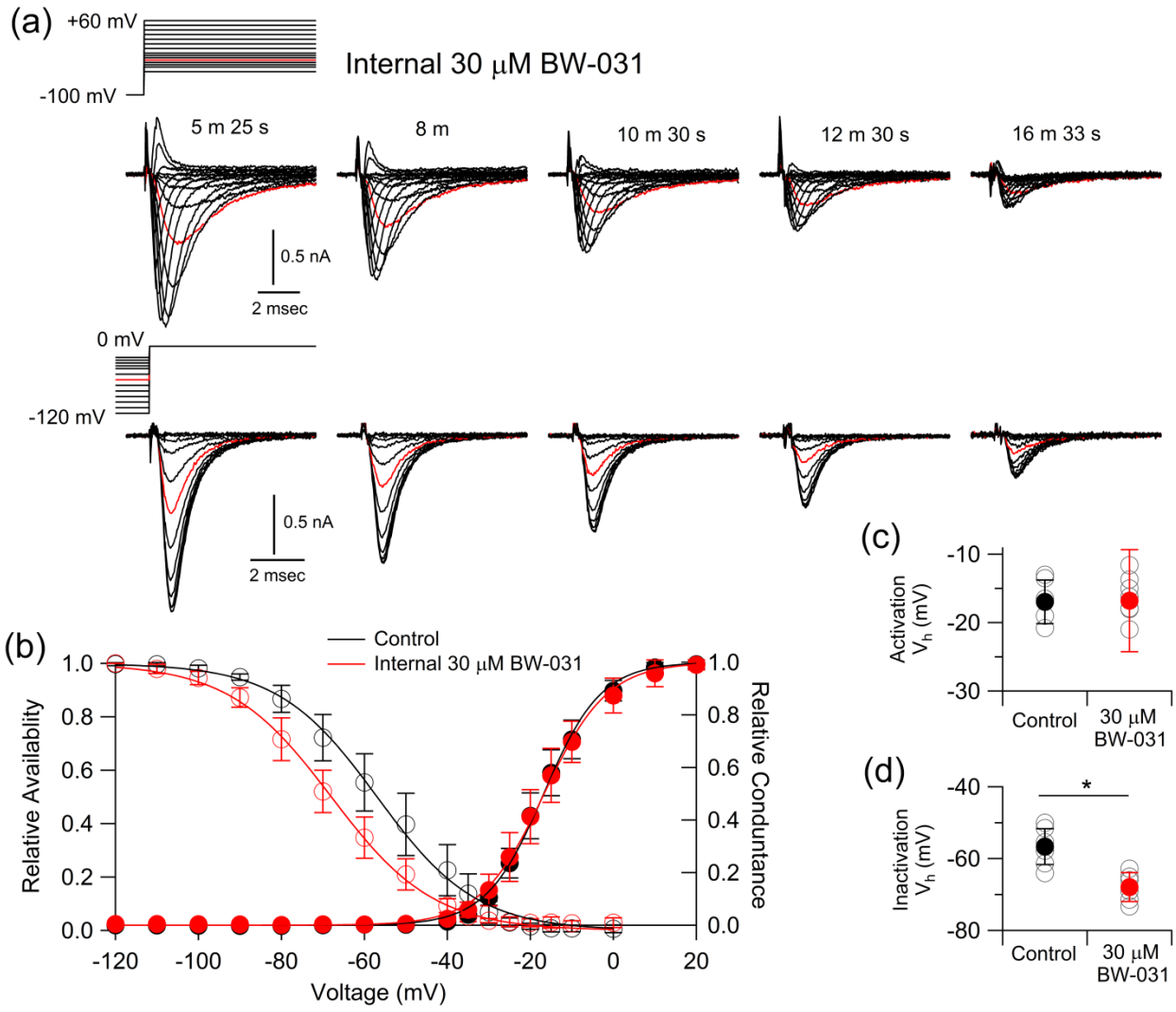


Supplementary Figure 1.

Effect of intracellular 30 μM BW-031 on voltage-dependence of activation and inactivation of hNav1.7 channels. (a), Top, voltage-dependence of activation was determined with 50-ms steps from -120 mV to +60 mV delivered from a holding potential of -100 mV. Families of current were measured at the indicated time after establishing whole-cell configuration. Panels show first 8 ms of test pulses from -50 to +60 (in 10 mV increments but with 5 mV increments between -40 and -10 mV). Red traces are steps to -25 mV. Currents were corrected for leak and capacity currents, determined by averaged 5-mV hyperpolarizing steps from -100 mV. The last, smallest current traces (at 16 m 33 s) were further corrected by subtracting current in the presence of 1 μM TTX. Bottom, voltage-dependence of inactivation determined by a 10-ms test pulse to 0 mV

delivered after 50-ms conditioning pulses to voltages between -120 mV and +60 mV (activation and inactivation determined with the same voltage protocol at the same time, with the 0 mV test pulse for inactivation following the 50-ms steps used to determine activation). Panels show test pulse currents at 0 mV following conditioning pulses ranging from -120 mV to -20 mV. Red traces are for conditioning pulses to -60 mV. (b), Voltage-dependence of activation and inactivation in cells recorded with intracellular 30 μM BW-031 compared with interspersed control cells recorded with BW-031-free internal solution. Voltage-dependence of activation (filled circles) was determined by peak sodium conductance normalized to maximal peak conductance, using a reversal potential of +45 mV. Data represent mean ± SD. For activation: Control, n=6; BW-031, n=8. For availability: Control, n=7; BW-031, n=6. Measurements in BW-031 were taken when peak sodium current evoked at 0 mV from -100 mV was inhibited by more than 50% from the initial peak current. Smooth traces drawn according to the Boltzmann function $1/(1+\exp(-(V-V_h)/k))$, where V_h is the voltage of half-maximal activation, V is test potential, and k is the slope factor. Control: V_h =-16.9, k =6.4. BW-031: V_h =-16.8, k =7.5. Availability (open circles) was determined by peak sodium current during the test pulse to 0 mV following 50-msec conditioning pulses, normalized to that from a conditioning pulse of -120 mV. Data fit by the Boltzmann function $1/(1+\exp(V-V_h)/k))$, where V_h is the voltage of half-maximal availability, V is conditioning potential, and k is the slope factor. Control: V_h =-56.7 mV, k =11.4 mV, n=7. BW-031: V_h =-67.9 mV, k =12 mV, n=6. Values of V_h and k for smooth curves in each condition were calculated as means of fits to data from individual cells. (c), Collected values for midpoint of activation with control internal solution (-16.9 ± 3.2 mV, n=6) and with 30 μM BW-031 (-16.8 ± 3.3 mV, n=8). (d), Collected values for midpoint of availability with control internal solution (-56.7 ± 5.0 mV, n=7) and with 30 μM BW-031 (-67.9 ± 4.0 mV, n=6). Data represent mean ± SD. Asterisk, P<0.05.

Detailed statistics for Figure data

Figure 1e: hNav1.7 cells. Control cells with intracellular solution without compound: 0.93±0.07, n=37. Intracellular 100 μM BW-031: 0.14±0.09, n=6. Intracellular 100 μM QX-314: 0.40±0.18, n=6. Extracellular 100 μM BW-031: 0.86±0.09, n=6. Comparison of intracellular 100 μM BW-031 with intracellular 100 μM QX-314, p=0.008, two-tailed Mann Whitney Test. Comparison of intracellular 100 μM BW-031with extracellular 100 μM BW-031, p=0.008, two-tailed Mann Whitney Test).

Figure 1g: iPSC-derived nociceptors. Control cells with intracellular solution with no compound: 0.95±0.07, n=4. 100 μM intracellular BW-031: 0.13±0.09, n=5. 100 μM intracellular QX-314: 0.43±0.15, n=5. 100 μM intracellular BW-031 vs. 100 μM intracellular QX-314, p=0.01, two-tailed paired t-test.

Figure 2b: Comparison of collected data by two-tailed Mann-Whitney Test. Asterisks indicate p<0.05.

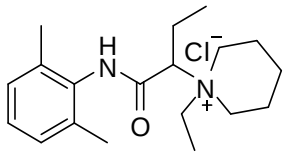
Figure 3a: Homogeneity of variance for data was achieved by transforming raw data (x+1 and then log 10 of the new value) prior to using ANOVA. The resulting Bartlett's tests were non-significant indicating success of the transformation method. Then 1-way ANOVA was calculated, [F(3, 42)=4.3], p=0.01, with Tukey's post-hoc test.

Figure 3b: 1-way ANOVA, [F(3, 39)=0.33], p=0.81.

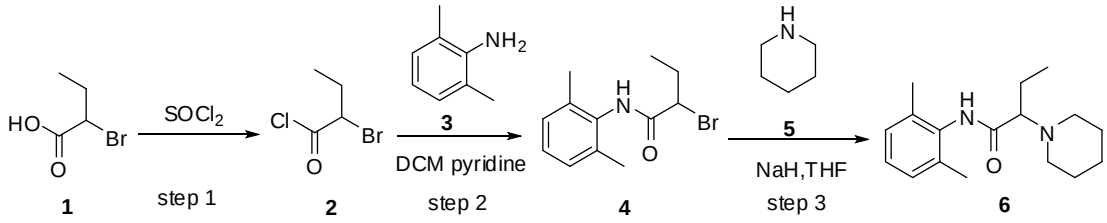
Figure 4a: Repeated measures two-way ANOVA with treatment as the between groups factor and time as the within groups factor, with assumption of sphericity (Greenhouse-Geisser epsilon value of 0.99). Treatment [F(2, 27)=15], P<0.0001; time [F (2, 54) = 15], and treatment x time

interaction [F(4,54) = 6.8], all P<0.0001. Post-hoc Tukey's tests between treatment groups.
Treatment [F(2, 27)=15], time [F(2, 54)=15], and treatment x time interaction [F(4, 54)=6.8], all p<0.001
Figure 4b: Mixed-effects analysis with treatment as the between groups factor and time as the within groups factor, with assumption of sphericity (Greenhouse-Geisser epsilon value of 1.0). Treatment [F(1, 25)=36], time [F(6, 99)=45] and treatment x time interaction [F(6, 99)=13], all P<0.001. Post-hoc Bonferroni tests between treatment groups at each time point.
Figure 5a: Two-way repeated measures ANOVA with treatment as the between groups factor and time as the within groups factor, with Greenhouse-Geisser correction for deviation from sphericity (epsilon value of 0.63). Treatment [F(2, 27)=291], time [F(5, 135)=81] and treatment x time interaction [F(3.2, 84.5)=81], all P<0.001. Post-hoc Tukey's tests between treatment groups at each time point.
Figure 5b: Two-way repeated measures ANOVA with treatment as the between groups factor and time as the within groups factor, with Greenhouse-Geisser correction for deviation from sphericity (epsilon value of 0.5). Treatment [F(2, 21)=225], time [F(1, 21)=225] and treatment x time interaction [F(4, 42)=225], all P<0.001. Post-hoc Tukey's tests between treatment groups at each time point.
Figure 5c: Fisher's exact test (5 min time point), $p=4.1x10^{-6}$.
Figure 6b: Homogeneity of variance for data was achieved by transforming raw data (x+1 and then log 10 of the new value), then 1-way ANOVA was calculated [F(3, 44)=5], p=0.0057, with Tukey's post-hoc test.
Figure 7b: Each comparison between naïve and ovalbumin-sensitized groups satisfied normality following log 10 transformation and the transformed data were then analyzed by 2-tailed Student's t-test.
Unpaired two-tailed t-tests between naïve and ovalbumin-sensitized groups.
Figure 7c: 1-way ANOVA, [F(3, 44)=7.1], p=0.0005; Tukey's post-hoc.
Figure 8a: Kruskall Wallis ranks analysis.
Figure 8c: 1-way ANOVA, [F(12, 57)=2.9], p=0.0035; Dunnett's post hoc.
Supplementary Figure 1:
Solid lines in B indicate Boltzmann equations drawn according to mean V_h and k values calculated from fits of the Boltzmann equation to data from each cell. Activation: Control: V_h =-16.9 ± 3.2 mV, k =6.4 ± 0.7 mV, n=6. BW-031: V_h =-16.8 ± 3.3 mV, k =7.5 ± 0.7 mV, n=8. Availability: Control: V_h =-56.7 ± 5.0 mV, k =11.4 ± 0.8 mV, n=7. BW-031: V_h =-67.9 ± 4.0 mV, k =12 ± 0.7 mV, n=6. Mean ± SD. Only the midpoint of availability was statistically significant at P<0.05 (p=0.001, unpaired Student t-test) between data from cells with control or BW-031-containing internal solution.

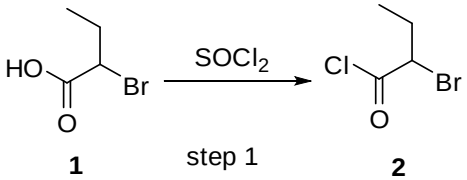
Synthesis of BW-031 (1-(1-(2, 6-dimethylphenylamino)-1-oxobutan-2-yl)-1-ethylpiperidinium)



Synthetic Scheme

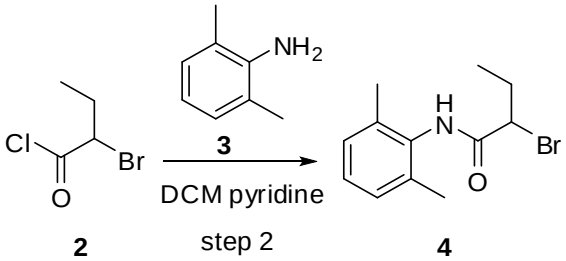


*Step 1：Preparation of intermediate **2***



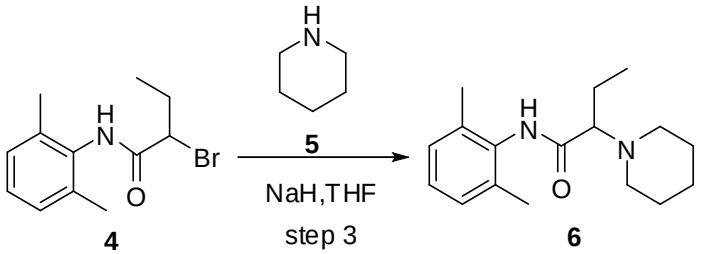
To a mixture of **1** (10.0g, 59.88mmol) was added $SOCl_2$ (60mL, c=1.0). The mixture was heated to reflux. After completion, the reaction mixture was concentrated under reduce pressure to give intermediate **2** (9.2g, yield=82.8%) as a yellow oil.

*Step 2：Preparation of intermediate **4***



To a solution of **3** (5.0g, 41.3mmol, 1.0eq) in DCM (100ml, c=0.5) was added pyridine (4.9g, 61.95mmol, 1.5eq). To the solution was added **2** (9.2g, 49.59mmol, 1.2eq) in DCM (40mL, c=1.2). The reaction mixture was stirred at room temperature overnight. Then to the solution was added water (50mL). The organic phase was washed with brine, dried over Na_2SO_4, filtered and concentrated under reduce pressure. The residue was washed with n-hexane to give intermediate **4** (7.8g, yield=70%, HPLC: 98.6%).

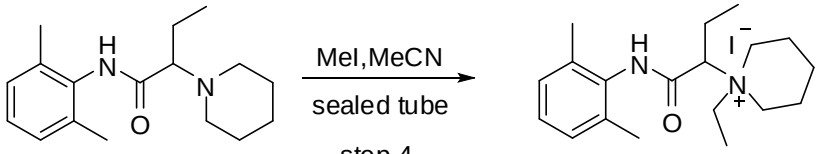
*Step 3：Preparation of intermediate **6***



4 **6**

To a solution of NaH (0.35g, 14.8mmol, 2.0eq) in THF (37mL, c=0.4) was added **5** (0.75g, 8.8mmol, 1.2eq). To the solution was added **4** (2.0g, 7.4mmol, 1.0eq) in THF (20mL, c=0.37). The reaction mixture was then stirred at room temperature overnight. To the suspension was added water (20mL) and EA (50mL). The organic phase was washed with water (50mL×2). Then the organic phase was adjusted to pH 2, extracted with EA(40mL×2). The aqueous fractions were combined and adjusted to pH 9, then extracted with EA (40×2). The combined organic fractions were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduce pressure. The residue was washed with n-hexane to give the intermediate **6** (0.48g, yield=24%, HPLC: 99.3%) as a solid.

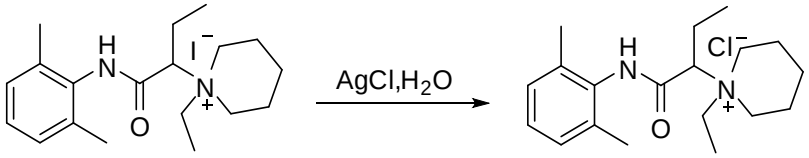
*Step 4：Preparation of intermediate **7***



6 **7**

Intermediate **6** (0.48g, 1.75mmol, 1.0 eq) and MeCN (9mL, c=0.2) was added in sealed tube. To this solution, EtI (2mL, 14.0 eq) was added. After addition, the reaction mixture was stirred at 90℃ for 10h. After completion, the reaction mixture was concentrated under reduce pressure. The residue was purified by column chromatography to give intermediate **7** (470mg, yield=62.6%, HPLC: 99%) as a solid.

*Step 4：Preparation of compound **8***



7 **8**

To a solution of 7 (200mg, 0.465mmol, 1.0 eq) in deionized water (3ml, c=0.15) was added AgCl (133mg, 0.93mmol, 2.0 eq). After addition, the reaction mixture was stirred at room temperature overnight. The suspension was then filtered and the filtrate was lyophilized to give compound 8 (141mg, yield=89.8%) as a solid. HPLC purity: at 220nm; Mass: M+1=339.4. 1H NMR (300 MHz, D2O): δ 7.117 (m, 3H), 4.056 (dd, J=8.1 Hz, 1H), 3.712~3.808 (m, 1H), 3.656 (m, J=13.2 Hz, 2H), 3.510~3.582 (m, 1H), 3.344 (m, 2H), 2.117 (s, 6H), 1.984~2.070 (m, 2H), 1.818 (m, 4H), 1.660 (m, 1H), 1.455 (m, 1H), 1.278 (t, J=7.2 Hz, 3H), 1.107 (t, 3H) ppm.