Modest CaV1.342-selective inhibition by compound 8 is β-subunit dependent

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Two voltage-gated calcium channel subtypes—CaV1.2 and CaV1.3—underlie the major L-type Ca2+ currents in the mammalian central nervous system. Owing to their high sequence homology, the two channel subtypes share similar pharmacological properties, and at high doses classic calcium channel blockers, such as dihydropyridines, phenylalkylamines and benzothiazepines, do not discriminate between the two channel subtypes. Recent progress in treating Parkinson’s disease (PD) was marked by the discovery of synthetic compound 8, which was reported to be a highly selective inhibitor of the CaV1.3 L-type calcium channels (LTCC). However, despite a previously reported IC50 of ~24 μM, in our hands inhibition of the full-length CaV1.342 by compound 8 at 50 μM reaches a maximum of 45%. Moreover, we find that the selectivity of compound 8 towards CaV1.3 relative to CaV1.2B15 channels is greatly influenced by the β-subunit type and its splice isoform variants.
Parkinson’s disease (PD) is a movement disorder arising from the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc). Although mutations of several genes have been associated with PD, 95% of PD cases are idiopathic. The over-reliance of CaV1.3 current for pacemaking in the adult SNc neurons was reported to aggravate mitochondrial oxidative stress, and consequently enhance the susceptibility of these neurons towards toxins such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and rotenone. Significantly, pharmacological blockade or genetic deletion of CaV1.3 channels confers protection against toxin induced neuronal damage. Interestingly, population studies also revealed significant reduction in the risk of developing PD among hypertensive subjects receiving dihydropyridines (DHPs), classical blockers of L-type calcium channels.

Voltage-gated calcium channels CaV1.2 and CaV1.3 underlie the majority of L-type calcium currents in the central nervous system. While CaV1.3 channels contribute only 20% of the total L-type currents in the central nervous system, the use of non-selective DHPs that also inhibits CaV1.2 channels could have profound neuro-physiological consequences. In addition, neuronal transcripts of CaV1.3 channels display extensive alternative splicing patterns, generating splice variants with different biophysical and pharmacological properties. The CaV1.3 splice variant with a truncated C terminus displayed different biophysical and pharmacological properties. The CaV1.3 current for effective inhibition of the CaV1.3 currents.

Recently, Kang et al. reported the identification of 1-(3-chlorophenethyl)-3-cyclopentylpyrimidine-2,4,6-(1H,3H,5H)-trione, also known as compound 8 as a pharmacological blocker highly selective for CaV1.3 (IC50 = 24.3 ± 0.7 μM) over CaV1.2 channels. In the current study, we evaluated compound 8 for its activity against different CaV1.3 channel splice isoforms. Unexpectedly, initial heterologous expression in human embryonic kidney 293 (HEK293) cells co-transfected with β2a-subunit revealed that compound 8 inhibited CaV1.2 more than either CaV1.3a or CaV1.3aβ2a channels. Replacing β2a-subunit with β1-β3- or β4-subunit on the other hand yielded results that showed modest selective inhibition of compound 8 against CaV1.3aβ2 over CaV1.2β1 channels. However, there was clearly no selective inhibition of compound 8 against CaV1.3aβ2a and CaV1.2 channels in the presence of any of the other three subunits. Based on the existing data, we conclude that the CaV1.3-selective inhibition by compound 8 is modest and is highly dependent on the composition of the CaV1.3 splice variant in association with a particular type of β-subunit.

Results

**Compound 8 is not selective against CaV1.3 with β2a-subunit.**

Compounds 1, 8 and PYT (1,3-bis-(4-chlorophenethyl)pyrimidine-2,4,6(1H,3H,5H)-trione; Fig. 1) were synthesized according to the procedure reported by Kang. et al. but with slight modifications (see Methods section). It has been reported that the newly discovered compound 8 inhibited ~30 and 60% of CaV1.3 current at concentrations of 5 and 50 μM, respectively, whereas CaV1.2 channels were weakly responsive as 50 μM of compound 8 inhibited only close to 10% of its peak current.

We have recently discovered that alternative splicing at the C terminus regulated the sensitivity of CaV1.3 channels towards DHPs; while the long-form CaV1.3aβ2 channel exhibited high sensitivity towards DHP; truncation of the C terminus immediately after the IQ domain via alternate use of exon 42a yielded the less sensitive CaV1.3aβ2a channel. To determine whether the compound 8 may also have differential effects on different C-terminal splice forms, we first tested the sensitivity of rat CaV1.3aβ2 and CaV1.3aβ2a. In the presence of β2a co-transfected with the CaV1.3 channels, the channel isoforms displayed very little run-down with repeated 1 s square pulse depolarization at test potential of 10 mV from a holding potential of −70 mV at the frequency of 0.05 Hz (Figs 2b and 3b). Surprisingly, 5 μM of compound 8 failed to significantly inhibit the peak Ca2+ current (IC50) of either CaV1.3aβ2 or CaV1.3aβ2a (Figs 2 and 3) as compared with the untreated controls. Moreover, 50 μM of compound 8 only inhibited 29.51 ± 3.01 and 23.57 ± 2.14% of the peak ICa of CaV1.3aβ2 or CaV1.3aβ2a respectively (Figs 2 and 3). In addition, CaV1.3aβ2 channels were blocked to a similar level with compound 8 obtained from an additional source (Supplementary Fig. 1). In comparison, 78.85 ± 2.63% of CaV1.3 current and 68.14 ± 3.43% of CaV1.3aβ2 current could be robustly inhibited by 5 μM of nimodipine (Figs 2 and 3). As compound 8 has been reported to have little effect on CaV1.2 current, we tested its inhibition on the rat CaV1.2B15 channels. As compared with the CaV1.3, a slightly enhanced run-down effect (10.14 ± 3.98%) was observed for CaV1.2B15 channels (Fig. 4b). However, 34.97 ± 4.39 and 44.03 ± 4.67% of CaV1.2B15 current could be inhibited by 5 and 50 μM of compound 8, respectively, indicating a stronger inhibitory effect of compound 8 on CaV1.2 than CaV1.3 channels. As expected, 80.66 ± 2.82% of CaV1.2B15 current was blocked by 5 μM nimodipine (Fig. 4).

In addition, we evaluated the inhibitory activities of PYT and compound 1 against CaV1.3 and CaV1.2 channels. Kang et al. reported that PYT was the original scaffold that displayed an eightfold selectivity for CaV1.3, whereas compound 1 was one of the PYT analogues that was 28 times more selective for CaV1.3 channels. However, in our experiments, 5 μM of PYT and compound 1 did not significantly inhibit the peak ICa of CaV1.3aβ2 (Supplementary Fig. 2). In addition, 5 μM of PYT inhibited 19.90 ± 2.59% of CaV1.3aβ2 current, whereas 5 μM of compound 1 had no effect on CaV1.3aβ2 channels (Supplementary Fig. 3). In comparison, PYT and compound 1 also appeared to be more selective for CaV1.2B15 showing 43.52 ± 4.83% and 31.69 ± 4.83% inhibition, respectively, (Supplementary Fig. 4). The effect could not be a result of current run-down as little current decay was observed for the untreated CaV1.2 current upon prolonged pulses, and the current density of the recorded cells under all treatment groups were not significantly different in the study (Supplementary Fig. 5).

Furthermore, we noted that the use of the original rat CaV1.3aβ2 (ref. 12) (Genbank accession no. AF370010) by Kang et al. contained three mutations, including G244S, A1104V and E1212V, that have been shown to drastically alter both the biophysical and pharmacological properties of the channel. To test whether the use of uncorrected CaV1.3aβ2 channel, named here as CaV1.3aβ2_UC, could account for the lack of selectivity...
of compound 8 against CaV1.3 channels in the presence of β2a-subunit, we measured the sensitivity of CaV1.342_UC to either 5 and 50 μM compound 8 or 5 μM nimodipine with instead shorter 100 ms depolarizing pulse that was used by Kang et al.10 While 5 μM compound 8 has little effect on CaV1.342_UC channels as compared with control treatment, only 15.75 ± 3.25% of the peak current could be inhibited by 50 μM compound 8, and expectedly 73.10 ± 3.48% of the current was blocked by 5 μM nimodipine (Fig. 5). Hence, it was consistent that in the presence of β2a-subunit, CaV1.2 displayed higher sensitivity to compound 8 as compared with CaV1.3 channels.

**β-subunit influences compound 8 selectivity.** Although it has been shown that the presence of different β-subunits did not overtly affect the sensitivity of L-type channels towards DHP15, the use of different β-subunits such as β3 in Kang et al.10 and β2a in the current study might account for the opposite selectivity of compound 8 on CaV1.2 and CaV1.3 channels observed in these two studies. To test this possibility, we first measured the effect of 50 μM compound 8 on CaV1.342, CaV1.342_UC, CaV1.2B15 and CaV1.342a channels co-transfected with β3-subunit. Interestingly, while ~40% of the peak ICa of CaV1.342 and the uncorrected CaV1.342_UC were similarly inhibited by 50 μM compound 8, CaV1.2B15 and CaV1.342a channels displayed a slightly but significantly weaker sensitivity in comparison with CaV1.342 channels as 23.47 ± 2.60 and 29.05 ± 2.48% of respective peak currents were inhibited (Fig. 6 and Supplementary Fig. 6). Additional experiments done using either β1- and β4-subunits produced results that demonstrated enhanced inhibitory effect of compound 8 on CaV1.342 as compared with CaV1.342_UC, CaV1.2B15 and CaV1.342a channels. However, CaV1.2B15 and CaV1.342a channels were inhibited to similar level by 50 μM compound 8 in the presence of β1-, β3- and β4-subunits (Fig. 6 and Supplementary Fig. 6). Significantly, in the presence of β2a, CaV1.2B15 still displayed higher sensitivity as compared with CaV1.342, CaV1.342_UC and CaV1.342a, even when...
The constant Ca\(^{2+}\) influx via the CaV1.3 channels enhances mitochondrial oxidative stress that could contribute towards the pathogenesis of PD. The presence of other possible pacemaking current underlied by hyperpolarization-activated and cyclic nucleotide-gated cation (HCN) channel suggested that it might be feasible to block CaV1.3 current without grossly affecting the normal physiological functions of SNC neurons\(^5\). The availability of a CaV1.3 channel-specific antagonist that could eventually replace non-selective L-type blockers such as DHPs would therefore be ideal for the therapeutic management of PD.

In contrast to the IC\(_{50}\) of 24.3 ± 0.7 μM as determined using patch-clamp electrophysiology by Kang et al.\(^{10}\), our initial data with β2α-subunits revealed that the percentage inhibition of compound 8 on CaV1.3 channels at 50 μM was only 30% while at 5 μM no significant inhibition was observed. In contrast to the published report, the inhibition of CaV1.2 channels at 50 and 5 μM were 44% and 35%, respectively, indicating selectivity of compound 8 against CaV1.2 channels.

The initial failure to observe any selectivity of compound 8 in inhibiting CaV1.3 channels led us to investigate the effect of compound 8 with other β-subunits. However, while the CaV1.3\(_{2α}\) current was indeed more sensitive than CaV1.2 in the presence of the currents were repeatedly recorded with a shorter 100-ms depolarizing square pulse (Fig. 6 and Supplementary Fig. 6). Finally, we performed analysis of the correlation of the % of inhibition with the current density for each of the CaV1.3\(_{42},\) CaV1.3\(_{42\_UC},\) CaV1.2\(_{15},\) and CaV1.3\(_{12α}\) channels. Only weak correlations were observed as indicated by the following \(R^2\) values: 0.0121 for CaV1.3\(_{42},\) 0.0859 for CaV1.3\(_{42\_UC},\) 0.2439 for CaV1.2\(_{15},\) and 0.2047 for CaV1.3\(_{12α}\). Therefore, the inhibitory effect of compound 8 on the CaV1.2 and CaV1.3 channels are not influenced by the amplitudes of the currents. (Supplementary Fig. 7).

**Discussion**

PD is a debilitating movement disorder currently with no cure. The constant Ca\(^{2+}\) influx via the CaV1.3 channels enhances mitochondrial oxidative stress that could contribute towards the pathogenesis of PD. The presence of other possible pacemaking current underlied by hyperpolarization-activated and cyclic nucleotide-gated cation (HCN) channel suggested that it might be feasible to block CaV1.3 current without grossly affecting the normal physiological functions of SNC neurons\(^5\). The availability of a CaV1.3 channel-specific antagonist that could eventually replace non-selective L-type blockers such as DHPs would therefore be ideal for the therapeutic management of PD.
In summary, the newly reported compound 8 is not a potent blocker of CaV1.3 channels as compared with the classical L-type blockers such as the DHPs. The selectivity of compound 8 on CaV1.3,2 over CaV1.2,15 channels could be influenced strongly by the presence of various β-subunits and the compound 8 was not selective for CaV1.3,2 over CaV1.2,15 channels in the presence of β1-, β3- or β4-subunit. Instead, in the presence of β2a, compound 8 strongly inhibits CaV1.2,15 channels over all the CaV1.3 splice variants tested. Given the differential distribution of β-subunits and the varied expression levels of CaV1.3 channel variants across various neuronal types, brain regions and organs, the use of compound 8 as a CaV1.3-selective antagonist needs to be further clarified. Nonetheless, the quest for discovering a highly selective and potent compound targeting CaV1.3 channels is a highly worthwhile goal.

β1-, β3- and β4-subunits, only 35–45% of the its peak currents could be blocked by 50 μM compound 8, and the percentage of inhibition on CaV1.2,15 channels was a modest 10–15% less as compared with CaV1.3,2 channels.

To complicate the story further, the short-form CaV1.3,2a channels displayed similar level of sensitivity as compared with CaV1.2,15 channels with either β1-, β3- or β4-subunit co-transfected. The CaV1.3,2a splice variant accounts for ~30% of the total CaV1.3 transcripts in the substantia nigra region7. While the roles of different CaV1.3 splice variants in neurodegeneration of SNc neurons in the pathogenesis of PD awaits further clarification, the larger current density and more hyperpolarized-shifted I-V relationship implied a greater involvement of CaV1.3,2a current in driving the pacemaking activity in the SNc neurons as compared with the full-length CaV1.3,2 channels8.

Figure 6 | Inhibition of CaV1.3,42, CaV1.3,42,UC, CaV1.2,15 and CaV1.3,2a current by 50 μM compound 8 in the presence of different β-subunits. (a) Averaged diary plot of effects of 50 μM 8 on CaV1.3,42 peak currents in the presence of β1-, β2a-, β3- and β4-subunit. The current was recorded by single square pulse from holding of −70 to 10 mV for over 100 ms with sweep interval of 20 s. (b-d) Averaged diary plot of effects of 50 μM 8 on the peak currents of CaV1.3,42,UC, CaV1.2,15 and CaV1.3,2a in the presence of different β-subunits. Within each β-subunit, the % inhibitions of CaV1.3,42,UC, CaV1.2,15, CaV1.3,2a were compared with that of CaV1.3,42. NS, non-significant, *P<0.05, **P<0.01, ***P<0.001 (Student’s unpaired t-test). Alternatively, P<0.001 within each group (one-way analysis of variance and Bonferroni’s test). The number of cells analysed is indicated in b-d. The data for each condition were collected from two to three transfections.
1-(4-chlorophenethyl)-3-cyclohexylpyrimidine-2,4,6(1H,3H,5H)-trione (1) (White powder; 1H NMR spectrum (CDCl3, 300 MHz): δ 7.21(t, J = 8.4 Hz, 2H), 7.12(d, J = 8.4 Hz, 2H), 4.54(m, 1H), 3.99(m, 2H), 3.55(s, 1H), 2.81(m, 2H), 2.17(m, 2H), 1.79(m, 2H), 1.58(m, 3H), 1.32(m, 3H); 13C NMR (CDCl3, 75 MHz): δ 164.59, 164.41, 150.92, 150.72, 152.33, 131.33, 128.48, 150.72, 128.96, 127.03, 126.81, 54.24, 42.48, 40.00, 33.55, 28.59, 25.42; melting point: 146.6-147.5°C (146-148°C; ref. 11); HRMS(ESI) calculated for C_{17}H_{16}Cl_{2}N_{2}O_{3}[M-H]−: 347.1168; found: 347.1168.

1-(3-chlorophenethyl)-3-cyclopyrpyridine-2,4,6(1H,3H,5H)-trione (2) (White solid; 1H NMR spectrum (CDCl3, 500 MHz): δ 7.23-7.18(m, 3H), 7.12(d, J = 4.14 Hz, 1H), 5.12(m, 1H), 4.05(s, 2H), 3.60(s, 2H), 2.86(m, 2H), 1.92(m, 4H), 1.84(m, 2H), 1.57(m, 2H); 13C NMR spectrum (CDCl3, 125 MHz): δ 164.68, 164.40, 150.79, 139.77, 134.18, 129.72, 128.96, 127.03, 126.81, 54.24, 42.48, 40.00, 33.55, 28.59, 25.42; melting point: 131.3-131.8°C (131-132°C; ref. 11); HRMS(ESI) calculated for C_{17}H_{16}Cl_{2}N_{2}O_{3}[M-H]−: 333.1011; found: 333.1007.

1,3-bis-(4-chlorophenethyl)pyridine-2,4,6(1H,3H,5H)-trione (PTT) (White solid; 1H NMR spectrum (CDCl3, 500 MHz): δ 7.28(d, J = 8.15 Hz, 4H), 7.17(d, J = 8.1 Hz, 4H), 4.06(l, J = 7.7 Hz, 4H), 3.66(l, 2H), 2.84(s, J = 7.95 Hz, 2H); 13C NMR spectrum (CDCl3, 125 MHz): δ 164.18, 150.98, 136.07, 132.57, 130.23, 128.67, 42.77, 39.46, 33.26; melting point: 171.8-172.9°C; HRMS(ESI) calculated for C_{18}H_{18}Cl_{3}N_{2}O_{3}[M-H]−: 403.0662; found: 403.0612.

Electrophysiological recordings and data analysis. Whole-cell patch-clamp electrophysiological recordings were used to characterize the recombinant rat Ca_{1.3,4,2} and of Ca_{1.3,4,3} channels, and rat Ca_{1.2,3,3} channel. The currents were recorded from transiently transfected mammalian HEK293 cells at room temperature with calcium phosphate method[17]. Outward I_Ks currents were blocked by Ca^{2+} in the internal and external solutions. Cells were transiently transfected with the respective Ca_{1.3} and Ca_{1.2,3,2} constructs with rat PNT, subunit and rat α5β-subunit using standard calcium phosphate transfection method. For whole-cell patch-clamp recording, the internal solution (patch-pipette solution) contained the following (in mM): 138 Cs-MSO5, 5 CsCl, 5.0 EGTA, 10 HEPS and 1 MgCl2 and 2 mg ml^{−1} Mg-ATP, pH 7.3 (adjusted with Ca(OH)2) and 290 mM osm with glucose. The external solution contained the following (in mM): 109 NaCl, 10 CaCl2, 1 MgCl2, 20 CsCl and 10 HEPS, (pH adjusted to 7.4 with CsOH and osmolarity to 290 mOsm with glucose). Pipettes of resistance in the range of 2-5 MΩ were used. Whole-cell currents, obtained under voltage clamp with an Axopatch 200B amplifier (Molecular Devices, Union City, CA), were filtered at 1-5 kHz and sampled at 5-50 kHz, and the series resistance was typically <5 MΩ after >80 compensation. A P/4 protocol was used to subtract on-line the leak and capacitive transients.

The currents were recorded by single square pulse from holding of −70 to 10 mV for over 1 s or 100 ms with sweep interval of 20 s. Data were acquired using the software pClamp9 (Molecular Devices), and analysed and fitted using GraphPad Prism V software (San Diego, CA) and Microsoft (Seattle, WA) Excel. Data are expressed as mean values ± s.e.m.

**Drug preparation.** Stock solutions were prepared by dissolving compound PYT, 1, 8 and nimodipine (RBI) in DMSO to make a 100 mM stock solution and stored at −20 °C in the dark. Respective concentrations were freshly prepared in the bath solution from stock, and perfused (1 ml per min) into the whole recording chamber by gravity during current recording. The cell selected for patch-clamp recording were positioned as close to the outlet as possible. The external solutions were protected from light throughout the experiment.

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patch-clamp experiment; and Y.L and T.W.S supervised the experiments and edited the manuscript.

**Additional information**

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

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