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Comparison of mibefradil and derivative NNC 55-0396 effects on behavior, cytochrome P450 activity, and tremor in mouse models of essential tremor

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ABSTRACT

NNC 55-0396 [(1*S*,2*S*)-2-(2-(*N*-[(3-benzimidazol-2-yl)propyl]-*N*-methylamino)ethyl)-6-fluoro-1,2, 3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride], is a mibefradil derivative that retains potent in vitro T-type calcium channel antagonist efficacy. We compared the two compounds for behavioral toxicity, effects on cytochrome P450 activity, and efficacy against tremor in the γ-aminobutyric acid type A ($GABA_A$) receptor subunit α1-null mouse, and the harmaline tremor model of essential tremor in wild-type mice. NNC 55-0396 was better tolerated than mibefradil in the horizontal wire test of sedation/motor function, with 3/6 failing at 300 and 30 mg/kg respectively. To assess for a potential interaction with harmaline, mice were given the drugs, followed by harmaline or vehicle, and tested 30 min later in the inverted wire grid test. Mibefradil exacerbated, whereas NNC 55-0396 ameliorated harmaline-induced test deficits. In mouse liver microsomes, NNC 55-0396 was a less potent inhibitor of harmaline O-demethylation than mibefradil (K_i: 0.95 and 0.29 μM respectively), and also less potent at inhibiting testosterone 6-β-hydroxylation (K_i: 0.71 and 0.12 μM respectively). In the $GABA_A$ α1-null model, NNC 55-0396 but not mibefradil, (each at 20 mg/kg), suppressed tremor while NNC 55-0396 at 12.5 mg/kg suppressed harmaline-induced tremor by half by 20–100 min, whereas mibefradil at the same dose did not significantly affect tremor. In contrast to mibefradil, NNC 55-0396 is well tolerated and suppresses tremor, and exerts less cytochrome P450 inhibition. These results suggest potential clinical utility for NNC 55-0396 or similar derivatives as a T-type calcium antagonist.

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1. Introduction

T-type calcium channel activity is a critical component of the spontaneous voltage oscillation of inferior olive neurons that occurs at a frequency similar to that of essential tremor (ET) (Llinás and Yarom, 1981). Administration of harmaline to animals induces rhythmic synchronous olivary burst-firing that is transmitted through the cerebellum, resulting in ET-like postural/kinetic tremor (Bernard et al., 1984). Harmaline-induced tremor in rodents is reduced by drugs that suppress tremor in ET and thus serves as a pre-clinical model (Martin et al., 2005; Stöhr et al., 2008). Another ET model is the γ-aminobutyric acid type A ($GABA_A$) receptor α1 subunit knockout mouse, which is also responsive to anti-ET agents (Kralic et al., 2005). Given the role of T-type calcium channels in olivocerebellar oscillatory circuits, a plausible hypothesis is that their activation is required for tremor expression. The field of T-type calcium channels has been hampered however by a lack of potent and specific compounds (Lory and Chemin, 2007).

Mibefradil, a potent T-type calcium antagonist, was approved by the FDA in June 1997 for the treatment of angina pectoris and hypertension, but had to be withdrawn one year later due to drug interactions (Po and Zhang, 1998). Mibefradil is a strong inhibitor of human CYP3A4, CYP2D6 and P-glycoprotein (Ernst and Kelly, 1998; Wandel et al., 2000). Another disadvantage is the failure to cross the blood–brain-barrier (Ertel and Clozel, 1997). It also possesses the significant drawback that intracellular hydrolysis removes the methoxyacetyl chain, creating a metabolite (Ro 40-5966) that is a specific L-type calcium channel antagonist (Wu et al., 2000). Despite these disadvantages, mibefradil has been widely employed in basic research as a T-type calcium channel antagonist.

Li's group substituted cyclopropane for the methoxy moiety, creating the mibefradil derivative NNC 55-0396 [(1*S*,2*S*)-2-(2-(*N*-[(3-

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benzimidazol-2-yl)propyl]-*N*-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphtyl cyclopropanecarboxylate dihydrochloride] (Fig. 1) (Huang et al., 2004; Li et al., 2005). NNC 55-0396 exerts no effect against high voltage calcium channels at 100 μM, but inhibits T-type channels in HEK293 cells with a potency comparable to that of mibefradil (IC_{50} 6.8 versus 10.1 μM). Despite this advantage, it has seldom been utilized for in vivo studies.

We therefore chose to compare the effect of mibefradil and NNC 55-0306 on tremor in the $GABA_A$ receptor subunit α1-null and harmaline mouse ET models. We found that whereas NNC 55-0396 was effective for tremor in both models and well tolerated, mibefradil was poorly tolerated and ineffective for tremor, indicating that the side chain modification markedly modifies the pharmacological profile of the derivative compared to mibefradil.

2. Materials and methods

2.1. Animals

$GABA_A$ receptor α1 heterozygous mice (+/−) of the F10+ generation on a mixed genetic background (approximately 25% C57BL/6J, 25% strain 129/Sv/SvJ, and 50% FVB/N) were generated in Pittsburgh as previously described (Vicini et al., 2001). Heterozygotes were shipped to the VA Greater Los Angeles and subsequently interbred to produce knockout offspring for the current studies, as identified through genotyping (Ortinski et al., 2004). These mice and male ICR mice (20–24 g, Harlan, Indianapolis, Indiana U.S.A.) for harmaline experiments were housed in groups with free access to rodent diet and water. Experiments were approved by the Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

2.2. Chemicals

Harmalol, testosterone, and 6-β-hydroxytestosterone were obtained from Sigma-Aldrich, mouse liver microsomes from BD Gentest (Franklin Lakes, New Jersey U.S.A.), and P450 reaction buffer mixtures containing the NADP(+)/H regeneration enzymes from Biocatalytics, Inc. (Pasadena, California U.S.A.). Ro 40-5966 was synthesized using alkaline hydrolysis of mibefradil according to Wu et al. (2000) with modifications (Bui et al., 2008). Complete hydrolysis was checked by HPLC. All HPLC solvents (HPLC grade) were from Sigma-Aldrich.

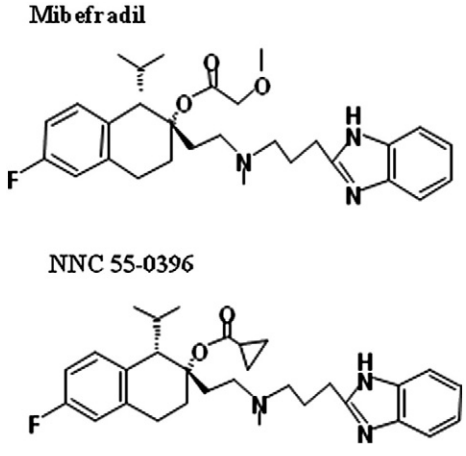

Fig. 1. Structures of mibefradil and NNC 55-0396.

2.3. Incubation of mouse liver microsomes with drugs

Assays were conducted in 1.5 ml Eppendorf tubes in duplicate. Enzyme buffer, 105 μl, was prepared that contained 1 mg/ml mouse liver microsomes and 2× reaction buffer (pH 7.5 NADPH/NADP+/ regenerating mixture). The enzyme buffer was pre-warmed for 3 min at 37 °C. The reaction was initiated by the addition of 95 μl of substrate (10 μM harmaline or 100 μM testosterone) along with vehicle (acetonitrile) or drug (mibefradil, NNC 55-0396, or Ro 40-5966). In preliminary studies of drug inhibition on harmaline metabolism, 100 nM (low concentration) and 10 μM (high concentration) were used. To determine IC_{50} and K_i values, 10 μM and eight successive 3:1 dilution drug concentrations were used. Reactions were terminated after 20 min by adding 200 μl ice-cold 2% acetic acid in acetonitrile. The solutions were placed in methanol/dry-ice for 5 min to precipitate proteins, followed by centrifugation at 13,000 *g* for 15 min. The supernatants were analyzed by HPLC.

2.4. HPLC analytical procedures

The HPLC system consisted of the Shimadzu (Kyoto, Japan) prominence series, including the LC-20 AT prominence LC pump, DGU-$20A_5$ degasser, CBM-20 prominence communications bus module, SPD-20A prominence UV/VIS detector, and RF-10AXL fluorescence detector (Shimadzu). Samples were separated with a C_{18} reversed-phase column (4.6 mm × 250 mm, 5 μm; Dupont, Wilmington, Delaware U.S.A.). For harmaline, solvents were held isocratic at 30% B from 0 to 8 min. The gradient was linear from 30% B to 70% B from 8 to 12 min and held at 70% until 17 min; before returning to starting conditions (solvent A: H_20, 0.1% trifluoric acid; solvent B: 100% acetonitrile; flow rate 1 ml/min). The harmaline metabolite harmalol was monitored with the fluorescence detector setting at 340 nm/495 nm (excitation/emission wavelengths), and eluted at 4.9 min. The separation of testosterone samples was performed using 40% B from 0 to 5 min, increasing to 80% B from 5 to 12 min, and then holding for another 17 min before returning to the starting condition (flow rate 1 ml/min). The testosterone metabolite, 6 β-hydroxytestosterone, was monitored at a wavelength of 240 nm and eluted at 5.7 min.

2.5. Drugs

Harmaline, mibefradil and NNC 55-0396 (Sigma-Aldrich, St. Louis, Missouri U.S.A.) were dissolved in sterile physiological saline. NNC 55-0396 and mibefradil were injected intraperitoneally (i.p.), harmaline was injected subcutaneously, in a volume of 4 ml/kg. Doses utilized for tremor suppression experiments were chosen based on the horizontal wire test and pilot experiments that sought to determine dosages capable of suppressing tremor by approximately 50% or 80%.

2.6. Horizontal wire test of behavioral toxicity

To assess for potential impairment of motor functioning we employed the horizontal wire test (Vanover et al., 1999). Following specific doses of mibefradil or NNC 55-0396, each mouse was picked up by the base of the tail and positioned so that the forepaws touched a 25-cm long, 2-mm diameter horizontal wire, inducing the mouse to grasp the wire. A mouse passed the test if it brought up at least one hind paw to touch the wire, and did not fall off, within 10 s of test initiation. Mice were tested at 10-min intervals for 1.5 h after drug administration, and had to pass all tests to be considered a pass.

2.7. Inverted grid test of behavioral toxicity

Mice were tested for the ability to hang upside down from a wire screen (Sango et al., 1996) using a modified wire cover to a rat tub cage. The wire bars were 1 mm in diameter and spaced 1 cm apart. A

10 cm × 18 cm section of the screen was bordered by tape in order to confine mice to this area. Each mouse was placed upright on the screen, then the screen waved gently in the air three times to induce the mouse to grip the wires. The screen was then immediately turned upside down, 20 cm above a large rodent housing cage with a sawdust floor. The latency to fall after turning the screen over was recorded using a stopwatch. Mice that fell in less than 10 s were given a second trial, and the longer time utilized. Mice can normally hang for 60 s or longer. A 90-second cut-off time was used. Mibefradil or NNC 55-0396, 20 mg/kg, or vehicle were injected i.p., followed 30 min later by s.c. harmaline, 20 mg/kg, or vehicle, then each mouse administered the test 30 min later.

2.8. Tremor measurement in the $GABA_A$ receptor α1-null model

Tremor was measured while the mouse was suspended by the base of the tail with a padded clip from the ceiling of a plexiglass box resting on a Convuls-1 Replacement Sensing Platform model 1335-1A (Columbus Instruments, Columbus, Ohio U.S.A.). This motion detector platform was connected to a Grass model P511 AC amplifier (Grass Instruments, West Warwick, Rhode Island U.S.A.) with 1 and 70 Hz filter settings. The tail suspension position was utilized as it most reliably elicits tremor (Kralic et al., 2005). Digitally recorded motion power was analyzed using Spike2 software (Cambridge Electronic Design, U.K.) to perform Fourier transformation of the data into frequency spectra. Tremor-associated motion power data (usually 22–27 Hz) were sampled at 454 Hz for 30 s, then the mouse returned to its cage. As the 5 Hz-wide tremor peak varies slightly between animals, preliminary readings served to identify the frequency band to be used for each mouse. Tremor was assessed in four pre-drug baseline assessments, each one min apart. After administration of NNC 55-0396, mibefradil or vehicle, four more tremor assessments were performed 30 min later. Each group utilized 5–6 mice.

2.9. Tremor measurement in the harmaline model

Motion activity was measured using the above-described equipment with the mouse sitting on the platform. Data were sampled at 128 Hz. The *motion power percentage* is the tremor bandwidth divided by overall motion power (10–16 Hz power)/(0–33 Hz power) × 100 (Martin et al., 2005). During baseline this measure is approximately 30–35%, representing normal movement within 10–16 Hz; after harmaline this value attains 60–80%. Baseline motion data were collected for 20 min, then harmaline, 20 mg/kg, was administered, followed by NNC 55-0396, mibefradil or vehicle 15 min later (time 0), when tremor had fully developed. Motion power was recorded for another 100 min. Each group utilized 5–6 mice.

2.10. Statistical analysis

Student's t tests were utilized to compare the effects of drugs on retention time on the wire grid. The P450 activities with and without drugs were measured by the area of products from the HPLC chromatograms. The K_i values were calculated with Prism software based on the equation $K_i = (IC_{50})/(1 + [\text{substrate}]/K_m)$. IC_{50} and K_m were determined using the non-linear regression model with one-site competition. The K_i values were compared using Student's t test (Prism software). Alpha was 0.05, two-sided.

The analysis measure for $GABA_A$ receptor α1-null experiments was the percent change in the mean tremor bandwidth motion power during four drug- or vehicle-treatment 30-second trials compared to the animal's mean power during four pre-treatment baseline trials. Student's t-test was employed to determine whether the percent tremor change relative to pre-treatment tremor was greater during NNC 55-0396 or mibefradil than for vehicle treatment.

A repeated measures ANOVA model was applied to motion power percentage values in the harmaline experiment followed by post-hoc Student's t-tests comparing mibefradil- and NNC 55-0396-treated groups with the vehicle-treated group under the model using the Tukey–Fisher significance criterion, employing JMP (SAS Inc., Cary, North Carolina U.S.A.). P values less than 0.05 were considered significant. The statistic *percent tremor reduction* was calculated as 100 × (1 − [(DT − DB)/(mean VT − mean VB)]), where DT is motion power percentage during a drug treatment epoch, DB is motion power percentage during the drug-treated group's baseline, mean VT is the saline vehicle group mean value for the same epoch, and mean VB is mean motion power percentage for the vehicle group during baseline. This measure is descriptive and involves the assumption that 10–16 Hz motion power attributable to normal movement is unchanged during harmaline-induced tremor. Statistical comparisons of groups were based on motion power percentage values, not the calculated percentage tremor reduction. A minimum of 5 mice per group were utilized in knockout and harmaline tremor experiments based on power analysis of prior data with NNC 55-0396 that indicated that in the doses used, there was 80% chance of detecting a 33% reduction in tremor.

3. Results

3.1. Horizontal wire test

Gross observations of NNC 55-0396 doses below 200 mg/kg in open fields did not reveal sedation or ataxia. We found that the maximal dose at which 6/6 mice passed the horizontal wire test was 200 mg/kg, whereas 3/6 failed the test at 300 mg/kg. Mibefradil doses that attained these benchmarks were 20 mg/kg and 30 mg/kg respectively. Thus compared to mibefradil, NNC 55-0396 was tolerated in doses an order of magnitude higher.

3.2. Inverted grid test

Mice pre-treated with mibefradil, 20 mg/kg, or NNC 55-0396, 20 mg/kg i.p., followed by saline vehicle, did not perform differently from vehicle/vehicle controls in maintaining their position on the inverted grid (P = 0.18, 0.36 respectively; left side of Fig. 2). In vehicle-pre-treated mice, harmaline, 20 mg/kg s.c., elicited a head/body tremor and reduced retention times on the wire grid compared to vehicle/vehicle controls (P = 0.004, Fig. 2).

Mibefradil-pre-treated mice administered harmaline displayed worse retention than mibefradil/vehicle mice (P = 0.0004) or vehicle/harmaline mice (P = 0.003, right side of Fig. 2). They did not exhibit

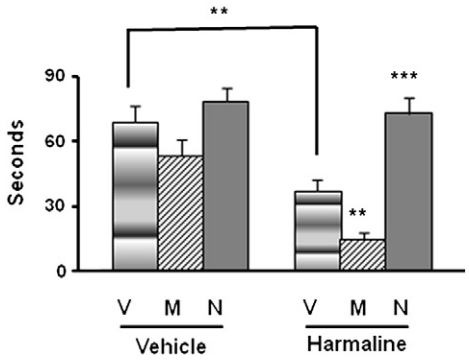

Fig. 2. Effect of drugs on retention time by mice on an inverted wire grid. Mice were pre-treated with saline vehicle (V), mibefradil (M), 20 mg/kg, or NNC 55-0396 (N), 20 mg/kg i.p., then 30 min later administered saline vehicle (n = 10, 14, 7 respectively) or harmaline, 20 mg/kg s.c. (n = 7, 11, 8 respectively). The duration of time mice held on to an inverted wire grid was assessed 30 min after vehicle or harmaline. Unless otherwise indicated, comparisons are with the vehicle-pre-treated subgroup of the same group. Means and S.E.M.s are shown. ** P < 0.01 *** P < 0.001.

more tremor than vehicle/harmaline mice, but appeared sedated. In contrast, NNC 55-0396/harmaline mice performed as well as NNC 55-0396/vehicle mice ($P = 0.585$), and better than vehicle/harmaline mice ($P = 0.001$, Fig. 2). They did not show as much tremor, and did not appear sedated.

Harmaline in high doses can induce catalepsy in animals (Pranzatelli and Snodgrass, 1987). thus we considered mibefradil-induced deterioration in harmaline-administered mice as possibly due to a metabolic interaction. The normalization of performance by NNC 55-0396 was associated with a visible reduction of tremor and, in contrast to mibefradil, the absence of sedation. We therefore investigated whether mibefradil and harmaline affect microsomal functions differently. Harmaline is metabolized mainly by CYP 2D6 and 1A2 in humans (Yu et al., 2003), but mice lack 2D6, and harmaline metabolism has not been well investigated in mice. We also wished to determine whether mibefradil inhibits CYP3A enzymes, as indicated by testosterone hydroxylation, and whether NNC 55-0396 differs in this property.

3.3. O-demethylation of harmaline

In a preliminary experiment, we examined the effect of two concentrations of mibefradil and NNC 55-0396 on O-demethylation of harmaline, as measured by the formation of harmalol. Compared to control samples, mibefradil and NNC 55-0396 at 100 nM did not affect O-demethylation (percentage of normal, means and S.D.s: 100.0 ± 20.9 and 103.7 ± 12.8 percent, respectively). At 10 μM, each inhibited O-demethylation, but mibefradil suppressed harmalol synthesis more than did NNC 55-0396 (percent of normal: 27.3 ± 2.1, 46.6 ± 5.3 respectively; $P = 0.04$).

On testing the effect of serially diluted drugs on harmaline O-demethylation in mouse microsomes, we found that the K_i for NNC 55-0396 was approximately three-fold higher than for mibefradil (Fig. 3; $P = 0.02$). Ro 40-5966, the hydrolyzed metabolite of mibefradil, also potently inhibits harmaline O-demethylation, with a K_i one-third that of NNC 55-0396 ($P = 0.02$, Fig. 3). These findings correlate with our behavioral observation that NNC 55-0396 caused less sedation in harmaline-administered mice than did mibefradil, in that the latter drug causes more inhibition of harmaline metabolism.

3.4. Hydroxylation of testosterone

We also examined the effect of mibefradil on 6-β-hydroxylation of testosterone in mouse microsomes, a CYP3A-mediated reaction. We found that mibefradil inhibited 6-β-hydroxytestosterone formation with a K_i of 122 nM (Fig. 4). By comparison, the K_i of NNC 55-0396 was over five-fold higher ($P = 0.01$), indicating that NNC 55-0396 is less prone than mibefradil to cause pharmacokinetic interactions with CYP3A-metabolized drugs.

3.5. NNC 55-0396 suppresses tremor in $GABA_A$ subunit α1-null mice

We measured tremor during the tail suspension position in a pre-treatment baseline, then again 30 min later, after vehicle, NNC 55-0396, or mibefradil administration. Fig. 5 displays motion power spectra for individual mice administered saline vehicle (panel A) or NNC 55-0396, 40 mg/kg (panel B). The suppression of the digital tremor motion power peak by NNC 55-0396 corresponded with visible tremor reduction. There was no emergence of a displaced tremor peak at a lower or higher frequency. NNC 55-0396 and mibefradil were then compared for their ability to suppress tremor at the maximum dose mice tolerate mibefradil in the horizontal wire test, 20 mg/kg. Whereas tremor increased slightly on re-test in mice administered saline vehicle (Fig. 5C), NNC 55-0396, 20 mg/kg, reduced tremor by 49.2% compared to baseline ($P = 0.00002$, Student's *t* test). In contrast, the same dose of mibefradil had no effect on tremor ($P = 0.92$).

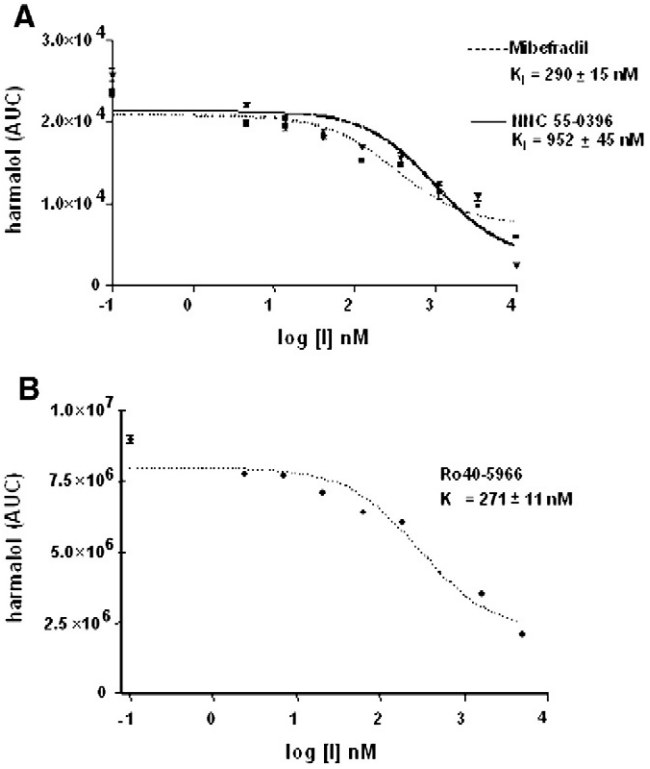

Fig. 3. Comparison of mibefradil and NNC 55-0396 effects on harmaline metabolism. A. Effect of mibefradil and NNC 55-0396 at various concentrations on harmaline O-demethylation to harmalol by mouse liver microsomes, as measured by the area under the curve (AUC) from HPLC chromatograms. The K_i of mibefradil inhibition of harmaline demethylation is less than that of NNC 55-0396 ($P = 0.02$). B. Effect of the mibefradil metabolite Ro 40-5966 on harmaline O-demethylation to harmalol. This K_i is also less than of NNC 55-0396 ($P = 0.02$), indicating that mibefradil and its primary metabolite are more prone to inhibit harmaline metabolism than NNC 55-0396. Each K_i, based on duplicate determinations, is expressed as the mean ± S.D.

3.6. NNC 55-0396 suppresses tremor in the harmaline model

We assessed motion power during a 20-min pre-treatment baseline, injected harmaline followed 15 min later by drug or vehicle, then measured motion power for 100 min. As described previously, harmaline induces a motion power peak at 10–16 Hz corresponding to tremor that lasts over 1.5 h in mice (Martin et al., 2005). Fig. 6A displays examples of mice administered harmaline plus vehicle, or

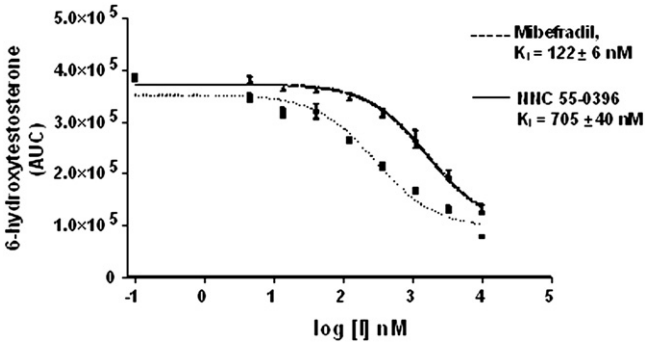

Fig. 4. Effect of mibefradil and NNC 55-0396 at various concentrations on testosterone 6-β-hydroxylation by mouse liver microsomes, as measured by the area under the curve (AUC) from HPLC chromatograms. The K_i of mibefradil inhibition is less than that of NNC 55-0396 ($P = 0.01$). Each K_i is based on duplicate determinations and expressed as the mean ± S.D.

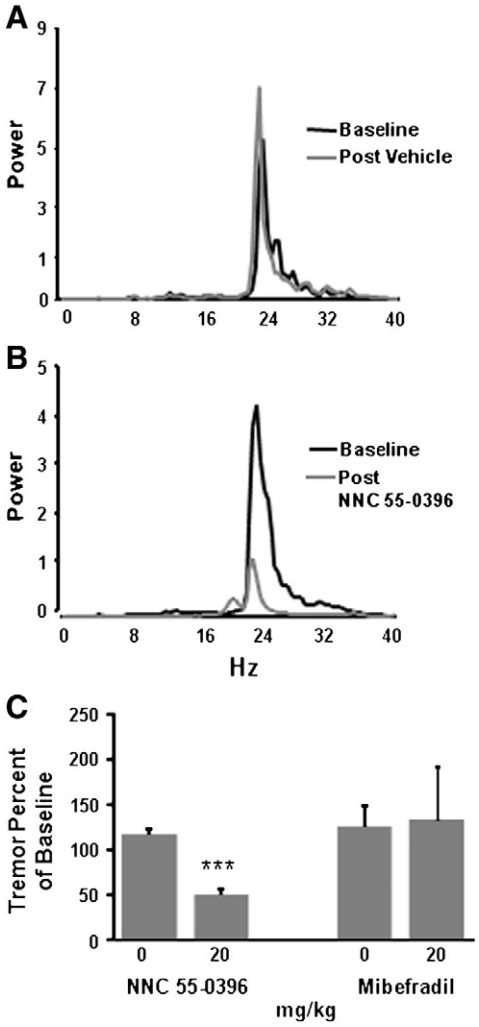

Fig. 5. Effect of NNC 55-0396 and mibefradil on tremor in the $GABA_A$ receptor α1 subunit-null mouse model. A. Spectral motion power in an α1-null mouse before and after saline vehicle. During a 30-second suspension by the tail, a tremor-associated motion power peak at 22–27 Hz occurs. After vehicle administration and retest 30 min later, a comparable tremor peak is reproduced. B. Before and after administration of NNC 55-0396, 40 mg/kg, i.p., in an α1-null mouse. On retest, NNC 55-0396 suppressed the tremor motion power peak that was present in baseline. C. Tremor after saline vehicle or NNC 55-0396, 20 mg/kg (n = 6 each group), or vehicle or mibefradil 20 mg/kg (n = 5 each group), expressed as a percentage of baseline tremor power. NNC 55-0396, but not mibefradil, significantly reduced tremor on re-test. Means and S.E.M.s are shown. Comparisons are with vehicle group, Student's t test. * $P<0.05$ ** $P<0.01$ *** $P<0.001$.

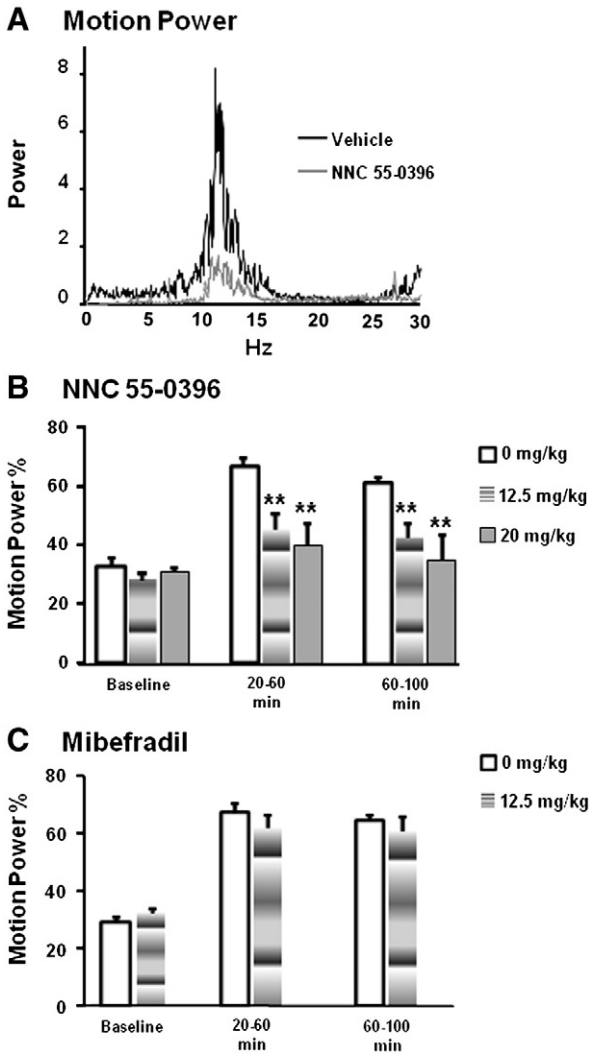

Fig. 6. Effect of NNC 55-0396 and mibefradil on tremor in the harmaline mouse model. A. Example motion spectra in mice given either harmaline, 20 mg/kg, plus saline vehicle or harmaline plus NNC 55-0396, 20 mg/kg. Whereas the control mouse exhibits a characteristic tremor-associated motion power peak at 10–16 Hz, the drug-treated mouse displays much less tremor-associated motion power. B. Motion power in the tremor bandwidth (10–16 Hz) as a percentage of overall 0–33 Hz power, during pre-harmaline baseline and at 20–60, 60–100 min after administration of vehicle or NNC 55-0396, 12.5 mg/kg or 20 mg/kg (n = 5 each group). C. Motion power during pre-harmaline baseline and at 20–60, 60–100 min after vehicle or mibefradil, 12.5 mg/kg (n = 6 each group). A dose of 20 mg/kg could not be utilized due to the behavioral interaction with harmaline depicted in Fig. 2. NNC 55-0396 suppressed tremor effectively whereas a comparable dose of mibefradil had no significant effect. Means and S.E.M.s are shown.

harmaline plus NNC 55-0396, 20 mg/kg, indicating NNC 55-0396 treatment was associated with less tremor.

Motion power in the tremor bandwidth (10–16 Hz) as a percentage of overall motion power (0–33 Hz) in mice administered NNC 55-0396, mibefradil, or vehicle is displayed in Fig. 6, panels B, C. During pre-harmaline baseline, 10–16 Hz motion power did not differ between groups, and approximated 30% of 0–33 Hz power, attributable to normal movement. After administration of harmaline plus vehicle, 10–16 Hz motion power increased to approximately 65% of 0–33 Hz motion power at 20–60 min after vehicle, due to the occurrence of tremor within this bandwidth. At 60–100 min, 10–16 Hz motion power in the harmaline plus vehicle group remained high, at approximately 60% of 0–33 Hz power. When harmaline administration was immediately followed by NNC 55-0396, 12.5 mg/kg, the generation of tremor was suppressed. Compared to the vehicle group, tremor generation was calculated as reduced by 48.5 ± 12.4% at 20–60 min post-drug (Fig. 6B, $P = 0.007$) and by 48.9 ± 13.6% at 60–100 min ($P = 0.006$). The dose 20 mg/kg suppressed tremor generation compared to vehicle-treated animals by 73.6 ± 20.1% and 85.1 ± 29.1% at 20–60 and 60–100 min respectively ($P = 0.011, 0.018$). Mibefradil could not be tested at 20 mg/kg due to the sedative interaction with harmaline noted in the wire grid test. Animals receiving mibefradil, 12.5 mg/kg, had 23.1 ± 3.9% and 20.8 ± 11.5% less tremor than vehicle-treated controls at 20–60 and 60–100 min, not statistically significant (Fig. 6C, $P = 0.10, 0.48$).

4. Discussion

Considerable physiological, neuroimaging and neuropathological evidence has implicated cerebellar dysfunction in ET (Axelrad et al., 2008; Köster et al., 2002; Kronenbuerger et al., 2007; Louis et al., 2002; Stolze et al., 2001). An important contributor to cerebellar physiology is the inferior olive, which projects climbing fibers to cerebellar neurons, and receives GABAergic feedback from deep cerebellar nuclei. The membranes of inferior olivary neurons undergo spontaneous voltage

oscillations at a tremor-like frequency that depend on T-type calcium currents (Llinás and Yarom, 1981). Harmaline administration to animals induces rhythmic synchronous olivary burst-firing that is transmitted through the cerebellum, resulting in postural/kinetic tremor (Bernard et al., 1984). These considerations lead to the hypothesis that T-type calcium channel activation is required for postural/kinetic tremor expression. Two fluoropiperidine T-type calcium channel antagonists have been found to suppress harmaline tremor (Shipe et al., 2008; Yang et al., 2008). Moreover we have observed suppression of experimental tremor by the T-type calcium channel antagonists ethosuximide, zonisamide, KYS05064 (3-Ethyl-2-(piperidin-1-yl)-4-[N-4-(p-toluenesulfonamido)benzylacetamido]-3,4-dihydroquinazoline), ECN ((3β,5α,17β)-17-hydroxyestrane-3-carbonitrile), as well as NNC 55-0396. Although these findings support a role of T-type calcium channels in tremor, mice deficient for Cav3.1, the dominant subtype found in the inferior olive, unexpectedly express harmaline-induced tremor normally (Handforth et al., 2010). This observation raises the possibility that T-type calcium channels outside the inferior olive may contribute to tremor expression, such as those in the deep cerebellar nuclei, where output neurons of the cerebellar system are located (Molineux et al., 2006). Compared to the harmaline model, even less is known about the anatomic substrate of tremor in the $GABA_A$ α1-subunit null model. Alterations in this subunit do not occur in ET (Deng et al., 2006). Tremor production may be due to GABA transporter down-regulation that occurs in these mice, associated with increased synaptic GABA levels (Ortinski et al., 2006). GABA transporter GAT1-null mice also exhibit tremor (Chiu et al., 2005), as well as humans given the GAT1 inhibitor tiagabine. Exacerbation of ET by tiagabine has been described (Zesiewicz et al., 2007). The mechanism by which GAT1 down-regulation and increased synaptic GABA levels lead to tremor is unknown, but prolonged GABA effect possibly promotes intra-cerebellar oscillations as in thalamic circuits. Harmaline as a chemical tremorigenic agent, and the $GABA_A$ α1-subunit knockout without a human genetic correlate, provide inferences for the pathophysiology of ET that have limitations, yet serve as useful pre-clinical models with pharmacologic response profiles similar to ET.

In our search for potent T-type calcium antagonists we were intrigued by the mibefradil derivative NNC 55-0396. Mibefradil was clinically marketed to treat hypertension and angina, but was withdrawn after serious interactions with other medications occurred (Welker et al., 1998). Li et al. (2005) noted that mibefradil is metabolized through intracellular hydrolysis of a methoxyester side chain to Ro 40-5966, an L-type calcium antagonist (Wu et al., 2000). NNC 55-0396 was created by substituting cyclopropane, successfully rendering the ester non-hydrolyzable (Huang et al., 2004). It was suspected that mibefradil lowers blood pressure through T-type calcium channel antagonism, so it was expected NNC 55-0396 would do the same. This assumption may be incorrect, as conditional Cav1.2 (L-type) knockout mice fail to show hypotensive responses to mibefradil (Moosmang et al., 2006). We did not measure blood pressure in our mice, but we observed that although the two compounds have comparable IC_{50}'s for T-type calcium channels, the dose at which half the mice failed the horizontal wire test was much greater for NNC 55-0396 (300 mg/kg) than that for mibefradil (30 mg/kg), and considerably above mibefradil doses reported to produce severe hypotension (30 mg/kg) (Dogrul et al., 2003). Thus NNC 55-0396's lack of activity on L-type calcium channels may underlie its superior tolerance compared to mibefradil.

Neither NNC 55-0396 nor mibefradil, 20 mg/kg, impaired performance in the inverted grid test compared to vehicle controls, but when combined with harmaline NNC 55-0396 reversed, whereas mibefradil exacerbated harmaline-induced deficits. Mice appeared sedated with the combination of mibefradil plus harmaline, but not NNC 55-0396 plus harmaline, leading us to wonder whether the drugs affected harmaline metabolism differently.

CYP2D6 is an important cytochrome P450 in humans, responsible for the metabolism of approximately 25% of prescribed medications (Nebert, 1997). We previously confirmed that mibefradil inhibits human CYP2D6 activity, but also showed that NNC 55-0396 is a more potent inhibitor, with a K_i one-fourth that of mibefradil (Bui et al., 2008). Mice and rats do not possess CYP2D6 and do not metabolize CYP2D6 substrates well (Masubuchi et al., 1997). The mouse cytochrome P450s that metabolize harmaline have not been studied, but the rate of harmaline O-demethylation by mice is low (Yu et al., 2003). In contrast to our previous findings with human microsomes, the present study with mouse liver microsomes showed that mibefradil and Ro 40-5966 each displayed a K_i for harmaline O-demethylation that was less than a third that of NNC 55-0396, indicating that they suppress harmaline metabolism more than NNC 55-0396. This provides a plausible explanation for our observation that mibefradil worsened harmaline-associated motor performance whereas NNC 55-0396 did not. However we did not measure harmaline levels in our mice, and other interactions may possibly be responsible for the sedation we observed with the drug combination.

Mibefradil is a potent inhibitor of human CYP3A4 (Ernst and Kelly, 1998; Wandel et al., 2000), a property contributing to numerous drug interactions, as CYP3A4 is responsible for metabolizing approximately half of prescribed medications. The effect of mibefradil on mouse CYP3A activity has not been studied. The predominant mouse CYP3A forms are 3a11, 3a13, 3a25, and 3a41, which together metabolize testosterone as does human CYP3A4 (Martignoni et al., 2006). We previously confirmed that mibefradil inhibits human recombinant CYP3A4 and inhibits 6-β-hydroxylation of testosterone in human liver microsomes, and also found that NNC 55-0396 had a 9- and 6-fold higher K_i in these systems respectively (Bui et al., 2008). The present study extends these observations by showing that in mouse liver microsomes the K_i of NNC 55-0396 is approximately 6 times higher than that of mibefradil.

These results indicate that the cyclopropane side chain modification that differentiates NNC 55-0396 and mibefradil not only prevents hydrolysis of the ester and formation of an L-type antagonist, but also influences the relative effects of these drugs on microsomal drug metabolism, including O-demethylation (predominantly by CYP2D6 in humans) and testosterone 6-β-hydroxylation (a CYP3A function). The reduced inhibition of human CYP3A4 by NNC 55-0396 is favorable, and raises the question whether other derivatives with appropriately modified side chains may be devoid of this undesirable effect.

In the GABA α1 genetic model of ET, NNC 55-0396 given i.p. in a dose of 20 mg/kg suppressed tremor by approximately 50%, whereas mibefradil had no effect at the same dose. In the harmaline model, NNC 55-0396, 12.5 and 20 mg/kg, suppressed tremor by approximately 50% and 80%, whereas mibefradil, 12.5 mg/kg, had no significant effect. The suppression of tremor in these models is consistent with previous findings (Handforth et al., 2010). A potential limitation of the harmaline experiment is that NNC 55-0396 was administered at 15 min after harmaline, and no harmaline-alone epoch was measured, raising the question whether NNC 55-0396 is effective on established tremor. However we have previously shown that NNC 55-0396, 12.5 mg/kg, administered after a 20-min harmaline-alone epoch, reduced tremor by approximately 60% in wild-type mice of C57BL/6 background (Handforth et al., 2010). The marked reduction of harmaline tremor by NNC 55-0396 likely contributed to the improvement in the inverted grid task by mice administered harmaline. The ratio of the NNC 55-0396 dose that induces 3/6 to fail the horizontal wire test to that which suppresses half the tremor in these models (300/12.5 = 24) is superior to that found with any of four other T-type calcium channel antagonists we tested (Handforth et al., 2010), and contrasts with the lack of efficacy and poor tolerance of the parent compound, mibefradil.

Mibefradil does not cross the blood–brain-barrier (Ertel and Clozel, 1997), but can influence some neural functions such as pain peripherally (Todorovic et al., 2002). Therefore, the failure of mibefradil to suppress tremor in either tremor model despite doses

and in vitro potency comparable to that of NNC 55-0396, whereas NNC 55-0396 does suppress tremor, suggests that NNC 55-0396's anti-tremor effect cannot be due to peripheral effects, and it likely enters the brain. This notion is supported by observations of anti-seizure efficacy in the pentylenetetrazol model (Handforth et al., 2010). The ability to enter the brain would represent yet another distinguishing feature rendered by the cyclopropane side chain substitution, so that NNC 55-0396 or derivatives with similarly modified side chains may exhibit efficacy for other T-type calcium channel-mediated cerebral processes.

In conclusion, the cyclopropane side chain substitution that differentiates NNC 55-0396 from mibefradil not only confers greater specificity for T-type calcium channels, but reduces in vivo toxicity, modifies the inhibition of microsomal O-demethylation and 6-β-hydroxylation of other drugs, and enables a central nervous system action: tremor suppression. These favorable properties, and the high efficacy to toxicity ratio, suggest that this compound or related derivatives may hold promise for clinical development.

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