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Antagonism of 4-substituted 1,4-dihydropyridine-3,5-dicarboxylates toward voltage-dependent L-type Ca^{2+} channels $Ca_V1.3$ and $Ca_V1.2$

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ABSTRACT

L-type Ca^{2+} channels in mammalian brain neurons have either a $Ca_V1.2$ or $Ca_V1.3$ pore-forming subunit. Recently, it was shown that $Ca_V1.3$ Ca^{2+} channels underlie autonomous pacemaking in adult dopaminergic neurons in the *substantia nigra pars compacta*, and this reliance renders them sensitive to toxins used to create animal models of Parkinson's disease. Antagonism of these channels with the dihydropyridine antihypertensive drug isradipine diminishes the reliance on Ca^{2+} and the sensitivity of these neurons to toxins, pointing to a potential neuroprotective strategy. However, for neuroprotection without an antihypertensive side effect, selective $Ca_V1.3$ channel antagonists are required. In an attempt to identify potent and selective antagonists of $Ca_V1.3$ channels, 124 dihydropyridines (4-substituted-1,4-dihydropyridine-3,5-dicarboxylic diesters) were synthesized. The antagonism of heterologously expressed $Ca_V1.2$ and $Ca_V1.3$ channels was then tested using electrophysiological approaches and the FLIPR Calcium 4 assay. Despite the large diversity in substitution on the dihydropyridine scaffold, the most $Ca_V1.3$ selectivity was only about twofold. These results support a highly similar dihydropyridine binding site at both $Ca_V1.2$ and $Ca_V1.3$ channels and suggests that other classes of compounds need to be identified for $Ca_V1.3$ selectivity.

1. Introduction

Voltage-gated Ca^{2+} channels (Ca_V) are important to a wide range of cellular functions including patterning of repetitive activity, neurotransmitter release, and gene expression.[1] Based on their pore-forming subunit, they are classified into three broad groups: Ca_V1, Ca_V2, and Ca_V3.[2] Members of each group play distinct roles in cellular functions.[2] Because of their diverse and important roles in cellular activity, Ca_V channels are important drug targets. The most commonly targeted channels are members of the Ca_V1 class because of their roles in the cardiovascular system.[3,4] Dihydropyridines are among the most therapeutically useful Ca_V1 Ca^{2+} channel antagonists;[5] these compounds reduce Ca^{2+} influx through $Ca_V1.2$ channels in vascular smooth muscle, diminishing muscle tone and blood pressure.[6] Nifedipine, nimodipine, and isradipine (Fig. 1) are widely prescribed 1,4-dihydropyridine antihypertensive drugs that antagonize $Ca_V1.2$ L-type Ca^{2+} channels.[4,7]

Adult dopaminergic (DA) neurons of the *substantia nigra pars compacta* (SNc) rely on L-type voltage-gated Ca^{2+} channels with a $Ca_V1.3$ pore for maintenance of rhythmic pacemaking.[8] This reliance on $Ca_V1.3$ Ca^{2+} channels increases with age and renders SNc DA neurons vulnerable to stressors thought to contribute to Parkinson's disease. Antagonism of $Ca_V1.3$ Ca^{2+} channels in adult SNc DA neurons by the antihypertensive drug isradipine (Fig. 1), a nonselective $Ca_V1.2/Ca_V1.3$ Ca^{2+} channel blocker, induces reversion of these adult neurons to a juvenile form of pacemaking that does not rely on Ca^{2+} flux, resulting in protection in mouse models of Parkinson's disease.[8] Hence, antagonism of $Ca_V1.3$ Ca^{2+} channels is a potentially neuroprotective strategy in the presymptomatic or early stages of Parkinson's disease. The problem with using antihypertensive drugs for Parkinson's disease, however, is that the optimal dose can produce hypotension. Even if this does not occur, it is known that during the course of Parkinson's disease hypotension is common;[9] administration of an antihypertensive drug would exacerbate this condition. What is needed is a drug that is selective for the $Ca_V1.3$ calcium channel to avoid undesirable cardiovascular

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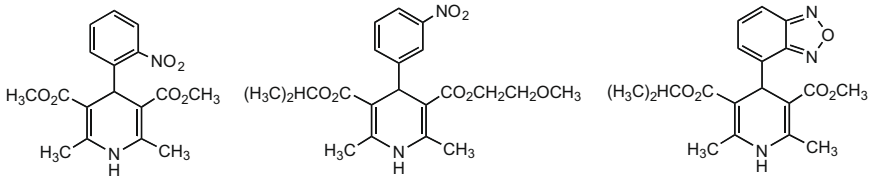


Figure 1. Structures of three $Ca_V1.2$ Ca^{2+} channel blockers.

effects produced by antagonism of the $Ca_V1.2$ calcium channel. However, $Ca_V1.2$ and $Ca_V1.3$ calcium channels are very similar, which makes it very difficult to identify compounds that show $Ca_V1.3$ selectivity.[10]

Although 1,4-dihydropyridines, such as isradipine, are potent antagonists of $Ca_V1.2$ channels, they are less effective antagonists of $Ca_V1.3$ channels underlying pacemaking in SNc DA neurons.[3,11,12] None of the Ca_V1 antagonists in clinical use preferentially antagonize $Ca_V1.3$ channels.[5,10–12] For example, the concentration for half-maximal antagonism (IC_{50}) by nimodipine for $Ca_V1.3$ channels is 20-fold higher than that for $Ca_V1.2$ channels.[11] Here, we describe the synthesis of a series of structurally diverse 4-substituted 1,4-dihydropyridines and their evaluation as antagonists for $Ca_V1.2$ and $Ca_V1.3$ Ca^{2+} channels in an attempt to identify compounds with increased potency and selectivity for $Ca_V1.3$ Ca^{2+} channels. Nifedipine (**1** in Table 1) was chosen as the lead compound because of its simplicity as an active dihydropyridine in these assays, and six of its parts were modified with structurally diverse substituents.

2. Results

2.1. Chemistry

Structural modifications were made at the R^1–R^5 positions of the skeletal structure, as shown in Figure 2; one additional compound was made that contained an *N*-methyl group at the nitrogen in the dihydropyridine ring. Modifications were initially made at the 4-position of 1,4-dihydropyridine ring. Many of the variations at R^1 were made to sample a relatively large chemical space, leaving the other substituents constant.

Starting from various aldehydes, β-keto esters, and 3-aminocrotonates or NH_4OAc, the target compounds, dialkyl (4-aryl or 4-alkyl)-2,6-dialkyl-1,4-dihydropyridine-3,5-dicarboxylates (**1–55**; see Table 1 for structures), were synthesized based on procedures previously described.[13,14] With only a few exceptions, the target compounds were synthesized by treatment of the aldehyde and β-keto ester with excess 3-aminocrotonates or NH_4OAc in one-pot reactions at 80 °C for 1–10 h. Attempts to obtain the trifluoromethyl-containing 1,4-dihydropyridines ($R^4 = R^5 = CF_3$) under the above-mentioned experimental conditions, however, were unsuccessful because of the failure of their intermediates to undergo dehydration. Therefore, the protocol was adjusted by the addition of a few drops of sulfuric acid, which dehydrated the intermediates and produced the final compounds (**53** and **54**).[15] Compound **55** was synthesized by alkylation of **12** with methyl iodide.[16]

To get more structural diversity at other positions of the 1,4-dihydropyridines, fourteen analogues (**56–69**; see Table 2 for structures) were prepared using modified Hantzsch conditions or chemical transformations from compound **65**, as shown in Figure 3. Modifications were made mostly at the 2-position of the 1,4-dihydropyridine ring. Starting from compound **65**,[17] acid hydrolysis afforded an aldehyde, which was then converted to conjugated systems by Wittig reactions (compounds **67–69**). Reduction of the aldehyde generated compound **61**. Acetyl and benzoyl protection of **61** generated **63** and **64**, respectively. Chlorination of **61** provided **62**, and further alkylation gave **66**. Lactonization of **61** afforded **117**. All chemical transformations were based on previously reported literature precedents.[17]

It has been reported that the two ester groups should be differentiated to obtain better interactions with the binding domains of calcium channels.[18] One ester group should be smaller and be able to fit into the active site; the other ester group should be large enough to interact with the lipophilic domain. Therefore, forty compounds (**70–109**; see Table 3 for structures) were prepared with two different ester groups (Fig. 4). The key intermediate **70** was synthesized from compound **72**, by deprotection of the allyl group.[19] Compound **70** was then coupled with various alcohols, by parallel synthesis, on the basis of literature precedence.[20]

Moreover, to gain more information about the influence of structural diversity on potency and selectivity, nine analogues (**110–118**; see Table 4 for structures) also were synthesized. In particular, three difluorophenyl 1,4-dihydropyridines (**110–112**) were prepared under Hantzsch conditions for comparison with the nitrophenyl 1,4-dihydropyridine as a bioisostere. Two 4,4-disubstituted 1,4-dihydropyridines (**115–116**) were prepared to investigate the conformational effects of the 1,4-dihydropyridine ring base.[21] One 4-phenyl-4-pyrane diester (**118**)[22] was synthesized to determine the importance of the dihydropyridine NH toward potency. Compounds **119–120** were made to see the effect of a 4-(2-naphthy) group and **120** contained a ketone in place of one of the esters. The last four compounds (**121–124**) were pairs of two enantiomers to determine if a stereochemical difference was sufficient to differentiate the two calcium channels. Therefore, we have modified all positions of the 1,4-dihydropyridines to obtain a structure–activity relationship for 4-substituted 1,4-dihydropyridines 3,5-dicarboxylates toward antagonism of $Ca_v1.3$ and $Ca_v1.2$ channels.

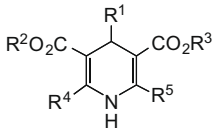
2.2. Biological results

Compounds **1–55**, which have a variety of substituents on the 1,4-dihydropyridine ring, were evaluated by a whole-cell patch-clamp recording assay with $Ca_V1.2$ and $Ca_V1.3$ Ca^{2+} channels (see Table 1). Results are presented as percent inhibition determined at specified concentrations, and the ratio of inhibition of $Ca_V1.3$ to $Ca_V1.2$ also is given. Compounds **56–124** were evaluated for activity with $Ca_V1.3$ to $Ca_V1.2$ Ca^{2+} channels using a FLIPR system and a Calcium 4 assay kit (see Tables 2–4). The IC_{50} values for each compound were determined by dose–response curves with 11 concentration points. The selectivity of antagonism of $Ca_V1.3$ relative to $Ca_V1.2$ was determined by calculating the inverse of the ratios of IC_{50} values with $Ca_V1.2$ to those with $Ca_V1.3$. This was done because of the inverse relationship of IC_{50} and potency. Therefore, ratios greater than 1.0 indicate preferential antagonism of $Ca_V1.3$.

3. Discussion

With nifedipine (**1**) as the lead compound, changes were made to the 4-substituent (R^1), to each of the ester groups (R^2 and R^3), to

Table 1
Antagonism of $Ca_V1.2$ and $Ca_V1.3$ Ca^{2+} channels by compounds **1–55** and the $Ca_v1.3/Ca_v1.2$ ratio of antagonism



No.	R^1	R^2	R^3	R^4	R^5	Concd (nM)	Antag $Ca_v1.3$[a] (%)	Antag $Ca_v1.2$[b] (%)	Selectivity 1.3/1.2[c]
1	2-NO_2-C_6H_4	CH_3	CH_3	CH_3	CH_3	100	46 ± 16	69 ± 16	0.67
2	3-NO_2–C_6H_4	CH_3	CH_3	CH_3	CH_3	100	34 ± 7	62 ± 5	0.55
3	4-NO_2–C_6H_4	CH_3	CH_3	CH_3	CH_3	1000	20 ± 5	35 ± 8	0.57
4	2-NO_2–C_6H_4	C_2H_5	CH_3	CH_3	CH_3	10	26 ± 19	41 ± 7	0.63
5	3-NO_2–C_6H_4	C_2H_5	CH_3	CH_3	CH_3	100	65 ± 3	78 ± 8	0.83
6	4-NO_2–C_6H_4	C_2H_5	CH_3	CH_3	CH_3	1000	35 ± 9	48 ± 14	0.73
7	3-NO_2–C_6H_4	*i*-Propyl	CH_3	CH_3	CH_3	200	89 ± 11	96 ± 1	0.93
8	3-NO_2–C_6H_4	*i*-Butyl	CH_3	CH_3	CH_3	200	90 ± 4	94 ± 1	0.96
9	3-NO_2–C_6H_4	*t*-Butyl	CH_3	CH_3	CH_3	200	85 ± 10	91 ± 1	0.93
10	C_6H_5	C_2H_5	C_2H_5	CH_3	CH_3	100	41 ± 8	48 ± 8	0.85
11	4-NO_2–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	1000	40 ± 6	48 ± 12	0.83
12	3-NO_2–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	10	43 ± 10	32 ± 8	1.34
13	3-F–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	100	48 ± 21	64 ± 22	0.75
14	3-Cl–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	100	59 ± 16	58 ± 11	1.02
15	3-Br–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	100	80 ± 8	82 ± 10	0.98
16	2-I–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	200	95 ± 1	96 ± 4	0.99
17	3-CF_3–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	200	84 ± 3	85 ± 4	0.99
18	3-$CH_3OC_6H_4$	C_2H_5	C_2H_5	CH_3	CH_3	200	44 ± 8	69 ± 7	0.64
19	4-(4′-Br–C_6H_3–CH_2O)–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	1000	28 ± 3	38 ± 15	0.74
20	3-NO_2–4-Cl–C_6H_3	C_2H_5	C_2H_5	CH_3	CH_3	200	66 ± 15	86 ± 9	0.77
21	3-NO_2–6-Cl–C_6H_3	C_2H_5	C_2H_5	CH_3	CH_3	200	89 ± 6	97 ± 1	0.92
22	3,5-Di-Br–C_6H_3	C_2H_5	C_2H_5	CH_3	CH_3	200	72 ± 14	74 ± 8	0.98
23	3,5-Di-CF_3–C_6H_3	C_2H_5	C_2H_5	CH_3	CH_3	200	38 ± 6	38 ± 17	1.00
24	2-Styryl	C_2H_5	C_2H_5	CH_3	CH_3	1000	31 ± 5	30 ± 3	1.03
25	2-C_6H_5–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	200	65 ± 11	73 ± 12	0.89
26	3-Pyridyl	C_2H_5	C_2H_5	CH_3	CH_3	200	14 ± 4	25 ± 4	0.56
27	4-Pyridyl	C_2H_5	C_2H_5	CH_3	CH_3	200	15 ± 2	27 ± 2	0.56
28	2-Furyl	C_2H_5	C_2H_5	CH_3	CH_3	200	48 ± 2	72 ± 4	0.67
29	3-Furyl	C_2H_5	C_2H_5	CH_3	CH_3	200	32 ± 4	56 ± 6	0.57
30	2-Thienyl	C_2H_5	C_2H_5	CH_3	CH_3	200	57 ± 9	65 ± 4	0.87
31	3-Thienyl	C_2H_5	C_2H_5	CH_3	CH_3	200	50 ± 5	54 ± 4	0.93
32	5-Nitro-2-furyl	C_2H_5	C_2H_5	CH_3	CH_3	200	41 ± 16	43 ± 10	0.95
33	5-Phenyl-2-thienyl	C_2H_5	C_2H_5	CH_3	CH_3	1000	42 ± 10	41 ± 9	1.02
34	5-(2'-Thienyl)-2-thienyl	C_2H_5	C_2H_5	CH_3	CH_3	1000	35 ± 2	35 ± 6	1.00
35	5-(4'-Methoxy-phenyl)-isoxazolyl	C_2H_5	C_2H_5	CH_3	CH_3	1000	16 ± 5	35 ± 7	0.47
36	2-Naphthyl	C_2H_5	C_2H_5	CH_3	CH_3	200	12 ± 3	16 ± 1	0.75
37	3-Benzothienyl	C_2H_5	C_2H_5	CH_3	CH_3	200	57 ± 11	80 ± 7	0.70
38	5-Bromo-2-indolyl	C_2H_5	C_2H_5	CH_3	CH_3	500	43 ± 21	42 ± 3	1.03
39	2-Chloro-8-methyl-3-quinolyl	C_2H_5	C_2H_5	CH_3	CH_3	1000	9 ± 3	12 ± 6	0.75
40	*n*-C_3H_7	C_2H_5	C_2H_5	CH_3	CH_3	200	36 ± 18	42 ± 9	0.86
41	Cyclohexyl	C_2H_5	C_2H_5	CH_3	CH_3	200	21 ± 8	26 ± 11	0.81
42	1-Phenyl-1-ethyl	C_2H_5	C_2H_5	CH_3	CH_3	1000	20 ± 3	28 ± 11	0.72
43	3-NO_2–C_6H_4	*i*-Propyl	*i*-Propyl	CH_3	CH_3	200	66 ± 9	77 ± 14	0.86
44	3-NO_2–C_6H_4	*i*-Butyl	*i*-Butyl	CH_3	CH_3	200	59 ± 1	52 ± 28	1.14
45	3-NO_2–C_6H_4	*t*-Butyl	*t*-Butyl	CH_3	CH_3	100	22 ± 4	32 ± 13	0.69
46	4-NO_2–C_6H_4	*t*-Butyl	*t*-Butyl	CH_3	CH_3	1000	21 ± 2	36 ± 8	0.58
47	3-NO_2–C_6H_4	C_2H_5	CH_3	C_2H_5	CH_3	100	55 ± 11	76 ± 8	0.72
48	4-NO_2–C_6H_4	C_2H_5	CH_3	C_2H_5	CH_3	1000	30 ± 3	28 ± 6	1.07
49	3-NO_2–C_6H_4	C_2H_5	C_2H_5	C_2H_5	CH_3	200	88 ± 3	93 ± 5	0.95
50	3-NO_2–C_6H_4	C_2H_5	C_2H_5	C_2H_5	C_2H_5	100	37 ± 9	52 ± 6	0.71
51	4-NO_2–C_6H_4	C_2H_5	C_2H_5	C_2H_5	C_2H_5	1000	60 ± 8	58 ± 13	1.03
52	3-NO_2–C_6H_4	C_2H_5	C_2H_5	*n*-C_3H_7	*n*-C_3H_7	100	27 ± 11	25 ± 12	1.07
53	3-NO_2–C_6H_4	C_2H_5	C_2H_5	CF_3	CF_3	200	38 ± 9	34 ± 6	1.11
54	3-CF_3–C_6H_4	C_2H_5	C_2H_5	CF_3	CF_3	200	67 ± 8	61 ± 10	1.10
55[d]	3-NO_2–C_6H_4	C_2H_5	C_2H_5	CH_3	CH_3	1000	16 ± 10	35 ± 7	0.46

The compounds are organized first by alkyl group of the ester (R^2 and R^3), then by alkyl substituents R^4 and R^5.
[a] Percent antagonism of $Ca_V1.3$.
[b] Percent antagonism of $Ca_V1.2$.
[c] Ratio of percent antagonism for $Ca_V1.2/Ca_V1.3$ (selectivity of antagonism of $Ca_V1.3$).
[d] Compound **55** is the product of alkylation of **12** with methyl iodide (N-methylation of **12**).

each of the alkyl groups at the 2- and 6-positions (R^4 and R^5), to the dihydropyridine nitrogen, and to the 4-position. The goal was to explore structurally diverse substituents at each position to gain insight into how to increase potency and selectivity toward $Ca_V1.3$ Ca^{2+} channels relative to $Ca_V1.2$ channels. Ideally, all of the assays would have been carried out at the same concentrations, but because of the difficulty of the whole-cell patch-clamp assay procedure, multiple concentrations were not evaluated for compounds **1–55**. Therefore, many of the conclusions are based on extrapolated estimates at tested concentrations. Nevertheless,

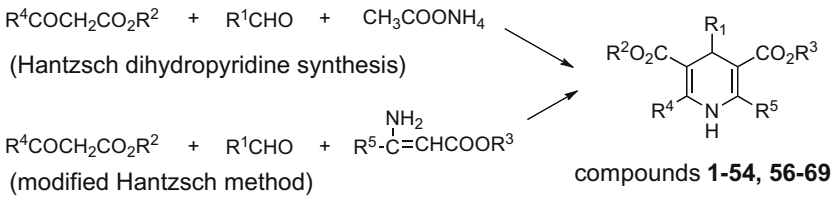
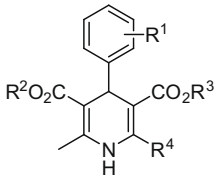
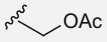
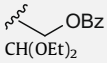
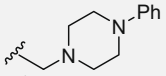
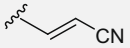
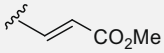
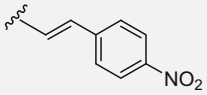


Figure 2. Hantzsch and modified conditions for the synthesis of target compounds **1**–**54**.

Table 2
IC_{50} values and the selectivity of antagonism of $Ca_V1.2$ and $Ca_V1.3$ Ca^{2+} channels by compounds **56**–**69**[a]



No.	R^1	R^2	R^3	R^4	IC_{50} (nM) $Ca_v1.3$	IC_{50} (nM) $Ca_v1.2$	Selectivity 1.3/1.2	Synthesis method
56	3-NO_2	C_2H_5	C_2H_5	*n*-Propyl	105	18	0.17	A
57	3-NO_2	C_2H_5	C_2H_5	*n*-Butyl	20	20	1.00	A
58	2-NO_2	C_2H_5	CH_3	CH_2OMe	790	1760	2.23	A
59	3-NO_2	C_2H_5	CH_3	CH_2OMe	96	14	0.15	A
60	3-NO_2	CH_3	CH_3	CH_2OMe	350	115	0.33	A
61	3-NO_2	C_2H_5	C_2H_5	CH_2OH	45	49	1.09	B
62	3-NO_2	C_2H_5	C_2H_5	CH_2Cl	53	10	0.19	B
63	3-NO_2	C_2H_5	C_2H_5		37	10	0.27	B
64	3-NO_2	C_2H_5	C_2H_5		170	210	1.24	B
65	3-NO_2	C_2H_5	C_2H_5	$CH(OEt)_2$	615	460	0.75	B
66	3-NO_2	C_2H_5	C_2H_5		610	630	1.03	B
67	3-NO_2	C_2H_5	C_2H_5		45	22	0.48	B
68[b]	3-NO_2	C_2H_5	C_2H_5		213	313	1.47	B
69	3-NO_2	C_2H_5	C_2H_5		2055	1940	0.94	B

The compounds are organized first by the position (R^1) of the nitro group on aromatic ring, and then alkyl groups in the esters (R^2 and R^3), then by alkyl substituents (R^4) at 2-position of the dihydropyridine ring.

[a] FLIPR calcium 4 assay kits were used. The percentage error [standard derivation/average] of the signal of $Ca_v1.2$ cells is 6.4% and that for $Ca_v1.3$ cells is 4.1% in the dose–response curves.

[b] A mixture of *trans*/*cis* (7/3) isomers. Synthesis Method A: prepared under Hantzsch or modified conditions; Synthesis Method B: prepared using the methods showed in Figure 3.

there is a consistent pattern of antagonism, suggesting that this is not a major limitation to the interpretation of the results. A high-throughput screen also was developed, and compounds **56–124** were assayed using a FLIPR from Molecular Devices, an industry-renowned instrument for monitoring ion channels.

As is apparent from compounds **1–3** (Table 1), moving the nitro group from the 2-(nifedipine) to 3- to 4-positions on the phenyl leads to a loss of potency and selectivity toward $Ca_V1.3$; para-substitution is strongly disfavored. The change of one methyl ester to an ethyl ester (**4**) led to a major increase in potency. This suggested that differentiating the two esters was the next step to pursue, which is presented in Table 3. Again, potency was a function of the placement of the nitro group on the phenyl ring (**4–6**). When the nitro group was at the 3-position, there was little, if any, difference in potency by varying one ester group from ethyl (**5**), to isopropyl (**7**), to isobutyl (**8**), or to *t*-butyl (**9**).

In the largest family of compounds investigated, two ethyl ester groups ($R^2 = R^3 = Et$) were held constant while other groups were varied. The unsubstituted phenyl analogue (**10**) was comparable to nifedipine (**1**) in $Ca_V1.3$ potency and again, there was a large change in potency for the 3-nitro analogue (**12**) compared to the 4-nitro analogue (**11**); compound **12** was quite potent (43% inhibition of $Ca_V1.3$ at 10 nM concentration) and had a selectivity for $Ca_V1.3$ channels of 1.34 (Table 1). Replacement of the 3-nitro group with 3-halogens (**13–15**) and 3-trifluoromethyl (**17**) showed a trend in the order Br–CF_3 > Cl > F in potency; the 2-I analogue (**16**) was slightly more potent than the 3-Br analogue. A strong electron-donating group at the 3-position (**18**) and especially at the 4-position (**19**) was detrimental to both potency and selectivity. Weaker electron-donating groups, such as vinyl (**24**), phenyl (**25**), or naphthyl (**36**) at the 2-position also were less potent relative to the 2-iodo analogue (**16**). Adding a second electron-withdrawing group (**20–23**) lowered the potency relative to a single electron-withdrawing group at the 3-position. Heteroaromatics (**26–31**) and substituted heteroaromatics (**32–35**, **37–39**) were much less potent than the phenyl series. Propyl (**40**), cyclohexyl

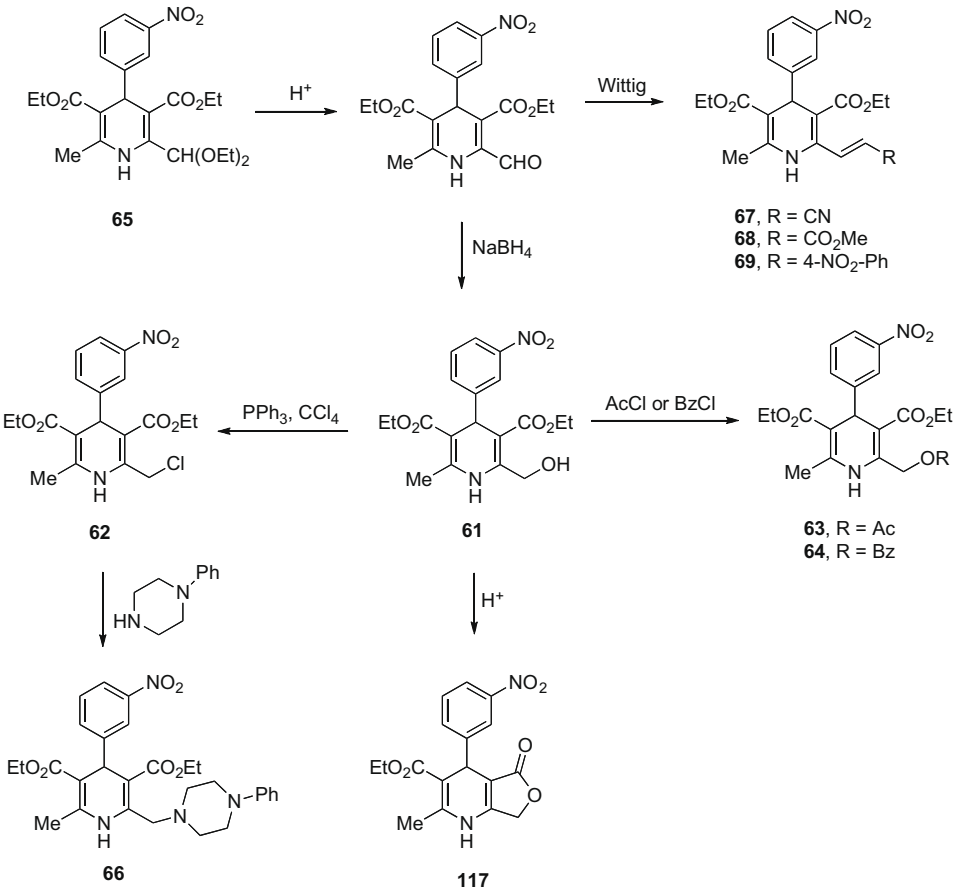


Figure 3. Synthesis of target compounds (**65**–**69** and **117**).[17]

(**41**), and 1-phenylethyl (**42**) also were much less potent than the heteroaromatic and substituted heteroaromatic analogues.

Bulkiness by the ester functionality appears to be important to potency but not selectivity, as evidenced by the trend that IC_{50} for isopropyl > isobutyl > *tert*-butyl esters for the 3-nitrophenyl series (**43–45**). It suggested again that the larger alkyl group in the esters might have better interaction with the channel and gain more potency, which is generally supported by compounds in Table 3. As observed with the other esters, substitution at the *para*-position of the 4-phenyl group gave a much less potent analogue (**46**).

Changing the two methyl groups at the 2- and 6-positions of the dihydropyridine ring (R^4 or R^5) (**12**) to ethyl decreased the potency of the 3-NO_2 compound (**50**), but slightly increased the potency of the 4-NO_2 analogue (**11** vs **51**). When $R^4 = R^5 = n$-propyl (**52**), the potency decreased further. Substitution of R^4 and R^5 by CF_3 (**53** and **54**), led to decreased potency as well. Steric effects at the 2- and 6-positions appear to be important to potency. Methylation of the dihydropyridine nitrogen (**55**) resulted in a large decrease of activity relative to the parent compound (**2**); the corresponding pyran (**118**) also showed low activity.

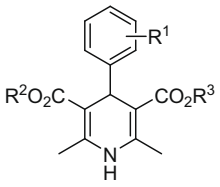
To broaden the scope of diversity, the two substituents at the 2- and 6-positions of the 1,4-dihydropyridine ring were amplified (Table 2). The compounds investigated retained one of the methyl groups and varied the other. Methoxymethyl analogues (**58–60**) showed decreased potency and selectivity. Slight modifications, such as hydroxyl (**61**), chloromethyl (**62**), and acetate (**63**), appear to retain the potency but not selectivity. Even among the analogues that contain hydrogen bond acceptors or other heteroatoms, steric hindrance (**64–66**) had the greatest impact on lowering potency. Changing to conjugated systems (**67–69**) confirmed that the larger the substituted group, the larger the reduction in potency. Modification at this position resulted in slightly better selectivity but not potency.

Subsequent compounds were designed to investigate what ester groups would give better potency and selectivity (Table 3). Carboxylic acid substitution at R^3 (**70** vs **2**) resulted in complete loss of potency. High potency (IC_{50} = 10–15 nM) was observed by changing the R^3 ester to an allyl group (**71** and **72**). A similar result was obtained in the ethyl ester series (**73** vs **5**), again supporting favorable properties with different ester groups. Slight modifications in the alkyl chain (**74–76**) still retained potency. An alkyl chain containing hydrophobic heteroatoms (**78**, **79**) appears to retain the potency (IC_{50} = ~20 nM) and increase the selectivity, but with hydrophilic groups, as in the case of methoxyethyl (**77**) and 2-cyanoethyl (**80**), the potency and selectivity decreased. An α,β-unsaturated ester (**83**) retained potency but not the trifluoroacetamide analogue (**84**).

The 3-nitrophenyl analogue with a methyl and benzyl ester (**85**) was potent and slightly $Ca_V1.3$ selective. Changing the benzyl ester to a formyl group and the methyl ester to ethyl ester (**86**), resulted in very poor potency for both calcium channels (micromolar), but selectivity was >2 in favor of $Ca_V1.3$. The compound with cyclohexenyl and methyl esters (**87**) was slightly more potent, but with lower selectivity, as the benzyl ester (**85**); however, **87** is a mixture of four isomers. Nitration (**88–89**), halogenation (**90–92**), methoxylation (**93–94**) and conversion to a benz-3,4-dioxole (**95**) of the benzyl ester ring lowered both potency and selectivity relative to the benzyl ester (**85**).

Heteroaromatic analogues, such as furfuryl (**96**) and 2-thiophenylmethyl (**97**) showed about the same potency (IC_{50} =

Table 3
IC_{50} values and the selectivity of antagonism of $Ca_V1.2$ and $Ca_V1.3$ Ca^{2+} channels by compounds **70**–**109**[a]



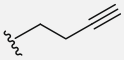
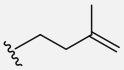
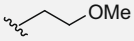
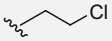
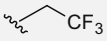
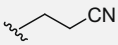
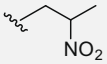
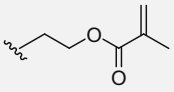
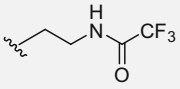
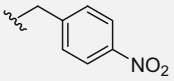
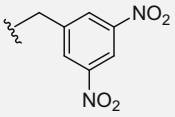
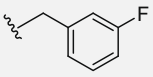
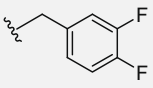
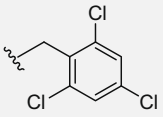
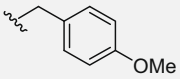
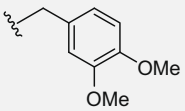
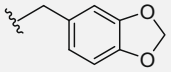
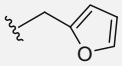
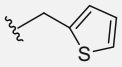
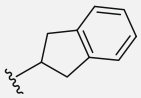
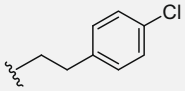
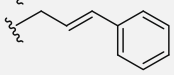
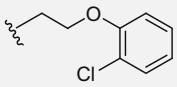
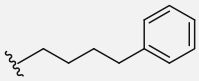
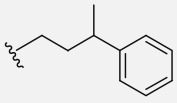
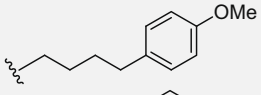
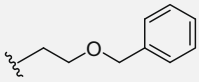
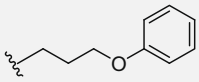
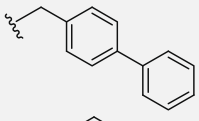
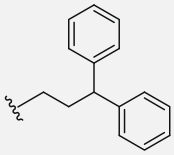
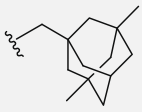
No.	R^1	R^2	R^3	IC_{50} (nM) $Ca_v1.3$	IC_{50} (nM) $Ca_v1.2$	Selectivity (1.3/1.2)	Method
70	3-NO_2	CH_3	H	930	900	0.97	B
71	2-NO_2	CH_3	Allyl	10	8	0.80	A
72	3-NO_2	CH_3	Allyl	15	15	1.00	A
73	3-NO_2	C_2H_5	Allyl	15	25	1.67	A
74	3-NO_2	CH_3		35	31	0.88	B
75	3-NO_2	CH_3		47	23	0.48	B
76	3-NO_2	CH_3		21	28	1.34	B
77	3-NO_2	C_2H_5		82	16	0.20	A
78	3-NO_2	CH_3		22	26	1.18	B
79	3-NO_2	CH_3		21	16	0.78	B
80	3-NO_2	CH_3		78	26	0.33	B
81	3-NO_2	CH_3		111	108	0.97	B
82	3-NO_2	C_2H_5	*n*-Pentyl	130	49	0.38	A
83	3-NO_2	C_2H_5		10	10	1.00	A
84	3-NO_2	CH_3		550	410	0.75	B
85	3-NO_2	CH_3	Bn	15	25	1.67	A
86	3-NO_2	C_2H_5	CHO	13,900	33,300	2.40	A
87	3-NO_2	CH_3		10	10	1.00	B
88	3-NO_2	C_2H_5		140	130	0.93	B
89	3-NO_2	CH_3		40	55	1.37	B
90	3-NO_2	CH_3		28	22	0.79	B
91	3-NO_2	CH_3		20	7	0.38	B
92	3-NO_2	CH_3		34	10	0.29	B
93	3-NO_2	CH_3		79	51	0.65	B
94	3-NO_2	CH_3		117	112	0.96	B

Table 3 (*continued*)

No.	R^1	R^2	R^3	IC_{50} (nM) $Ca_v1.3$	IC_{50} (nM) $Ca_v1.2$	Selectivity (1.3/1.2)	Method
95	3-NO_2	CH_3		22	13	0.57	B
96	3-NO_2	CH_3		15	20	1.33	B
97	3-NO_2	CH_3		23	31	1.34	B
98	3-NO_2	CH_3		43	72	1.66	B
99	3-NO_2	CH_3		39	8	0.21	B
100	3-NO_2	CH_3		37	37	1.00	B
101	3-NO_2	CH_3		46	19	0.42	B
102	3-NO_2	CH_3		38	10	0.27	B
103	3-NO_2	CH_3		11	6	0.57	B
104	3-NO_2	CH_3		38	17	0.43	B
105	3-NO_2	CH_3		12	9	0.74	B
106	3-NO_2	CH_3		11	9	0.82	B
107	3-NO_2	CH_3		74	69	0.94	B
108	3-NO_2	CH_3		270	100	0.37	B
109	3-NO_2	CH_3		5070	685	0.14	B

The compounds are organized first by the position (R^1) of the nitro group on the aromatic ring, and then alkyl groups in the esters (R^2 and R^3).

[a] FLIPR calcium 4 assay kits were used. The percentage error [standard derivation/average] of the signal of $Ca_v1.2$ cells is 6.4% and that for $Ca_v1.3$ cells is 4.1% in the dose–response curves. Method A: prepared under Hantzsch or modified conditions; Method B: prepared using the methods showed in Figure 4.

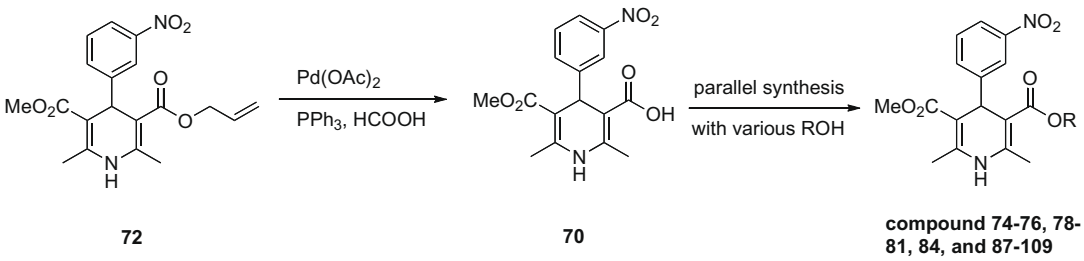


Figure 4. Parallel synthesis of various alkyl groups at one of the dihydropyridine esters.

Table 4
IC_{50} values and the selectivity of antagonism of $Ca_V1.2$ and $Ca_V1.3$ Ca^{2+} channels by compounds **110–124**[a]

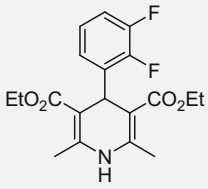
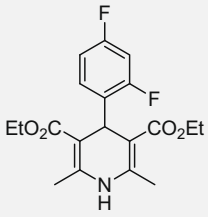
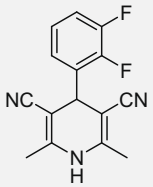
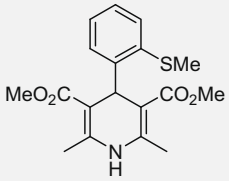
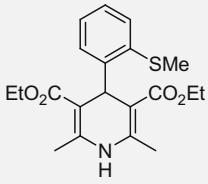
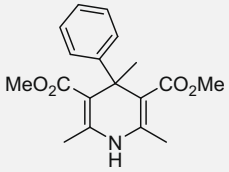
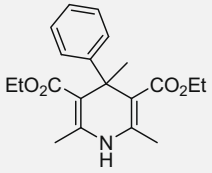
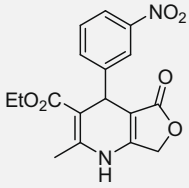
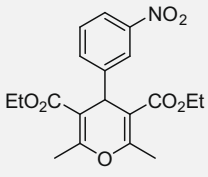
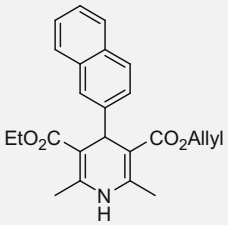
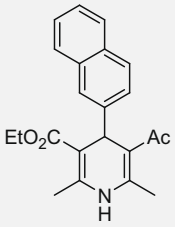
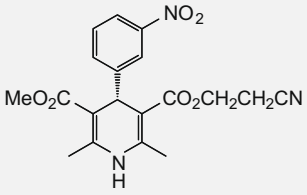
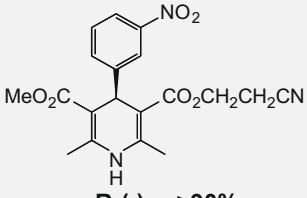
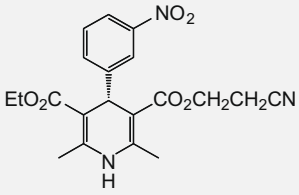
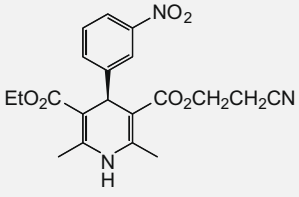
No.	Structures	IC_{50} (nM) $Ca_v1.3$	IC_{50} (nM) $Ca_v1.2$	Selectivity (1.3/1.2)	Method
110		10	15	1.50	A
111		58	20	0.34	A
112		1150	1610	1.40	A
113		35	8	0.23	A
114		15	8	0.53	A
115		2310	1470	0.64	Ref. 21
116		2140	630	0.29	Ref. 21
117		>4000	>4000	~1	See Figure 3
118		1925	>4000	>2	Ref. 22

Table 4 (*continued*)

No.	Structures	IC_{50} (nM) $Ca_v1.3$	IC_{50} (nM) $Ca_v1.2$	Selectivity (1.3/1.2)	Method
119		2161	1306	0.60	A
120		2315	2217	0.96	A
121	 **S-(+)- >90% ee**	589	143	0.24	B
122	 **R-(-)- >90% ee**	399	262	0.66	B
123	 **S-(+)- >90% ee**	211	51	0.24	B
124	 **R-(-)- >90% ee**	137	50	0.37	C

[a] FLIPR calcium 4 assay kits were used. The percentage error [standard derivation/average] of the signal of $Ca_v1.2$ cells is 6.4% and that for $Ca_v1.3$ cells is 4.1% in the dose–response curves. Method A: prepared under Hantzsch or modified conditions. Method B: prepared by the resolution of the racemate by chiral HPLC on a Chiralpak AS column (90% hexane, 5% *i*PrOH, 5% EtOH, 0.8 mL/min); Method C: synthesized by the esterification of optically active 1,4-dihydropyridine monoethyl esters.[23]

~20 nM) and selectivity (ratio ~1.3), as the benzyl analogue (**85**). Other arylalkyl-substituted esters in lieu of the benzyl ester (**98–102**, **104**) were about half to a third as potent as the benzyl ester analogue (**85**) with lower selectivity. When a branch (**103**) or an oxygen (**105**, **106**) was added to the arylalkyl chain, the potency increased to as good or better than **85**, but with lower selectivity; however, **103** also is a mixture of four isomers. Addition of a phenyl group to the benzyl ester (**107**) or to the phenylpropyl ester (**108**) decreased potency and selectivity. The bulky analogue, dimethyl adamantane-1-methyl (**109**) showed a complete loss of potency and selectivity.

Substitution of the nitrophenyl by difluorophenyl as a potential bioisostere, produced **110**, which had comparable potency and slightly better selectivity than the 3-nitrophenyl analogue (**12**). Movement of one of the fluorines to the 4-position (**111**) lowered both potency and selectivity. Conversion of the diethyl esters of

110 to cyano groups (**112**) dropped the potency by two orders of magnitude, but retained the slight selectivity. Methylthio analogues **113**, **114** were potent but not selective for $Ca_V1.3$. They were precursors in the synthesis of 4,4-disubstituted analogues **115**, **116**, respectively, which exhibited both poor potency and selectivity, presumably because of a different conformation of the dihydropyridine ring.[21] Conversion of one of the esters to a lactone (**117**) resulted in loss of potency and selectivity.

All of the compounds with two different ester groups or R_4 and R_5 groups are chiral molecules. To test whether one enantiomer was more potent and selective than the other, two compounds were resolved to greater than 90% ee (**121–124**). In both cases, the (*R*)-(−)-isomers were about 50% more potent and slightly more selective for $Ca_V1.3$ than the (*S*)-(+)-isomers, although neither was selective for $Ca_V1.3$.

4. Conclusions

Recently, a new target for potential antiparkinsonian drug therapy was reported.[8] Antagonism of the $Ca_V1.3$ Ca^{2+} channel by a dihydropyridine antihypertensive drug causes a reversion of adult neurons to a juvenile form of pacemaking, resulting in protection in mouse models from Parkinson's disease. To the best of our knowledge, there are no compounds reported that antagonize $Ca_V1.3$ greater than the closely related Ca^{2+} channel, $Ca_V1.2$. We have synthesized a library of 124 chemically diverse 4-substituted 1,4-dihydropyridines in search of structures that are potent antagonists of $Ca_V1.3$ with minimal antagonism of $Ca_V1.2$. A summary of our SAR of dihydropyridines toward Cav1.3 is showed in Figure 5. We have only been able to prepare dihydropyridines that show a modest preference for antagonism of $Ca_V1.3$ over $Ca_V1.2$. In general, the activity of the 4-substituted 1,4-dihydropyridines was as follows: substituted phenyl > thienyl > furyl > pyridyl > naphthyl > alkyl (cyclic alkyl) with substitution on the phenyl ring at the 2-position the most potent and substitution at the 4-position the least potent. Loss of activity and selectivity was observed when the hydrogen at the dihydropyridine nitrogen was replaced by methyl (**55**) or the NH was replaced by O (**118**). Although the introduction of a fluorine or trifluoromethyl group into organic molecules frequently results in compounds that display more potent activity than the parent,[24,25] in the present case, potency decreased when the 2- and 6-methyl groups were substituted by trifluoromethyl. Thirteen of the analogues exhibited IC_{50} values for $Ca_V1.3$ of $\leqslant$15 nM, which is quite potent; however, of those, the largest selectivity for $Ca_V1.3$ was 1.67-fold (**73** and **85**). The two most $Ca_V1.3$ selective analogues (**58** and **86)** were only 2.2- and 2.4-fold selective and both had micromolar potencies. These results support previous results[10] that the dihydropyridine binding sites of $Ca_V1.3$ and $Ca_V1.2$ channels are very similar, but not identical. Although highly potent $Ca_V1.3$ channel antagonists were identified, and an increase in $Ca_V1.3$ selectivity relative to nifedipine (100 and 0.67 nM selectivity) was accomplished, high selectivity for $Ca_V1.3$ with dihydropyridines was not attainable and seems unlikely. Consequently, we are currently carrying out a high-throughput screen to identify new scaffolds that act as highly selective $Ca_V1.3$ antagonists.

5. Experimental section

5.1. General methods

All starting reagents were purchased from Aldrich (Milwaukee, WI). Nifedipine was bought from Tocris Bioscience (Ellisville, MO). All melting points were taken on a Buchi B540 apparatus in open glass capillary tubes and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Inova 500-MHz or Varian Mercury 400-MHz NMR spectrometer. ^{19}F NMR (376.5 MHz) spectra were recorded on a Varian Mercury 400-MHz NMR spectrometer using $CFCl_3$ as the external standard. Chemical shifts are reported as values in parts per million downfield from TMS (δ = 0.0) as the internal standard in $CDCl_3$. Electrospray mass spectra were obtained on a Micromass Quattro II spectrometer. Thin-layer chromatography was carried out on E. Merck precoated Silica Gel 60 F254 plates. E. Merck Silica Gel 60 (230–400 mesh) was used for flash column chromatography, and spots were visualized with ultraviolet (UV) light. The purity of compounds was determined by elemental analysis (EA) from Atlantic Microlab, Inc.

5.2. General procedure for the synthesis of 4-aryl (alkyl)-1,4-dihydro-2,6-dialkyl-3,5-pyridinedicarboxylates (Hantzsch procedure)

A mixture of the aldehyde (3.3 mmol), β-ketoester (6.6 mmol), NH_4OAc (4.95 mmol) or a mixture of the aldehyde (3.3 mmol), β-ketoester (3.3 mmol), β-aminocrotonate (3.3 mmol) was stirred (or dissolved in 3 mL of ethanol if the aldehyde had a high melting point) at 80 °C for an appropriate time (generally 1–10 h). After completion of the reaction, as indicated by TLC, it was poured into

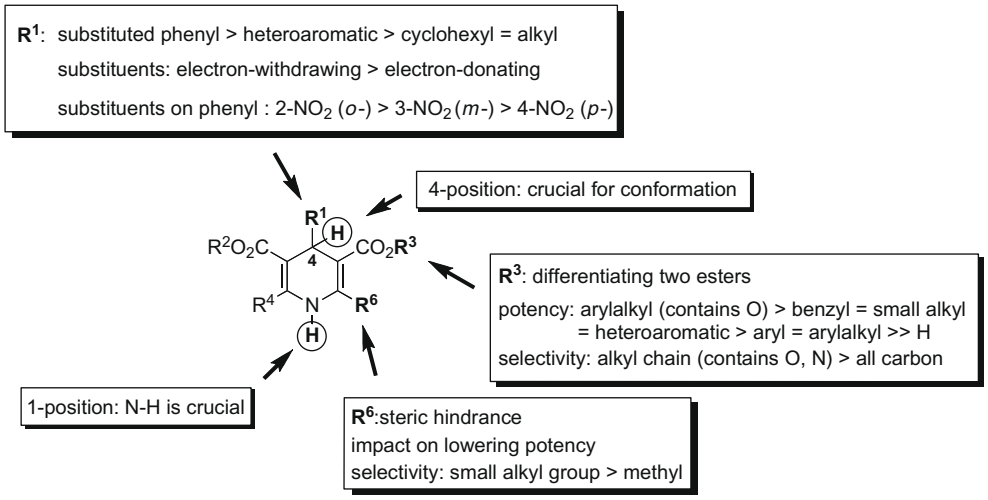


Figure 5. Summary of SAR of Dihydropyridines toward $Ca_V1.3$.

ice cold water and extracted with ethyl acetate (3 × 5 mL). The organic layer was washed with sodium thiosulfate, with water, dried, and concentrated in vacuo. The crude products were purified by column chromatography using silica gel (60–120 mesh) and eluted with ethyl acetate/hexane (1:3) to afford 1,4-dihydropyridines in 41–98% yields.

5.2.1. Methyl ethyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (4)

Yellow solid; mp 78–81 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.16 (t, J = 7.0 Hz, 3H, CH_3), 2.28 (s, 6H, $2CH_3$), 3.63 (s, 3H, OCH_3), 4.01–4.14 (m, 2H, OCH_2), 5.72 (s, 1H, CH), 5.79 (s, 1H, NH), 7.24–7.71 (m, 4H, C_6H_4); Anal. Calcd for $C_{18}H_{20}N_2O_6$ (360.13): C, 59.99; H, 5.59; N, 7.77. Found: C, 60.11; H, 5.40; N, 7.77.

5.2.2. Diethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (12)

Yellow solid; mp 158–160 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.22 (t, J = 7.2 Hz, 6H, $2CH_3$), 2.37 (s, 6H, $2CH_3$), 4.03–4.13 (m, 4H, $2OCH_2$), 5.09 (s, 1H, CH), 5.74 (s, 1H, NH), 7.36–8.13 (m, 4H, C_6H_4); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 14.5, 19.8, 40.2, 60.1, 103.5, 121.6, 123.2, 128.8, 134.8, 145.0, 148.1, 150.2, 167.4; HRMS (ES): m/z calcd for $C_{19}H_{21}N_2O_6$ ($M-H^+$): 373.1400, found 373.1396; Anal. Calcd for $C_{19}H_{22}N_2O_6$ (374.14): C, 60.95; H, 5.92; N, 7.48. Found: C, 60.98; H, 5.98; N, 7.36.

5.2.3. Diethyl 2-methyl-6-*n*-propyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (56)

Yellow solid; mp 97–98 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.00 (t, J = 7.0 Hz, 3H, CH_3), 1.20–1.25 (two t overlapped at 1.22 and 1.24, J = 7.0 Hz, 6H, $OCH_2CH_3 \times 2$), 1.58–1.72 (m, 2H, CH_2), 2.36 (s, 3H, CH_3). 2.65–2.75 (m, 2H, CH_2), 4.05–4.15 (m, 4H, $OCH_2CH_3 \times 2$), 5.11 (s, 1H, CHAr), 5.95 (br s, 1H, NH), 7.38 (t, J = 8.0 Hz, 1H. Ar-H), 7.65 (d, J = 8.0 Hz, 1H, Ar-H), 8.00 (d, J = 8.0 Hz, 1H, Ar-H), 8.14 (s, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.0, 14.2, 14.3, 19.6, 2.0, 34.6, 39.9, 60.0, 102.9, 103.1, 121.3, 123.1, 128.6, 134.5, 144.9, 148.1, 149.2, 150.0, 166.8, 167.2; HRMS (ESI): m/z calcd for $C_{21}H_{27}N_2O_6$ ($M+H^+$): 403.1864; found: 403.1874; Anal. Calcd for $C_{21}H_{26}N_2O_6$ (402.17): C, 62.67; H, 6.51; N, 6.96. Found: C, 62.61; H, 6.64; N, 6.88.

5.2.4. Ethyl *n*-pentyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (82)

Yellow viscous oil; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.84–0.90 (m, 3H, $OCH_2CH_2CH_2CH_2CH_3$), 1.19–1.32 (m, overlapped with t at 1.24, J = 8.0 Hz, 7H, $CH_2 \times 2$ and CH_3), 1.56–1.62 (m, 2H, $OCH_2CH_2CH_2CH_2CH_3$), 2.36 (s, 3H, CH_3), 2.37 (s, 3H, CH_3), 3.98–4.10 (m, 4H, $OCH_2 \times 2$), 5.10 (s, 1H, CHAr), 6.04 (br s, 1H, NH), 7.38 (t, J = 8.0 Hz, 1H, Ar-H), 7.65 (d, J = 8.0 Hz, 1H, Ar-H), 8.01 (d, J = 8.0 Hz, 1H, Ar-H), 8.13 (s, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.0, 14.2, 19.6, 22.3, 28.2, 28.3, 39.9, 60.0, 64.2, 103.2, 103.3, 121.3, 123.0, 128.6, 134.5, 144.9, 145.0, 148.1, 149.9, 167.1, 167.2; HRMS (ESI): m/z calcd for $C_{22}H_{29}N_2O_6$ ($M+H^+$): 417.2026; found: 417.2026; Anal. Calcd for $C_{22}H_{28}N_2O_6$ (416.19): C, 63.45; H, 6.78; N, 6.73. Found: C, 63.18; H, 6.75; N, 6.65.

5.2.5. Dimethyl 2,6-dimethyl-4-(2-methylthiophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (113)

Compound 113: White solid; mp 168–170 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.31 (s, 6H, $CH_3 \times 2$), 2.49 (s, 3H, SCH_3), 3.62 (s, 6H, $OCH_3 \times 2$), 5.46 (s, 1H, CHAr), 5.66 (br s, 1H, NH), 7.06 (t, J = 8.0 Hz, 1H, Ar-H), 7.10 (t, J = 8.0 Hz, 1H, Ar-H), 7.26 (d, J = 8.0 Hz, 1H, Ar-H), 7.32 (d, J = 8.0 Hz, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 18.10, 19.6 × 2, 37.1, 50.9 × 2, 104.6 × 2, 126.0, 126.8, 128.1, 129.9, 136.6, 143.7×, 147.8, 168.1 × 2; HRMS (ESI): m/z calcd for $C_{18}H_{22}NO_4S$ ($M+H^+$): 348.1270; found: 348.1267; Anal. Calcd for $C_{18}H_{21}NO_4S$ (347.11): C, 62.23; H, 6.09; N, 4.03. Found: C, 62.06; H, 5.95; N, 3.89.

5.3. General procedure for the synthesis of various 4-aryl (alkyl)-1,4-dihydro-2,6-dialkyl-3,5-pyridinedicarboxylates (parallel synthesis in Table 3).[20]

To a suspension of compound **70** (200 mg) in 3 mL of dichloromethane was added acetic anhydride (171 μL) at room temperature. The reaction mixture was stirred at room temperature for 2 h followed by addition of two drops of acetyl chloride and then 1.1 equiv of various alcohols. The reaction mixture was stirred overnight (18 h) and then purified by flash chromatography to afford the desired product.

5.3.1. Ethyl methoxyethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (77)

Yellow solid; mp 83–85 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.22 (t, J = 7.5 Hz, 3H, CH_2CH_3), 2.36 (s, 6H, $CH_3 \times 2$), 3.35 (s, 3H, OCH_3), 3.51–3.56 (m, 2H, OCH_2), 4.04–4.12 (m, 2H, OCH_2CH_3), 4.12–4.22 (m, 2H, OCH_2), 5.11 (s, 1H, CHAr), 5.99 (br s, 1H, NH), 7.38 (t, J = 8.0 Hz, 1H, Ar-H), 7.67 (d, J = 8.0 Hz, 1H, Ar-H), 8.00 (d, J = 8.0 Hz, 1H, Ar-H), 8.13 (s, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.2, 19.5, 19.6, 39.9, 58.8, 60.0, 63.0, 70.5, 103.0, 103.4, 121.3, 123.1, 128.6, 134.7, 144.7, 145.3, 148.1, 149.9, 167.0, 167.1; HRMS (ESI): m/z calcd for $C_{20}H_{25}N_2O_7$ ($M+H^+$): 405.1662; found: 405.1666; Anal. Calcd for $C_{20}H_{24}N_2O_7$ (404.15): C, 59.40; H, 5.98; N, 6.93. Found: C, 59.33; H, 6.11; N, 6.83.

5.3.2. Methyl cyanoethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (80)

Yellow solid; mp 134–137 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.38 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 2.65–2.69 (m, 2H, OCH_2CH_2CN), 3.66 (s, 3H, OCH_3), 4.21–4.31 (m, 2H, OCH_2CH_2CN), 5.10 (s, 1H, CHAr), 6.04 (br s, 1H, NH), 7.41 (t, J = 7.5 Hz, 1H, Ar-H), 7.68 (d, J = 7.5 Hz, 1H, Ar-H), 8.02 (d, J = 7.5 Hz, 1H, Ar-H), 8.10 (s, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 18.1, 19.5, 20.0, 39.6, 51.2, 58.4, 102.0, 103.6, 117.1, 121.6, 122.8, 128.9, 134.4, 144.7, 146.6, 148.4, 149.3, 166.3, 167.4; HRMS (ESI): m/z calcd for $C_{19}H_{20}N_3O_6$ ($M+H^+$): 386.1352; found: 386.1354; Anal. Calcd for $C_{19}H_{19}N_3O_6$ (385.12): C, 59.22; H, 4.97; N, 10.90. Found: C, 59.25; H, 5.04; N, 10.73.

5.4. Method for transfection of tsA201 cells with $Ca_V1.3$ and $Ca_V1.2$

5.4.1. Constructs

Rat Cav1.3α1D, Ca_Vβ3, and Ca_Vα2δ-1 cDNA were gifts of Dr. D. Lipscombe, Brown University, Providence, RI. Sequence alignments and RT-PCR from brain tissue revealed a few point mutations in Ca_V1.3 α1D, which were corrected by site-directed mutagenesis. Rabbit Ca_V1.2 α1C cDNA was a gift of Dr. Johannes Hell, University of Iowa.

5.4.2. Transfection of tsA201 cells

tsA201 cells were maintained in D-MEM medium supplemented with 10% fetal bovine serum (Invitrogen) without antibiotics. A mixture of Ca_V1.3 α1D or Ca_V1.2 α1C, Ca_V β3, and Ca_Vα2δ-1 cDNA at a molar ratio of 1:1:1 together with 1/40 (w/w) GFP cDNA (Invitrogen) were transfected into tsA201 cells using Geneporter reagent (Genetic Therapy Systems, San Diego, CA) according to the manufacturer's protocol. Cells were trypsinized 48 h later and plated on poly-D-lysine-coated coverslips. GFP-labeled cells were recorded after attachment.

5.4.3. Stable $Ca_V1.2$ and $Ca_V1.3$ cell lines for FLIPR screens

HEK 293 cells were maintained in D-MEM medium supplemented with 10% fetal bovine serum (Invitrogen) without antibiotics. $Ca_V1.2$ and $Ca_V1.3$ cell lines were created in two steps. First, $Ca_V\beta3$ and $Ca_V\alpha2\delta$-1 constructs were co-transfected into HEK 293 using the Geneporter reagent (Genetic Therapy Systems, San Diego, CA) according to the manufacturer's protocol. Forty-eight hours after transfection, 200 μg/mL zeocin and 100 μg/mL hygromycin were added to the medium to select antibiotic resistant colonies. The colonies developed were transferred to 48-well plates and subsequently tested for the expression of $Ca_V\beta3$ and $Ca_V\alpha2\delta$-1 by RT-PCR and Western blotting. One of the colonies with high levels of expression of $Ca_V\beta3$ and $Ca_V\alpha2\delta$-1 was designated as the $\alpha2\delta$-1/$\beta3$ cell line and used for the following experiments. $Ca_V1.2$ $\alpha1C$ or $Ca_V1.3\alpha1D$ constructs were transfected into the $\alpha2\delta$-1/$\beta3$ cell line. Geneticin (600 μg/mL) or blasticidin (4 μg/mL) in addition to zeocin and hygromycin were used for the selections of $Ca_V1.2$ and $Ca_V1.3$ colonies, respectively. Cell lines containing functional channels were selected by calcium imaging with the Fluo-4 NW Calcium Assay Kit (Invitrogen). KCl (90 mM) was used to stimulate the culture in the imaging protocol. Live images were acquired at 1-second intervals using an Olympus DSU spinning disc Confocal microscope. Expressions of $Ca_V1.2$ $\alpha1C$ or $Ca_V1.3\alpha1D$ were verified by RT-PCR.

5.5. Whole-cell patch-clamp recording assay

The external bath solution contained the following (in mM): 110 NaCl, 1 $MgCl_2$, 10 $BaCl_2$, 10 HEPES, 10 glucose, 20 CsCl at pH 7.4. The test compound stock solutions in DMSO (10 mM or just DMSO) were diluted with the external bath solution to the desired concentration (1000 nM to 10 nM), which was perfused into the cell while measuring the calcium currents. Calcium currents were measured from whole-cell voltage patch-clamp recordings using the Pulse 8.4 software data acquisition system (HEKA, Germany). Signals were low-pass filtered at 1 kHz, digitized (sampled) at 1 kHz, and were amplified with an Axopatch 200B patch-clamp amplifier (Axon Instruments). Calcium currents were evoked by a voltage pulse from a holding potential of −60 mV to +10 or 0 mV in the presence of tetrodotoxin (0.5 mM) at room temperature (about 22 °C). Patch pipettes were pulled from borosilicate glass and had a resistance of approximately 3–5 MΩ. Internal pipette solutions contained the following (in mM): 180 NMG (*N*-methyl-D-glucosamine), 40 HEPES, 4 $MgCl_2$, 12 phosphocreatine, 2 Na_2ATP, 0.5 Na_3GTP, 0.1 leupeptin, 5 BAPTA, pH 7.2–7.3. Electrophysiological signals were analyzed using Clampfit 9.2 (Axon Instruments).

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Supplementary data

Supplementary data (compound characterization) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.038.

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