Research Paper

Ligand-Based Virtual Screening to Identify New T-Type Calcium Channel Blockers

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Abstract

T-type calcium channels are involved in the generation of rhythmical firing patterns in the mammalian central nervous system and in various pathological alterations of neuronal excitability such as in epilepsy or neuropathic pain. In the search for new T-type calcium channel blockers that would help to treat these disorders, we have followed a bi-dimensional pharmacophore-based virtual screening approach to identify new inhibitors. Nineteen molecules extracted from AurSCOPE Ion Channels knowledgebase were used as query molecules to screen an external database. This in silico approach was then validated using electrophysiology. Interestingly, 16 compounds out of 38 distinct molecules tested showed more than 50% blockade of the CaV3.2 mediated T-type current. Two series of compounds show chemical originality compared with known T-type calcium channel blockers.

Introduction

Neuronal voltage-gated Ca2+ channels play a central role in the control of cellular excitability and in a number of Ca2+-dependent cellular functions including gene transcription and transmitter release. While subtypes of high voltage-activated Ca2+ channels are important for synaptic transmission in the nervous system, the main proposed functions of low voltage-activated or transient (T-type) Ca2+ channels include promotion of Ca2+-dependent burst firing, generation of low-amplitude intrinsic neuronal oscillations, elevation of Ca2+ entry, and boosting of dendritic signals. These events, in turn, contribute to neuronal pacemaker activity, wakefulness, seizure susceptibility, or integration of sensory information including pain.3–5 Three structurally and functionally distinctive T-type calcium channels pore-forming subunits have been cloned and denoted CaV3.1, CaV3.2 and CaV3.3 in the current nomenclature. Of these isoforms, CaV3.2 has been identified as a good target to identify new classes of analgesic drugs for pathological pain syndromes.3–5 Several organic molecules such as flunarizine, U-92032, nicardipine and mibebradil (Fig. 1) have been reported as active T-type Ca2+ channel blockers; however, only limited progress has been made to date in the quest to identify both potent and selective T-type channel blockers.

Mibebradil, the first marketed selective T-type Ca2+ channel inhibitor blocks T-type Ca2+ channels 10–30 times more potently that L-type Ca2+ channels. It was finally withdrawn due to its pharmacokinetic interactions with other drugs metabolized by cytochromes P450 3A4 and 2D6. Therefore, new T-type Ca2+ channel blockers having innovative chemical scaffolds are necessary to understand the exact role of T-type Ca2+ channel in cellular functions. In the course of identifying new ligands, Doddareddy et al generated the first 3D pharmacophore model by using eight highly-active compounds including mibebradil.5 Later, this 3D model was used to screen commercially available databases leading to the discovery of new potent T-type calcium channel blockers.5,6 Inversely, we report the application of ligand-based virtual screening using bidimensional fingerprints as another approach to identify a new series of T-type calcium channel blockers (TCCBs). Biological activity of selected hits was assessed by using a functional patch clamp assay on the CaV3.2 isoform transfected in tsA 201 cells.

Methods

Dataset selection. The chart of Figure 2 summarizes the strategy followed during this study. The first step was to collect all pertinent chemical and biological information related
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to the TCCBs. All of the collected literature was then integrated into AurSCOPE Ion Channels database described in the companion paper in this issue of Channel. The chemical structures of ligands as well as a precise description of their targets and biological activities were collected from more than 5,200 published articles and patents. Biological activities are classified according to biological protocols, such as binding, in vivo, electrophysiology, isolated organs, and flux (uptake and release). Aureus’s query system AurQUEST was used to extract a data set of the most active non-peptidic ligands tested in binding or electrophysiology protocols on T-type calcium channels. Biological activity threshold, expressed in terms of Ki and IC50, was set to retrieve affinities or activities less than 10 μM. At the time of this analysis (April 2005), the query resulted in the identification of 45 molecules. Some of these molecules are depicted in Figure 3. The sulfonamido-3,4-dihydroquinazoline derivative (compound 1) has potent blocking effect but shows less selectivity for T-type channel over N-type channel. Recently, Doddareddy et al. used this series of compounds to generate a 3D pharmacophore with three hydrophobic regions, one positive ionizable center, and two hydrogen bond acceptor groups. Virtual screening using this pharmacophore lead to the identification of new potent and selective T-type blockers. Compound 2 is one of these identified molecules with IC50 value of 100 nM. The compound 3 (SUN N5030) has potent blocking effect on both Na+ and T-type Ca2+ channels and also extremely low affinity for dopamine D2 receptors. The diphenyl ether group has been shown to play a crucial role for the blockade of ion channels. Compound 4 is a DHP (dihydropyridine) Nifedipine analog. The pentadecyl chain on the aryl ring which is absent in Nifedipine is most likely to be responsible for the inhibitory effects of this compound series on T-type calcium channel activity. The morpholin-2-one derivative (compound 5) is a member of chemical series reported to be selective T-type Ca2+ channel blockers (T-type/
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N-type from 2 to 37)\textsuperscript{12} SR 141716A (compound 6) has been the focus of several studies conducted on CB\textsubscript{2} receptor antagonism or inverse agonism, but was also shown to inhibit T-type channels.\textsuperscript{13} As can be seen, these compounds present a large chemical diversity and possibly different binding modes. Subsequently, these molecules were clustered using variable-length Jarvis-Patrick clustering. Central molecules of resulting clusters and singletons constituted a 19-molecule-query data set for virtual screening analysis.

Virtual screening. Over the past few years, virtual screening has emerged as a complementary approach to high throughput screening and has become an important in silico technique in the pharmaceutical industry.\textsuperscript{14} It includes ligand- and structure-based methods. Pharmacophore modeling is one such approach; in which known active molecules are analyzed for common steric and electronic features responsible for drug-receptor interactions. Alternatively, protein-ligand crystal structure complexes can be used to construct specific receptor-based pharmacophore models. Here a ligand-based approach was adopted to quickly identify novel TCCBs using bidimensional (2D) pharmacophoric fingerprints (FP).

First, an external molecular database was created and maintained. More than 2 million compounds coming from different commercial catalogs were uploaded into the database. After applying Lipinski drug-like filters,\textsuperscript{15} subsequent processing included salt removal, duplicate suppression and standardization. Next, the chemical space representation of these molecules was assessed by calculating the pharmacophoric fingerprints. Two sets of fingerprints were computed: ChemAxon’s PF (Pharmacophoric Fingerprints)\textsuperscript{16} and CCG’s GpiDAPH3 fingerprints implemented in MOE suite.\textsuperscript{17} Particularly for PF, several parameters have to be optimized for their generation.

Biological assay. tsA 201 cells, cultivated in DMEM, Glutamax, 10% Fetal Calf Serum, were transfected with the human Ca\textsubscript{v}3.2 isoform inserted in the pcDNA3 expression vector using JetPEI (QBiogen) following standard protocol. Cells were cotransfected with a CD8 expression plasmid (1/10 ratio) to allow optimized identification of the transfected cells using CD8 dynabeads (Dynal) applied prior to electrophysiological recordings. Whole-cell calcium currents were recorded at room temperature 2 to 3 days after transfection using an Axopatch 200A amplifier (Axon Instruments, CA, USA). Extracellular solution contained (in mM): 2 CaCl\textsubscript{2}, 135 NaCl, 20 TEACl, 1 MgCl\textsubscript{2}, and 10 HEPES (pH to 7.4 with TEAOH). Bovine Serum Albumine (BSA 0.5mg/ml) was added to the external solution to prevent drug fixation to the perfusion tubing. Borosilicate glass pipettes that had a typical resistance of 1.5–2 M\textohm were filled with an internal solution containing (in mM): 130 CsCl, 10 HEPES, 10 EGTA, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 4 Mg-ATP and 0.3 GTP (pH to 7.3 with CsOH), estimated free calcium concentration of 35 nM.

Figure 3. Some representatives of T-type calcium channels blockers used as query molecules for 2D virtual screening. For each molecule, the apparent affinity (expressed as IC\textsubscript{50}, Kd, or concentration giving maximal T-type current inhibition) is given as reported in the manuscripts cited.

Figure 4. Functional screening of 38 selected hits. Histogram showing averaged block (mean ± SEM of 3 to 8 cells) of Ca\textsubscript{v}3.2 mediated T-type current in the presence of 10 μM of the different drugs. Compounds considered as positive correspond to the bars crossing the 50% inhibition red dotted line.
The compounds were purchased and tested at 10 μM for their ability to affect the functional activity of recombinant human CaV3.2. As depicted in Figure 4, of the 38 distinct compounds tested, 16 showed more than 50% blockade of the CaV3.2 mediated T-type current and were therefore considered as active hits (bars above the red dotted line, Fig. 4).

To illustrate these active compounds, representative effects obtained with compound 3 and 14 are shown in Figure 5A and B. As seen from the time course, application of compound 3 leads to a large inhibition of the peak current amplitude that is reversible upon wash out. Concomitant to this inhibition, there is a slowing of the current kinetics (5A inset). In contrast, application of 14 leads to a rapid complete block that is not reversible upon wash out. In between these two extreme situations, the analysis of the inhibitory action of the sixteen hits shows that most of the compounds exhibit a reversible action without affecting the current’s biophysical properties, while a few produce a total blockade scarcely reversible. Structurally, compounds 3 and 14 are derivatives of piperazine or amino piperidine, respectively. A generic core structure of these compounds is depicted on Figure 6. Both phenyls are substituted.

**DISCUSSION**

In this study, a virtual screening procedure incorporating various bidimensional chemical and pharmacophoric fingerprints was successful in the identification of new T-type calcium channel blockers from a structurally diverse dataset of known active compounds. It is important to note the high hit rate obtained using this 2D methodology. Of the 36 compounds identified computationally as potential T-type calcium channel blockers, biological evaluation confirmed high potency of more than 10 compounds. To control the bioassays, a known blocker (Fluspirilene, compound 20) has been added as a reference compound. This compound is far from to be selective due to its large spectrum of biological activities (Table 1). Also, two identical compounds (5 and 28) purchased from different suppliers were tested and resulted in identical biological results.

Analyses of electrophysiology data demonstrate important differences in the mode of action of the active compounds. Most of the drugs produce a reversible inhibition, but some were irreversible. Differences were also noted in the rates of block and washout, indicating differences in affinities for CaV3.2. As pointed out for compound 3, there was an observed alteration of current kinetics with a slowing of the activation as well as the deactivation tail current upon repolarization of the membrane after the test pulse. These characteristics are reminiscent of an effect on the channel activation gating. For other active compounds, evidence for an apparent preferential action on the channel's inactivation state were observed.
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![Core structure of compounds 3 and 14](image)

Figure 6. Core structure of compounds 3 and 14. These compounds are piperazine or amino piperidine derivatives, respectively. Both phenyls are substituted.

### Table 1 Biological spectrum of Fluspirilene

| Biological Protocol | Species | Activity (nM) |
|---------------------|---------|---------------|
| **Binding**         |         |               |
| Alpha1              | Rat     | K<sub>i</sub>: 102 |
| Alpha2A             | Human   | K<sub>i</sub>: 570 |
| Alpha2B             | Human   | K<sub>i</sub>: 48 |
| Alpha2C             | Human   | K<sub>i</sub>: 62 |
| Alpha2              | Rat     | K<sub>i</sub>: >5E3 |
| Beta1               | Human   | K<sub>i</sub>: 590 |
| Beta2               | Human   | K<sub>i</sub>: 510 |
| Ca channel          | Rat     | K<sub>i</sub>: 7.5 |
| D1                  | Rat     | K<sub>i</sub>: 450 |
| D2                  | Rat     | K<sub>i</sub>: 1.5 |
| D2S                 | Human   | K<sub>i</sub>: 1.2 |
| D3                  | Human   | K<sub>i</sub>: 3.9 |
| D3                  | Rat     | K<sub>i</sub>: 1.1 |
| D4                  | Human   | K<sub>i</sub>: 3.5 |
| H1                  | Guinea pig | K<sub>i</sub>: 540 |
| H1                  | Human   | K<sub>i</sub>: 12 |
| Mu                  | Rat     | K<sub>i</sub>: 77 |
| Muscarinic          | Rat     | K<sub>i</sub>: >SE3 |
| Sig1R               | Guinea pig | K<sub>i</sub>: 150 |
| Na channel          | Rat     | K<sub>i</sub>: 130 |
| S-HT1A              | Human   | K<sub>i</sub>: 56 |
| S-HT1A              | Rat     | K<sub>i</sub>: 110 |
| S-HT1B              | Human   | K<sub>i</sub>: >SE3 |
| S-HT1B              | Rat     | K<sub>i</sub>: >SE3 |
| S-HT1D              | Human   | K<sub>i</sub>: 1.12E3 |
| S-HT1E              | Human   | K<sub>i</sub>: 2.23E3 |
| S-HT2A              | Human   | K<sub>i</sub>: 9.5 |
| S-HT2A              | Rat     | K<sub>i</sub>: 3.5 |
| S-HT2C              | Pig     | K<sub>i</sub>: 1.83E3 |
| S-HT3               | Mouse   | K<sub>i</sub>: >SE3 |
| S-HT7               | Mouse   | K<sub>i</sub>: 35 |
| **Electrophysiology** |         |               |
| Kv11.1              | Human   | IC<sub>50</sub>: 2.34E3 |
| T-type Ca           | Human   | IC<sub>50</sub>: 220 |

Data were extracted from AurSCOPE Ion Channels knowledgebase.7

suggesting that a careful analysis of the state-dependent block should be performed in the future on the most interesting and original chemical structures identified.

Regarding the chemical diversity of identified hits, two series of compounds were found to show chemical originality compared to known blockers. A first series of three compounds exhibits a low inhibition activity (50–55%). Compounds in the second series of two active compounds (% inhibition > 80%) have a significantly different molecular backbone compared to query molecules, and can be considered as starting compounds for optimization phase. Structure-Activity Relationships analysis of the available congeneric compounds of these two new T-type calcium blockers will be undertaken in the next phase of this project.

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