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3D pharmacophore based virtual screening of T-type calcium channel blockers

Munikumar Reddy Doddareddy,[a] Hyunah Choo,[a] Yong Seo Cho,[a] Hyewhon Rhim,[a] Hun Yeong Koh,[a] Jung-Ha Lee,[b] Seong-Woo Jeong[c] and Ae Nim Pae[a,*]

[a]*Life Science Division, Korea Institute of Science and Technology, PO Box 131, Cheongryang, Seoul 130-650, Republic of Korea*
[b]*Department of Life Sciences, Sogang University, Shinsu-1-Dong, Seoul 121-742, Republic of Korea*
[c]*Department of Physiology, Yonsei University, Wonju College of Medicine, Ilsan-Dong 162, Wonju, Kangwon-Do 220-701, Republic of Korea*

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Abstract—Virtual screening of the commercial databases was done by using a three dimensional pharmacophore previously developed for T-type calcium channel blockers using CATALYST™ program. Biological evaluation of 25 selected virtual hits resulted in the discovery of a highly potent compound VH04 with IC_{50} value of 0.10 μM, eight times as potent as the known selective T-type calcium channel blocker, mibefradil. Search for similar compounds yielded several hits with micro-molar IC_{50} values and high T-type calcium channel selectivity. Based on the structure of the virtual hits, small molecule libraries with novel scaffolds were designed, synthesis and biological evaluation of which are currently in progress. This result shows a successful example of ligand based drug discovery of potent T-type calcium channel blockers.

1. Introduction

Calcium channel blockers (CCBs) have been used since the early 1960s in the treatment of variety of cardiovascular diseases. They are widely used in the treatment of patients with angina pectoris, hypertension, and variant angina.[1–3] CCBs are a heterogeneous group of drugs that inhibit inward calcium channel current to a variable degree in different tissues including the vascular smooth muscle, myocardium, and sinus and atrioventricular (AV) nodes.[1,2] The different subtypes of voltage-dependent Ca^{2+} channels have been extensively investigated and classified into two main classes, one that responds to strong depolarization also called as high voltage activated (HVA) and the other which responds to weak depolarization also called as low voltage activated (LVA). Based on pharmacological studies, HVA Ca^{2+} channels are again divided into L-, N-, P-, Q-, and R-types and LVA Ca^{2+} channel is also called as T-type. L-type (long-lived) and T-type (transient) calcium channels both coexist in neurons, heart, vascular smooth muscle and endocrine cells. Traditional CCBs such as nifedepine, diltiazem, and verapamil all block L-type calcium channels and are classified as dihydropyridine (e.g., nifedipine) or non-DHP agents such as phenylalkylamines (e.g., verapamil) and benzothiazepines (e.g., diltiazem). The second and third generation CCBs are either slow-release or long acting formulations of the first generation CCBs, examples such as amlodipine or felodipine. But most of the CCBs used in the therapy of hypertension and angina pectoris feature to some extent unwanted effects such as negative inotropism, atrioventricular blockade or neurohormonal activation,[1,3] which often limit their therapeutic use. These unwanted side effects are absent in case of T-type calcium channel blockers. Blockade of T-type calcium channels which play an important role in the initial depolarization of sinus and AV nodes[4,5] slows the sinus rate and prolongs AV nodal conduction, in addition to causing vasodilation, without adverse negative inotropic or positive chronotropic cardiac actions.[6,7] In addition many reports have shown that T-type calcium channels are involved in the pathogenesis of epilepsy and neuropathic pain.[8–11]

Keywords: Virtual screening; Pharmacophore; T-type calcium channel blockers.
* Corresponding author. Tel.: +82 2 958 5185; fax: +82 2 958 5189; e-mail: anpae@kist.re.kr

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In spite of the importance of the T-type calcium channels in the physiological process, limited progress is made in the related research due to lack of potent and selective T-type calcium channel blockers. In addition, mibefradil one of the most important examples of selective T-type calcium channel blockers was withdrawn from the market due to its pharmacokinetic interactions[4] with other drugs metabolized by cytochromes P-450 3A4 and 2D6 (antihistamines, such as astemizole) and postmarketing data showing an increase in mortality in the elderly who were also taking β-blockers and DHP CCBs, and reports of rhabdomyolysis with concomitant simvastatin therapy.[12,13] As a result many research groups nowadays are involved in the development of selective T-type calcium channel blockers and are successful to some extent.

Previously, we have reported a three dimensional pharmacophore[14] generated using eight highly active compounds available for us at that time. We made an attempt to identify the hypothetical 3D ligand based pharmacophore model by using common feature hypothesis generation approach (HipHop) implemented in CATALYST[15] program. HipHop algorithm finds common feature pharmacophore models among a set of highly active compounds thus carrying out a 'qualitative model' without the use of activity data, which represents the essential 3D arrangement of functional groups common to a set of molecules for interacting with a specific biological target. HipHop does not require the selection of a template, as each molecule in the dataset is treated as template itself. Different 3D arrangement of functional groups is identified in the template molecule using a pruned exhaustive search which starts with small sets of features and extends them until no larger 3D arrangement is found. Each 3D arrangement or hypothesis is then compared with the remaining molecules to identify configurations that are common to all molecules. The resulting hypotheses are ranked using a combination of how well the molecules in the training set map onto the hypothesis and the rarity of the model. In HipHop the user can select chemical functions to be present in the pharmacophore model based on the atom types present in the molecule. Molecules which are to be used in pharmacophore generation and which are to be ignored can be specified by using 'Principal number' option given in the program. Partial hits, that is, molecules that can map incompletely to the hypothesis can be controlled by using misses, feature misses, and complete misses options. This partial mapping allows one to identify larger, more diverse, more significant hypotheses, and alignment models without the risk of missing compounds that do not map to all of the pharmacophore features. The generated pharmacophore can be used to search databases for the molecules satisfying the chemical features present in the pharmacophore. This can be accomplished by fast flexible search option or best flexible search option given in view database workbench. In the present work we used a pharmacophore previously developed[14] for screening commercially available ion channel[16] and Maybridge2001[17] databases, selected hits of which were tested for blocking effect on T-type calcium channel. Based on the information obtained from the initial results, we developed a new pharmacophore, which was again used to screen databases, results of which were also discussed.

2. Results and discussion

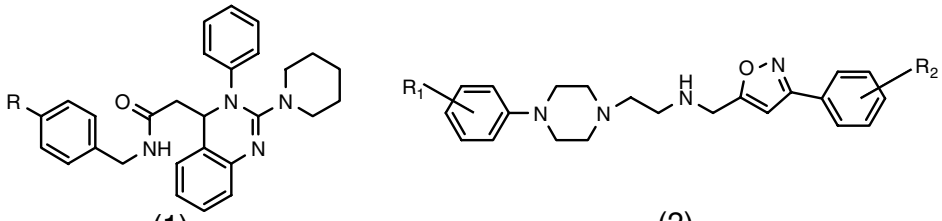
2.1. Pharmacophore model generation

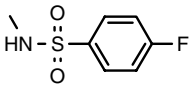
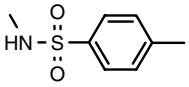
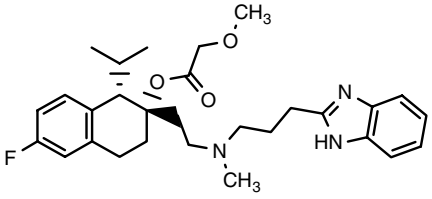
Three dimensional pharmacophore[14] was generated by using eight molecules including mibefradil[18] representing the most interesting compounds available for us at that time. Three compounds (**1a**, **1b**, and **1c**) belonging to 3,4-dihydroquinazoline series,[19] four compounds (**2a**, **2b**, **2c**, and **2d**) belonging to piperazinylalkylisoxazole series[20] and mibefradil were used as the dataset (Table 1). The biological activities (IC_{50}) on HEK293 cell with stabilized α_{1G} T-type calcium channel were also shown. In hypothesis generation, on the basis of the atom types in the molecules, the following chemical functions were selected in the feature dictionary of the catalyst: hydrogen bond acceptor, positive ionizable, and hydrophobic groups. The generated pharmacophore contained three hydrophobic regions, two hydrogen bond acceptors and one positive ionizable group (Fig. 1). The detailed description about the pharmacophore generation was published elsewhere.[14]

2.2. Virtual screening of commercial databases with generated phamacophore

Commercially available ion channel database which contains about 8222 compounds and Maybridge2001 database which contains about 55,273 compounds were screened using the generated pharmacophore. In CATALYST, we can search a chemical database for compounds that fit certain criteria. The search criteria here is the hypothesis or pharmacophore which represents the essential 3D arrangement of functional groups common to a set of molecules for interacting with a specific biological target, that is, T-type calcium channel. Both the databases were searched using best flexible search option given in CATALYST. Screening resulted in total 2445 virtual hits, of which 1846 are from ion channel database and 609 are from Maybridge database. Using a 'Best fit' cutoff value of 3, hits were filtered to 525 and 159 in case of ion channel database and Maybridge database respectively. Our main aim of this work was not to find all the possible active compounds that are present in the databases but to find some lead molecules with novel scaffolds. Furthermore economic constraints restricted us to select few compounds for real biological evaluation. Based on the knowledge of existing T-type calcium channel blockers and expert visual inspection, we selected 25 compounds, 19 from ion channel database and 6 from Maybridge database for real biological testing. These compounds were bought from ChemDiv[16] and Maybridge,[17] respectively, and % inhibition of calcium channel current was measured in *Xenopus* unfertilized oocytes expressed with T-type calcium channel α_{1H} ($Ca_v3.2$) by a two-electrode voltage clamp method[21] as a preliminary test. The ID number, structure, and % inhibition data of the selected compounds are given in

Table 1. Structures of the compounds used for pharmacophore generation[14]



No	Compound	R		IC_{50}[a] (μM)
1	**1a**			0.20
2	**1b**			0.25
3	**1c**	H		0.90
		R_1	R_2	
4	**2a**	3-Trifluoromethyl	2-Trifluoromethyl	1.02
5	**2b**	2,4-Dimethyl	2-Trifluoromethyl	1.53
6	**2c**	2,4-Dimethyl	2-Methoxy	2.02
7	**2d**	3-Trifluoromethyl	2-Methoxy	2.04
8	Mib			0.84

[a] On HEK293 cell stabilized with α_{1G} T-type calcium channel.

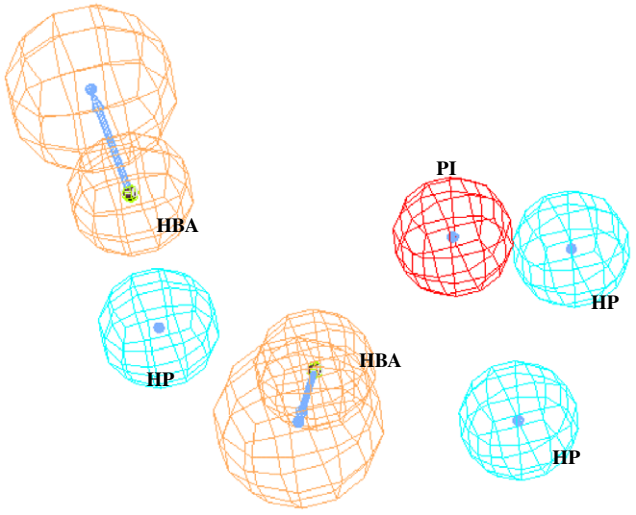


Figure 1. Published 6-feature hypothesis for T-type calcium channel blockers14. HBA, hydrogen bond acceptor; HP, hydrophobic; PI, positive ionizable.

Table 2. Four compounds namely VH02, VH04, VH09, and VH21 showed more than 50% inhibition on T-type calcium channel, particularly compound VH04 which is obtained from ion channel database showed high % inhibition of 98.33. IC_{50} value of VH04 was determined using the dose–response curve and was found to be 0.10 μM, which is about 8 times as potent as mibefradil (IC_{50} = 0.84 μM). Furthermore, VH04 contained a thioxodihydroquinazolinone group as novel scaffold, so we selected this compound for further study. To understand more about the structure activity relationship of these compounds, ion channel database was screened for compounds containing thioxohydroquinazolinone moiety. Fifteen of the selected compounds were screened for inhibitory effect on T-type calcium channel current in the same way as previously mentioned. Only two compounds namely VS05 and VS07 with similar structure like that of the parent hit VH04 showed high % inhibition of 72.93 and 84.11, respectively (Table 3). Another compound which showed more than 50 % inhibition was VS13 which has little different structure with bromine in the place of 4-chlorobenzyl group of VH04. On careful observation we found that these three virtual hits have one striking similarity with compounds **1a**, **1b** and **1c** (Table 1) from which original pharmacophore was generated. All these compounds contained a positive ionizable group, one of the most important pharmacophoric features at terminal position in the form of piperidine or pyrrolidine. In case of other compounds (series 2 and mibefradil) the positive ionizable group is in the center of the structure. The positive ionizable group or the basic nitrogen is thought to bind with conserved repeat III and IV glutamates of the pore region thereby blocking the channel.[22] The higher activity of series 1 compounds and VH04 compared to other

Table 2. Percentage inhibition data of 25 selected hits

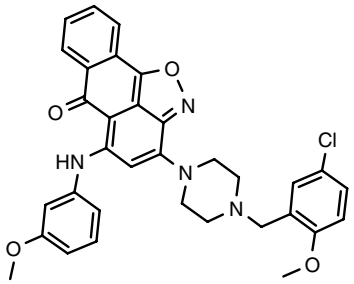
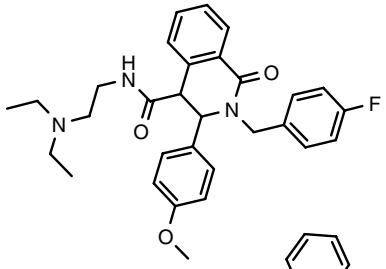
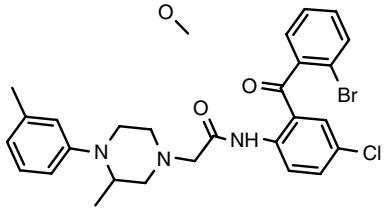
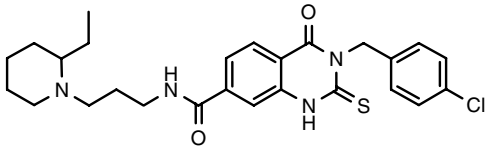
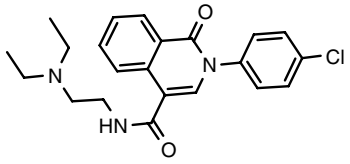
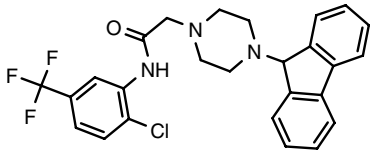
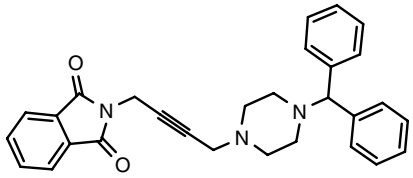
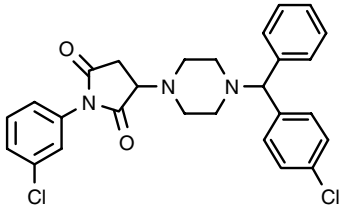
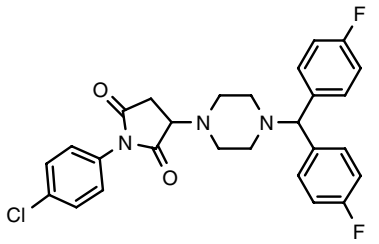
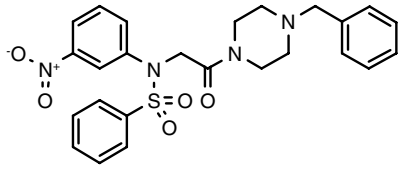
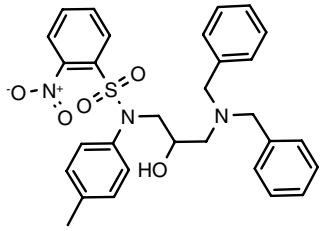
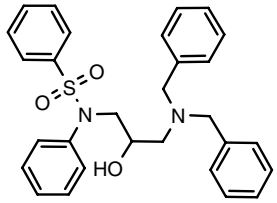
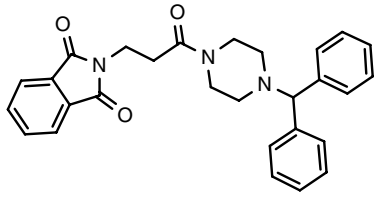
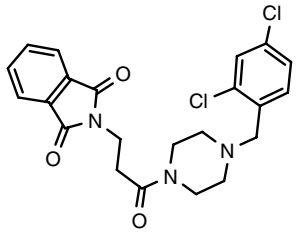
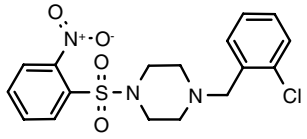
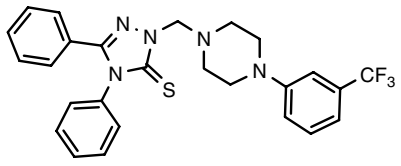
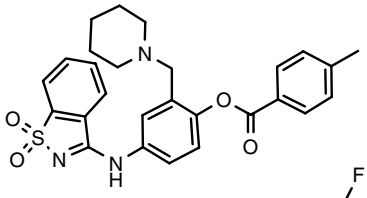
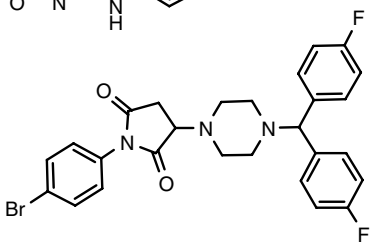
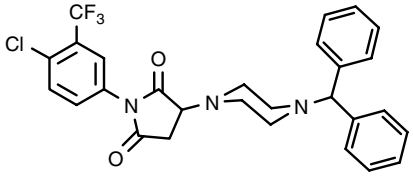
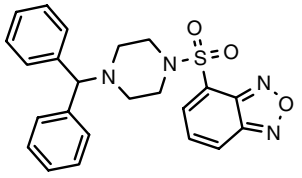
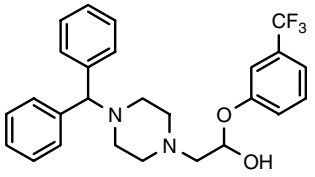
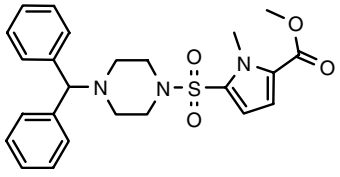
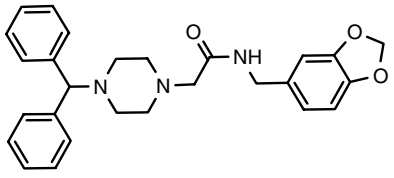
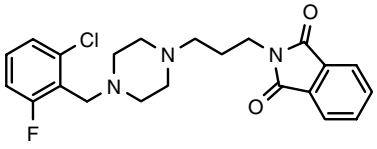
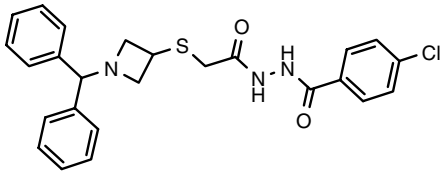
No	ID	Structure	% INH[a]
1	VH01		16.13
2	VH02		52.20
3	VH03		9.23
4	VH04		98.33
5	VH05		13.00
6	VH06		No block
7	VH07		No block
8	VH08		16.65

Table 2 (*continued*)

No	ID	Structure	% INH[a]
9	VH09		51.76
10	VH10		42.46
11	VH11		34.69
12	VH12		15.19
13	VH13		43.32
14	VH14		12.00
15	VH15		7.57
16	VH16		11.41

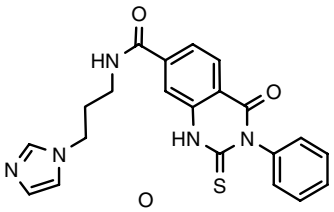
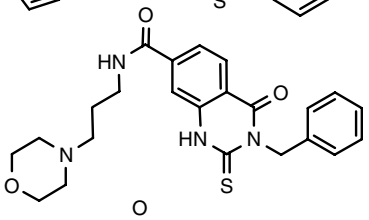
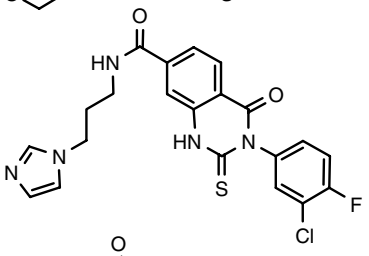
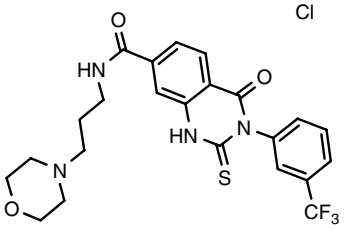
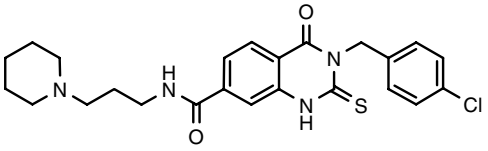
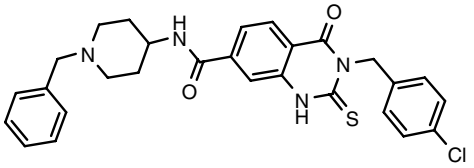
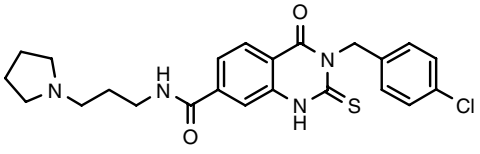
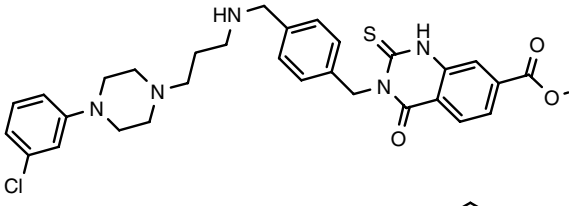
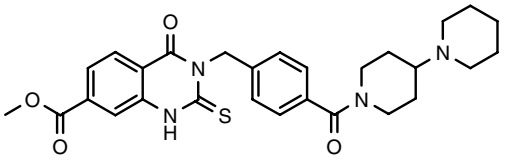
(*continued on next page*)

Table 2 (*continued*)

No	ID	Structure	% INH[a]
17	VH17		13.52
18	VH18		37.14
19	VH19		41.82
20	VH20		15.20
21	VH21		58.28
22	VH22		No block
23	VH23		12.52
24	VH24		8.10
25	VH25		28.27

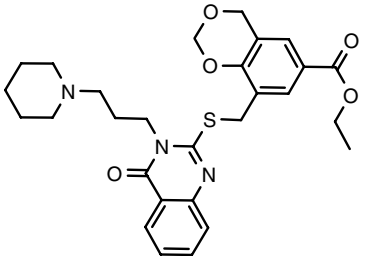
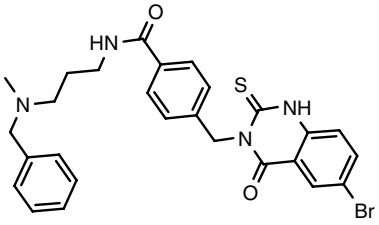
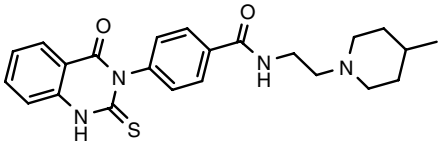
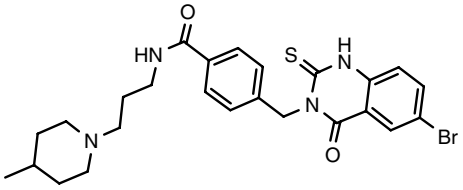
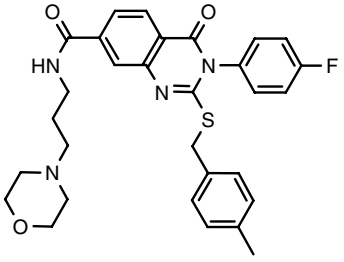
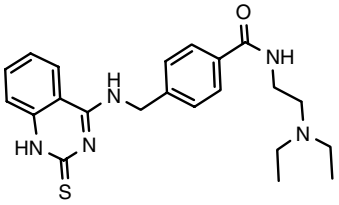
[a] % inhibition measured in *Xenopus* oocytes expressed with α_{1H} T-type calcium channel at 100 μM concentration.

Table 3. Percentage inhibition data of thioxodihydroquinazolinones

No	ID	Structure	% INH[a]
1	VS01		2.47
2	VS02		15.93
3	VS03		No block
4	VS04		15.90
5	VS05		72.93
6	VS06		No block
7	VS07		84.11
8	VS08		11.36
9	VS09		10.31

(*continued on next page*)

Table 3 (*continued*)

No	ID	Structure	% INH[a]
10	VS10		35.29
11	VS11		25.69
12	VS12		No block
13	VS13		62.58
14	VS14		No block
15	VS15		9.54

[a] % inhibition measured in *Xenopus* oocytes expressed with α_{1H} T-type calcium channel at 100 μM concentration.

inhibitors suggests that the presence of positive ionizable group at terminal position may be more advantageous and also indicates the possibility of different binding mode for these compounds. For this reason we tried to generate different pharmacophore for series 1 compounds and VH04 to get an idea about SAR of thioxohydroquinazolinone compounds. Three compounds namely **1a**, **1d**, and VH04 (Fig. 2) were used for the generation of the pharmacophore using the same Hiphop module in CATALYST program. Compound **1d** also belongs to 3,4-dihydroquinazoline group (series 1) which was recently reported[23] and has high selectivity for T-type Ca^{2+} channel with no effect on N-type channel.

2.3. Generation of new pharmacophore model

Structures of the three selected compounds were generated using 2D/3D editor sketcher in CATALYST 4.11

software package and are minimized to the closest local minimum using the CHARMm-like force field implemented in the program.[24] A stochastic research coupled to a poling method[25] was applied to generate conformers for each compound by using 'Best conformer generation' option with 20 kcal/mol energy cutoff (20 kcal/mol maximum compared to the most stable conformer). In hypothesis generation, same chemical functions which were presented in original pharmacophore were selected in the feature dictionary of the catalyst: hydrogen bond acceptor, positive ionizable and hydrophobic groups. Hypothesis generation was done by assuming that 'all compounds are equally important and all contain important features', this was achieved by putting 2 in principal number which ensures that all of the chemical features in the compound will be considered in building hypothesis space and 0 in maximum omitting features column which forces mapping of all features for all compounds. Except these, all other parameters were kept at default. Table 4 shows the summary of hypothesis run. All the 10 hypotheses generated contained three hydrophobic regions (H), one positive ionizable region (P) and two hydrogen bond acceptor groups (A), same as that in original pharmacophore (Fig. 1). Direct hit mask value of '1' for all hypotheses indicates that all the molecules of the dataset mapped to all features of the hypotheses. The partial hit mask value of '0' for all hypotheses indicates there is no partial mapping or missing of any feature in the data set molecules. The generated hypotheses have a small range of ranking scores ranging from 58.17 to 56. This small range of ranking score and same features in all hypotheses suggests that the same six features are spatially arranged almost similar way in all hypotheses. The higher the ranking, the less likely it is that the molecules fit the hypothesis by a chance correlation.

Statistically best hypothesis (Fig. 3) with ranking score of 58.17 is selected to understand the structure–activity relationship of the compounds in Table 3. Figure 4 shows the mapping of VH04 to the selected six feature

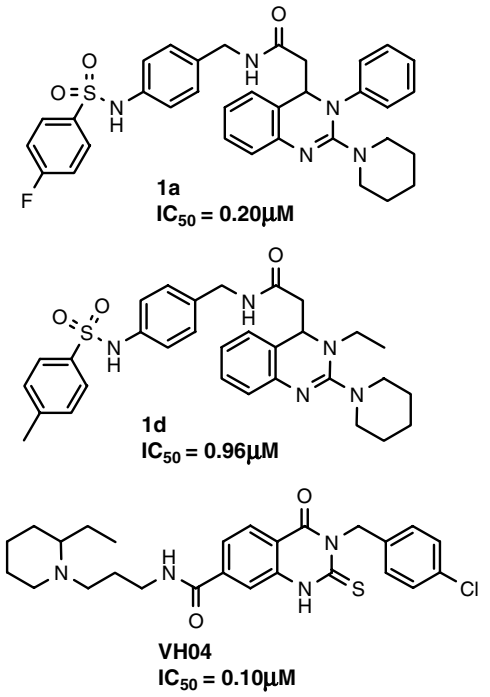


Figure 2. Compounds used to generate pharmacophore for thioxodihydroquinazolinone compounds.

Table 4. Summary of hypothesis run

No	Composition	Ranking score	Direct hit mask	Partial hit mask
1	PHHHAA	58.17	111	000
2	PHHHAA	58.17	111	000
3	PHHHAA	57.21	111	000
4	PHHHAA	56.98	111	000
5	PHHHAA	56.80	111	000
6	PHHHAA	56.48	111	000
7	PHHHAA	56.30	111	000
8	PHHHAA	56.22	111	000
9	PHHHAA	56.20	111	000
10	PHHHAA	55.99	111	000

P, positive ionizable; H, hydrophobic; A, hydrogen bond acceptor. Direct hit mask indicates whether (1) or (0) not a training set molecule mapped every feature. Partial hit mask indicates whether (1) or (0) not a molecule mapped all but one feature.

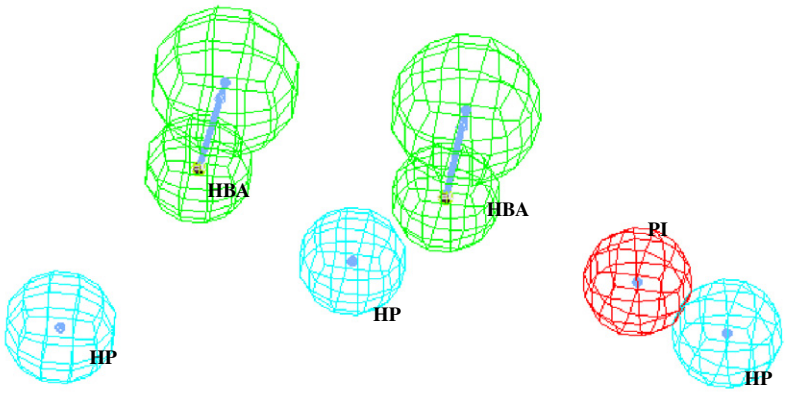


Figure 3. Pharmacophore developed for thioxodihydroquinazolinone compounds. HBA, hydrogen bond acceptor; HP, hydrophobic; PI, positive ionizable.

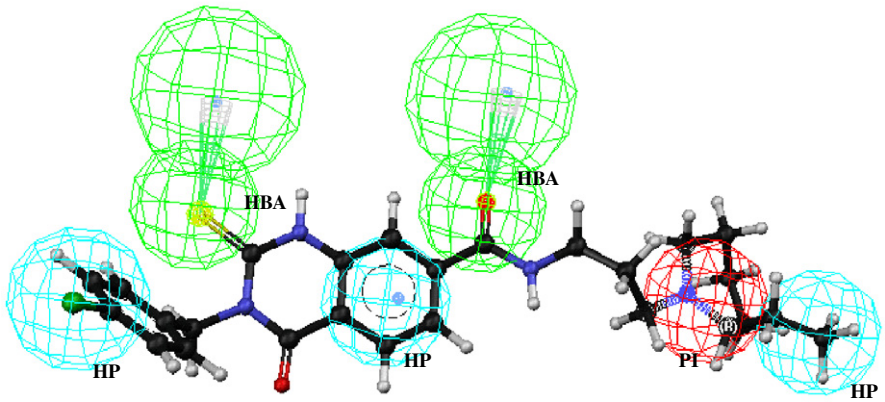


Figure 4. VH04 mapping to new pharmacophore. HBA, hydrogen bond acceptor; HP, hydrophobic; PI, positive ionizable.

hypothesis. One hydrophobic region is occupied by 4-chlorobenzyl group, one to the phenyl group of thioxohydroquinazolinone, and the last one to ethyl group on piperidine ring, one hydrogen bond acceptor is occupied by O of the amide group and another to S of thioxohydroquinazolinone moiety, and the single positive ionizable region is occupied by N of the piperidine group. Most of the compounds of Table 3 that did not showed appreciable inhibition lacked one or more of these pharmacophoric important features. Compounds VS01 and VS03 donot have the terminal positive ionizable group as shown in the pharmacophore (Fig. 3). Even though VS02 and VS04 have the terminal positive ionizable group, hydrophilic O of the morpholine moiety is present in the position of hydrophobic region of the pharmacophore and may be showing unfavorable effect.

VS05 and VS07 perfectly fitted to the pharmacophore and showed appreciable inhibition of 72.93% and 84.11%, respectively. In case of VS06, VS08, and VS11, the presence of larger hydrophobic group, that is, phenyl ring directly connected to basic nitrogen may be causing steric hindrance and restricting its binding to the pore region glutamates. VS09 which showed just 10% inhibition has a hydrophilic ester group present in the place of hydrophobic region of the pharmacophore. Similarly other compounds missed one or more of the important features or have incompatible groups at some positions.

2.4. Virtual screening of the commercial databases with new pharmacophore

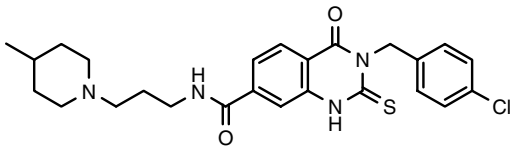
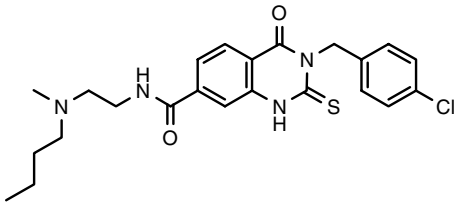
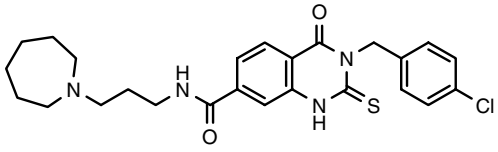
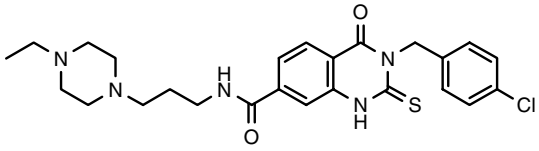
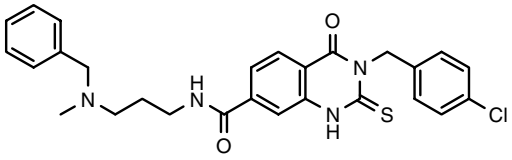
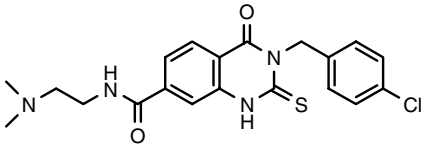
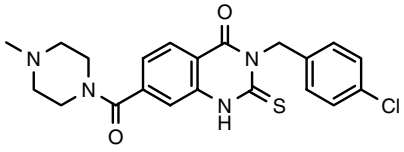
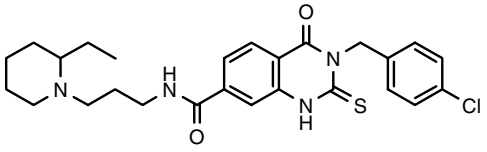
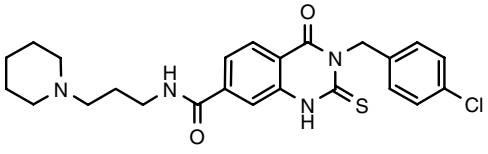
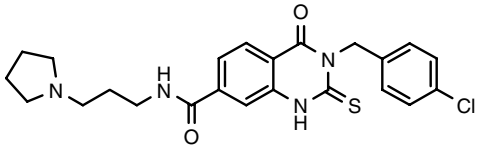
The new pharmacophore which was generated using the virtual hit VH04 and two other known compounds namely **1a** and **1d** (Fig. 2) is used to screen the databases. Based on the knowledge obtained from the SAR of thioxohydroquinazolinone compounds of the Table 3, we tried to search the ion channel database for compounds that contain thioxohydroquinazalinone group and also have all the features of the pharmacophore (Fig. 3). Ten of the selected thioxohydroquinazalinone compounds with all features of the pharmacophore were tested for the blocking effects on α_{1G} T-type calcium channels expressed in HEK293 cells at 10 μM concentration by whole-cell patch–clamp method.[26]

Percentage inhibition and IC_{50} data of the 10 selected thioxohydroquinazalinone compounds are given in Table 5. As expected most of the compounds showed appreciable inhibition with some showing comparable IC_{50} value to that of known selective T-type calcium channel blocker, mibefradil. For the evaluation of the ion channel selectivity, the top six compounds with micro-molar IC_{50} values were screened against α_{1B} N-type calcium channels (high voltage activated Ca^{2+} channels). The IC_{50} values of these six compounds for α_{1G} (T-type Ca^{2+} channels) and α_{1B} (N-type Ca^{2+} channel) with their selectivity ratios are given in Table 6. All the compounds showed higher blocking effect on T-type calcium channel (α_{1G}) than N-type (α_{1B}) with selectivity ranging from 3 to 20 times. Compounds VH04 and VS07 showed highest α_{1G}/α_{1B} selectivity of 21 and 15 times, respectively.

The new phamacophore (Fig. 3) which was used for searching thioxohydroquinazalinone compounds (Table 6) was also used to search compounds with different scaffolds. Percentage inhibition of the 10 selected compounds was measured in *Xenopus* unfertilized oocytes expressed with T-type calcium channel α_{1H} as a preliminary test (Table 7). Two compounds namely VD09 and VD10 showed high percentage inhibition of about 55.95% and 86.50%, respectively. Further study on these compounds is in progress. Figure 5 shows the flowchart of the entire virtual screening process.

As a next step in the drug discovery process, we designed two small molecule libraries to get compounds with good pharmacokinetic properties. Based on the structure of the virtual hits (VH04) and the important chemical features mapping to pharmacophore, two novel scaffolds were designed, the library construction and biological evaluation of which are currently in progress and results of which were expected soon.

Table 5. Percentage inhibition and IC_{50} data of thioxodihydroquinazolinone compounds satisfying all the features of the pharmacophore

No	ID	Structure	% INH	IC_{50} (μM)
1	SS01		89.2	1.1
2	SS02		34.3	11.37
3	SS03		85.4	1.41
4	SS04		78.9	2.53
5	SS05		37.5	4.69
6	SS06		43.5	53.5
7	SS07		51.4	11.39
8	VH04		88.3	0.10
9	VS05		34.3	2.08
10	VS07		85.4	1.16

Percentage inhibition and IC_{50} were measured in HEK293 cell stabilized with α_{1G} T-type calcium channel at 10 μM concentration.

Table 6. T/N selectivity of selected thioxodihydroquinazolinone compounds

No	ID	α_{1G}[a]	α_{1B}[b]	Sel[c]
1	VS05	2.08	8.1	3.9
2	VS07	1.16	17.2	14.8
3	VH04	0.10	2.1	21
4	SS01	1.1	6.4	5.8
5	SS03	1.41	5.8	4.1
6	SS04	2.53	7.1	2.8

[a] IC_{50} measured in HEK293 cell stabilized with α_{1G} T-type calcium channel at 10 μM concentration.
[b] IC_{50} measured in HEK293 cell stabilized with α_{1B} N-type calcium channel at 10 μM concentration.
[c] T/N (α_{1G}/α_{1B}) selectivity.

Table 7. Percentage inhibition data of virtual hits with different scaffolds

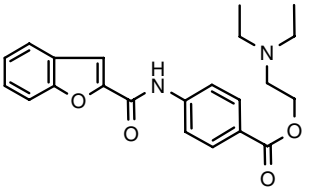
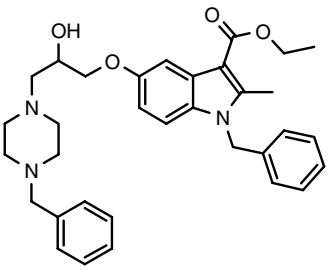
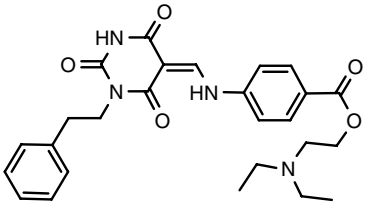
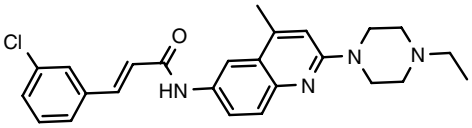
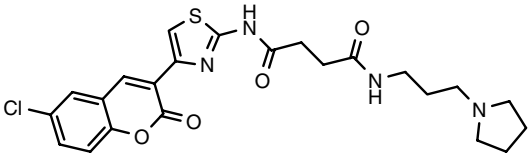
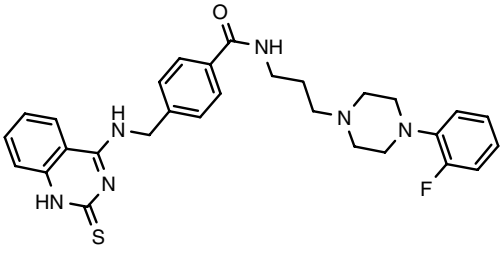
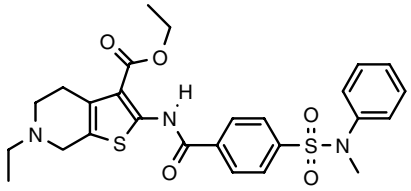
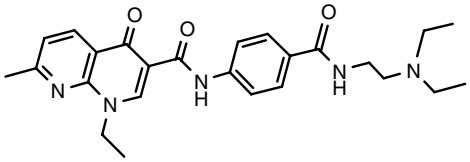
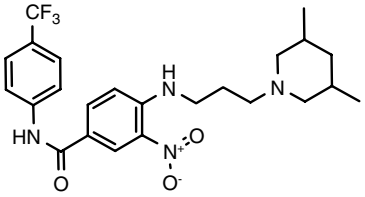
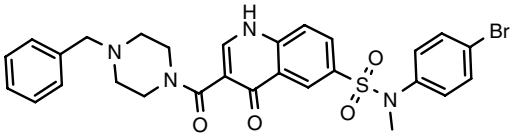
No	ID	Structure	% INH[a]
1	VD01		34.37
2	VD02		8.83
3	VD03		No block
4	VD04		No block
5	VD05		43.91
6	VD06		No block

Table 7 (*continued*)

No	ID	Structure	% INH[a]
7	VD07		22.03
8	VD08		41.91
9	VD09		55.95
10	VD10		86.50

[a] % inhibition measured in *Xenopus* oocytes expressed with α_{1H} T-type calcium channel at 100 μM concentration.

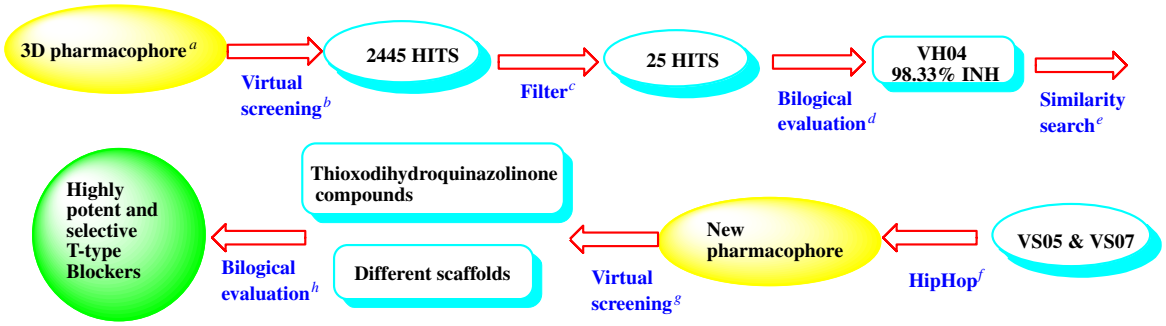


Figure 5. Flowchart of the virtual screening process. [a]Published 3D pharmacophore,[14] [b]virtual screening of ion channel,[16] and Maybridge[17] databases, [c]hits were filtered based on best fit value, Lipinski rule and visual inspection, [d]% inhibition in *Xenopus* oocytes,[21] [e]search for thioxodihydroquinazolinone compounds, [f]new pharmacophore using **1a**, **1d**, and VH04, [g]screening for compounds with thioxohydroquinazolinone scaffold and different scaffolds, [h]IC_{50} measured in HEK293 cells.[26]

3. Conclusions

Virtual screening of commercially available ion channel and Maybridge2001 databases was done by using a three dimensional ligand based pharmacophore developed for T-type calcium channel blockers using CATALYST program. Biological evaluation of 25 selected virtual hits resulted in the discovery of a highly potent compound VH04 with IC_{50} value of 0.10 μM, eight times as potent as the known selective T-type calcium channel blocker, mibefradil. Search for similar compounds with thioxohydroquinazalinone scaffold in the parent ion channel database yielded several hits with micro-molar IC_{50} values and high T-type calcium channel selectivity, particularly compounds VH04 and VS07 showed high T/N selectivity (α_{1G}/α_{1B}) of 21 and 15 times, respectively. Based on the structure of the virtual hits small molecule libraries with novel scaffolds were designed to improve the pharmacokinetic properties. The discovery of potent and selective T-type calcium channel blockers by ligand based 3D pharmacophoric virtual screening indicates the efficiency of this kind of approach in drug discovery program which is economical and saves considerable amount of labor and time.

4. Biological assays

4.1. Preparation of unfertilized *Xenopus* oocytes and cRNA synthesis of α_{1H} T-type calcium channel[21]

In order to express a gene encoding T-type calcium channel α_{1H} ($Ca_v3.2$) in unfertilized *Xenopus* oocytes,

vector (pGEM-HEA) was treated with restriction enzyme *Afl*II to obtain a DNA fragment containing 5′-terminal region having the T-type calcium channel cDNA (AF051946), and cRNA having a corresponding sequence to that of the fragment was synthesized using T7 RNA polymerase according to the manufacturer's instruction (mMESSAGE mMACHINE kit, Ambion, Austin, USA). The synthesized cRNA was quantified by measuring the OD value with a spectrophotometer. At this time, unfertilized oocytes were prepared from female *Xenopus laevis* (Xenopus I, USA) according to the following method. After the frog's abdomen was incised by about 1 cm, three to four lobes were detached therefrom with scissors and separated into small pieces to which several oocytes attached. The small pieces were hydrolyzed in OR solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes, pH 7.6) supplemented with collagenase type IA (Sigma, USA) to remove defolliculation. After selecting healthy oocytes with a dissecting microscope, they were soaked in SOS solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, 2.5 mM pyruvate, and 50 μM/mL gentamicin, pH 7.6) for 3–4 h to revitalize them. cRNA (2–5 ng) was injected into each oocyte using a nano-injector, and the oocytes were subjected to test for examining the electrical properties of the channel expressed there from 4 to 5 days after the injection with maintaining at 18 °C.

4.2. Examination of electrophysiological property of α_{1H} T-type calcium channel using a two-electrode voltage clamp method[21]

Current of the calcium channel expressed from the *Xenopus* unfertilized oocytes was measured according to a two-electrode voltage clamp method. Current was measured in 10 mM Ba^{2+} solution [10 mM $Ba(OH)_2$, 90 mM NaOH, 1 mM KCl, 0.1 mM EDTA, and 5 mM Hepes, pH was adjusted to 7.4 with methanesulfonic acid]. At this time, voltage clamp and current measurements were regulated with an amplifier for unfertilized oocytes (Model OC-725C, Warner Instrument Corp., USA), analog signals were converted into digital signals using Digidata 2000A (Analog-Digital converter, Axon Instrument), and acquisition, storage, and analysis of all data were recorded in Pentium IV computer via pCLAMP8. The data were mainly collected at 5 kHz and filtered at 1 kHz (Model 902 filter; frequency devices located at Harverhill, MA, USA). The generation of T-type current was occurred by imposing test electric potential of −20 mV every 15 s on the unfertilized oocytes whose potential was fixed at −90 mV, and a blocking percentage was calculated by comparing the potentials before and after the drug treatment.

4.3. Methods for culturing HEK293 cells and measuring T- and N-type calcium channel activity using an electrophysiological method[26]

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (v/v) in 36.5 °C humidified incubator (95% air–5% CO_2). The culture solution was replaced with a fresh medium every 3–4 days, and the cultured cells were subjected to subculture every week. At this time, the culture solution was treated with G-418 (0.5 mg/mL) solution so that only HEK293 cells expressing α_{1G} T-type calcium channel can grow. The cells used for T-type calcium channel activity assay were cultured on a coverslip coated with poly-L-lysine (0.5 mg/mL) whenever sub-cultured, and their calcium channel activity was recorded 2–7 days after the cultivation. Current of the T-type calcium channel at a single cell level was measured according to an electrophysiological whole-cell patch–clamp method using EPC-9 amplifier (HEKA, Germany). At this time, a cell exterior solution [140 mM NaCl, 2 mM $CaCl_2$, and 10 mM Hepes (pH 7.4)] and a cell interior solution [130 mM KCl, 10 mM Hepes, 11 mM EGTA and 5 mM MgATP (pH 7.4)] were employed. Inward current caused by the T-type calcium channel activation which occurred when the cells were converted into a whole-cell recording mode by stabbing a microglass electrode having 3–4 MΩ resistance which was filled with the cell interior solution into a single cell and depolarized at −30 mV (50 ms duration period) every 10 s with fixing membrane potential to −100 mV was measured according to a T-type calcium channel protocol activated at low current. In the case of N-type, Ca^{2+} currents were measured in HEK293 cells expressing α_{1B} Ca^{2+} channel using a cell exterior solution [151 mM TEACl, 5 mM $BaCl_2$, 10 mM Hepes, 1 mM $MgCl_2$, and 10 mM glucose (pH 7.4)] and a cell interior solution [100 mM CsCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM BAPTA, 3.6 mM MgATP, 0.1 mM GTP, 14 mM phosphocreatine, and 50 U/mL creatine phosphokinase (pH 7.4)]. The currents were evoked every 15 s by a 200 ms depolarizing voltage step from −80 to 0 mV.

4.4. Method for screening T-type calcium channel blockers using an electrophysiological method

In order to confirm whether the cells and methods used above are a suitable screening system for selecting T-type calcium channel blockers, the results obtained above were compared with those of α_{1G} T-type calcium channel study reported in a public document.[26] As a result, it has been confirmed that since the screening system of the present invention showed: (1) the maximum activation at low voltage of −30 mV, (2) the fast activation–inactivation of the activated current, and (3) the same IC_{50} as those of Ni^{2+} and Mibefradil known as T-type calcium channel blockers, it is suitable for screening T-type calcium channel blockers. Thus, the candidate compounds were subjected to test for their inhibitory effects on the T-type calcium channel according to the screening system of the present invention as follows: each compound was dissolved in 100% dimethylsulfoxide (DMSO) to prepare 10 mM stock solution, and the inhibitory effect on the T-type calcium channel current was examined in 10 μM sample solution (containing 0.1% DMSO) prepared by diluting the stock solution by 1000-fold. The cells were treated with each compound

at a concentration of 10 μM for 30–60 s with the cell exterior solution. Then, the inhibition level of peak current caused by the compound was calculated as a percentage.

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