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Lead discovery and optimization of T-type calcium channel blockers

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Abstract—A series of compounds were designed as T-type calcium channel blocker containing 6 or 5 pharmacophore features from structure-based virtual screening. To optimize the suggested structure, over 130 derivatives were synthesized and their inhibitory activities on T-type calcium channel were assayed using in vitro screening system with $\alpha 1_G$ and $\alpha 1_H$ clones. For the compounds with higher activities in FDSS assay system, the efficacy was measured by patch–clamp method. Among the library with 5 features, alkaneamide derivatives (**7b**, **9j**, **11b**, **11g**, **11h**) with 4-arylsubstituted piperazine showed better IC_{50} values than Mibefradil.

1. Introduction

Voltage-gated calcium channels are transmembrane proteins involved in the regulation of calcium influx in a number of cell types.[1,2] A number of different types of calcium channels have been identified in native tissues and divided into various categories based on functional and pharmacological criteria.[3,4] High-voltage-activated (HVA) channels which can be subdivided further into L-, N-, P/Q-, and R-types[5,6] require strong depolarization for activation, whereas low-voltage-activated or T-type (transient) channels first activate at relatively more negative voltage range and rapidly inactivate.[7–9] The main structural component of the voltage-gated calcium channel is the α_1 subunit, which forms the pore and the channel gate. So far, 10 α_1 subunits have been identified by molecular cloning. α_{1A}–α_{1E} and α_{1S} encode HVA channels Ca^{2+}, whereas α_{1G}–α_{1I} encode T-type channels. Pharmacological agents that have selective activity on α_1 subtypes have been key in studying calcium channel function in physiological systems, but a selective antagonist of T-type calcium channel is not yet available. Mibefradil, which blocks T-type Ca^{2+} channels at concentration lower than that needed to block L-type Ca^{2+} channels,[10,11] appeared to be a promising drug for the treatment of hypertension, and angina pectoris.[12–14] Unfortunately, the drug had to be withdrawn by the manufacturer due to its interaction with the cytochrome P-450 3A4 enzyme, an effect unrelated to T-type Ca^{2+} channel blockade. T-type Ca^{2+} channels participate in cardiac pacemaking,[15] regulation of vascular tone and hormone secretion.[16,17] Involvement of T-type Ca^{2+} channels in cell growth and proliferation has been proposed.[18] The distribution and properties of T-type Ca^{2+} channels have been reported to be altered in pathophysiological conditions such as ventricular hypertrophy[19,20] and cardiomyopathy.[21] Thus, T-type Ca^{2+} channels are now considered to be novel therapeutic targets for the treatment of various cardiovascular disorders such as heart failure, arrhythmia, and hypertension and neuronal disorders such as epilepsy and pain. Even with these properties as promising drug target, the speed of development is surprisingly slow compared to other areas. It is because of non-availability of X-ray structure and existence of several subtypes of the channel. To obtain a lead compound as a T-type calcium channel blocker, the strategy we used involved the pharmacophore model generated from structure-based virtual screening and hypothetical mapping with the designed structure.

The first set of compounds with 6 pharmacophore features (**5**) was prepared and their activities were assayed with calcium imaging screening system. Disappointingly, they showed weaker activity than

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mibefradil. Then we need to understand the relative importance of the pharmacophores by comparing the activities of the compounds with 5 features without one hydrogen bond acceptor or the linker carbon length is varied. The 2nd set of libraries (**7**, **13**, **15**) in which the number and position of the proposed features were varied have been designed and synthesized. The synthetic procedure of the compounds with 5 features without one carbonyl group was simplified and the library was easily prepared. The FDSS results of this library showed relatively better activity than that of the compounds with initial 6 features. The 3rd set of libraries (**9**, **11**, **17**, **19**, **21**) in which the number of rotatable carbon and halogen of aniline moiety are varied were prepared. The in vitro screening assays to figure out the selectivity and hERG channel blocking activity are still going on.

2. Results and discussion

2.1. Chemistry

The synthesis of suggested compound which generated from statistically best 6-pharmacophore features hypothesis was not successful. Then it was modified to fit the 6-features (Fig. 1) and increase conformational rigidity. A series of compounds with 6 features were prepared by routine procedures shown in Scheme 1. To obtain the derivatives easily, the common intermediates from aniline moiety were used as starting material and the piperazine derivatives were introduced at final step. All the steps showed acceptable yields (40–80%) and purity. The 2nd set of library was designed to fit the essential 5 features (Fig. 2) and synthesized easily from piperazine derivatives and bromovaleryl chloride (Scheme 2) or aniline derivatives and piperazine units(Scheme 3). To evaluate the importance of distance of two features and number of halogens in aniline moiety, the 3rd set was prepared as same as Scheme 2.

2.2. Biological data and structure–activity relationships

As shown in Table 1, the in vitro activities of initial **5** series were assayed with T-type channels stably expressed in *Xenopus* oocytes ($\alpha 1_H$). After setting up the HTS system utilizing calcium imaging plate reader system (FDSS), the primary assay was performed with HEK293 cells ($\alpha 1_G$). For the compounds with high inhibition value in FDSS assay, the efficacy was measured as IC_{50} value by patch–clamp method. The in vitro activities of 1st set of compounds (**5**) were weaker than expected. Also the synthetic procedure was not enough

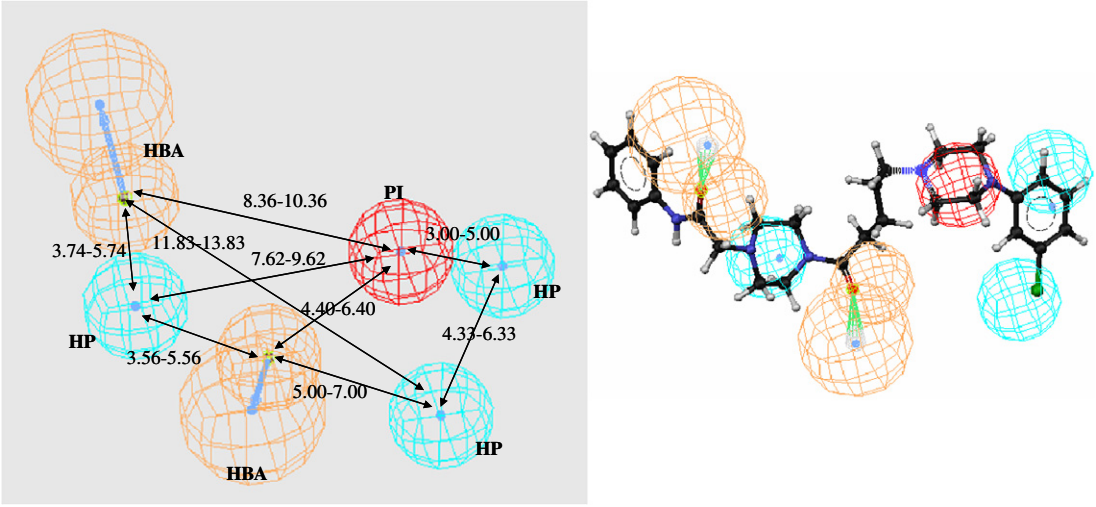
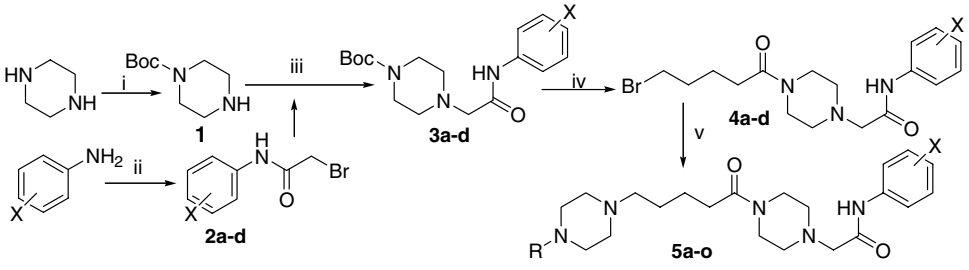


Figure 1. (L) 6-feature hypothesis for T-type calcium channel blockers with distance constraints in armstrong units (Å) (HBA, hydrogen bond acceptor; HP, hydrophobic; PI, positive ionizable). (R) Mapping of **5a** with 6-feature hypothesis.



Scheme 1. Reagents and conditions: (i) $(Boc)_2O$, TEA, CH_2Cl_2, 0 °C to rt; (ii) bromoacetyl chloride, NEt_3, 0 °C to rt; (iii) NEt_3, CH_2Cl_2, rt; (iv) TFA, CH_2Cl_2, rt; (v) 1-substituted piperazine derivatives, NEt_3, 0 °C to rt.

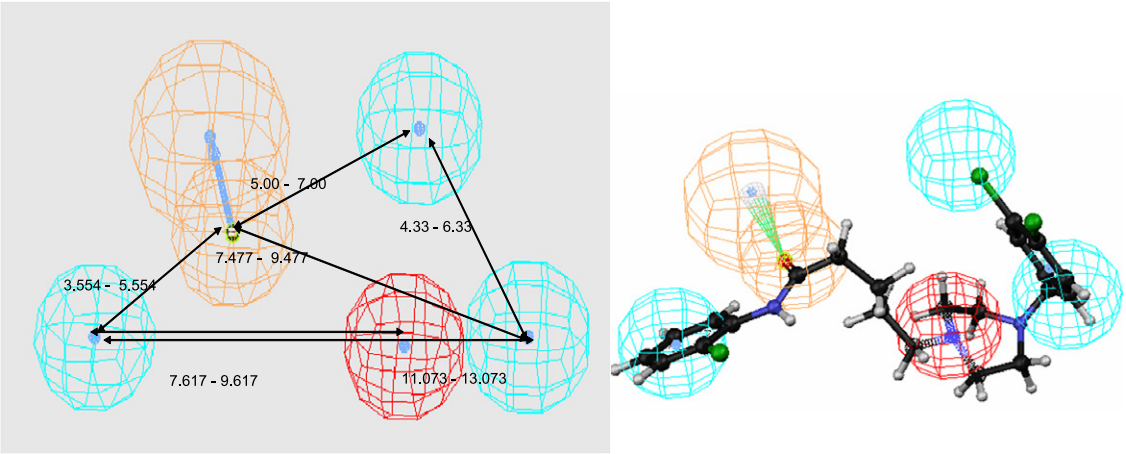
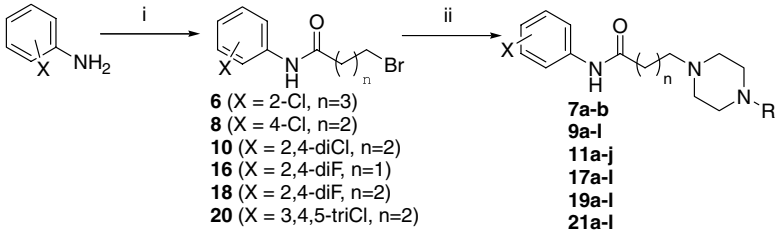
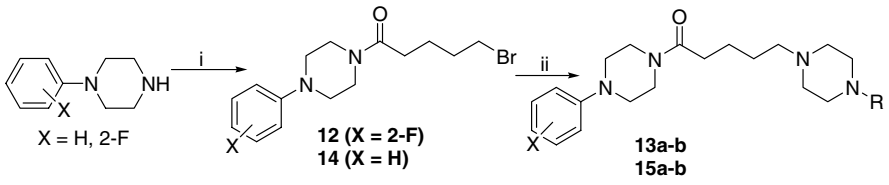


Figure 2. (L) 5-feature hypothesis for T-type calcium channel blockers with distance constraints in Armstrong units (Å) (HBA, hydrogen bond acceptor; HP, hydrophobic; PI, positive ionizable). (**R**) Mapping of **11g** with 5-feature hypothesis.



Scheme 2. Reagents and conditions: (i) *n*-bromoalkyl chloride, benzene, rt; (ii) 1-substituted piperazine derivatives, NEt_3, CH_2Cl_2, reflux.

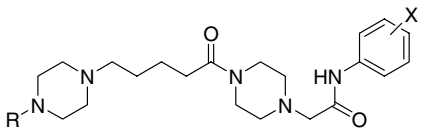


Scheme 3. Reagents and conditions (i) 4-bromovaleryl chloride, benzene, rt: (ii) 1-substituted piperazine derivatives, NEt_3, CH_2Cl_2, reflux.

efficient to obtain a library easily. If one of the hydrogen bond acceptors were omitted to have 5 features, the synthetic procedure will be simplified. Also, we need to figure out the relative importance of two hydrogen bond acceptors, the distance of features, and number of halogens, the suggested structure was modified and they fitted the 5-feature model previously suggested by Dr. Pae's modeling group in the same institute.[22] From the activity data in Table 2, the compounds with 5 features showed higher activity than those with 6 features and some of them has better IC_{50} value than mibefradil. Among the two carbonyl groups in the 6 features, that of the amide contributed more in terms of activity than that attached to piperazine ring. Examination of the substituents of R group of compounds **7**, **9**, **11** (Table 1) reveals a moderate structure–activity relationship: the halogen substituents on the phenyl or benzyl ring increase the efficacy of these compounds. The halogen substitution at the same position (para) of phenyl and benzyl ring is preferred, because the hydrophobic feature is well matched with para position of aromatic ring. And the optimal length of linear carbons including carbonyl is 4 or 5 carbons. If it is shorter than 3, the activities were slightly decreased as shown in Table 3. The in vitro activities of compound **19** series were better than those of **17** series and the halogen (Cl) substituent increases the activities. Also the general tendency was found that the derivatives with one or two chlorines in aniline moiety had higher activities than three-chlorine derivatives as in Table 4.

In conclusion, the new scaffolds starting from the hit compound by virtual screening were designed to fit the 5- or 6-pharmacophore hypothesis and evaluated as T-type calcium channel blocker. Even with the preliminary biological data, this approach has proved its usefulness in designing new ligands especially when the crystal structure of the target protein is unknown. The derivatization

Table 1. In vitro calcium channel blocking effects of derivatives with 6 features



5

Compound	X	R	*Xenopus* oocyte (T-type $\alpha 1_H$) % inhibition[a] (100 μM)	HEK 293 cell (T-type $\alpha 1_G$) % inhibition[a] (10 μM)
5a	3-Chloro	Phenyl	75.35	
5b	3-Chloro	Methyl	No block[b]	
5c	3-Chloro	Ethyl	71.61	
5d	3-Ttrifluoromethyl	Phenyl	52.98	
5e	3-Trifluoromethyl	Methyl	No block	
5f	3-Trifluoromethyl	Ethyl	23.60	
5g	3-Methyl	Ethyl		−1.36
5h	2-Chloro	Phenyl		25.17
5i	2-Chloro	2-Fluorophenyl		22.13
5j	2-Chloro	3-Methoxyphenyl		26.11
5k	2-Chloro	2-Cyanophenyl		7.38
5l	2-Chloro	4-Chlorobenzyl		26.24
5m	2-Chloro	3-Chlorobenzyl		40.94
5n	2-Chloro	4-Fluorophenyl		34.89
5o	2-Chloro	Benzyl		15.89
	Mibefradil		86.0	78.17

[a] % Inhibition value was obtained by FDSS assay.
[b] No block means the inhibition was less than 1%.

with **9j**, **11g**, and **11h**, and IC_{50} assay for the compounds with higher value in FDSS assay are in progress to increase the number of hit compounds and diversity.

3. Experimental

3.1. Chemistry

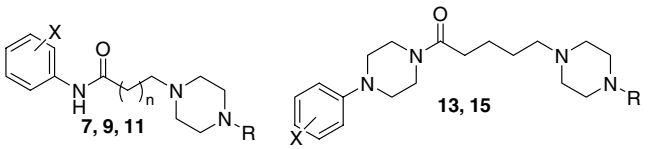
3.1.1. Materials and methods. All commercially available chemicals were of reagent grade and used as purchased unless stated otherwise. All reactions were performed under an inert atmosphere of dry argon or nitrogen using distilled dry solvents. Reactions were monitored by TLC analysis using Merck silica gel 60 F-254 thin layer plates. Flash column chromatography was carried out on Merck silica gel 60 (230–400 mesh) by preparative LC system. ^1H and ^{13}C NMR spectra were recorded on a spectrometer operating at Bruker 400 and 100 MHz, respectively. High-resolution mass spectra were recorded on a 4.7 T Ion Spec ESI-FTMS or a Micromass LCT ESI-TOF mass spectrometer.

3.1.2. 5-Bromo-*N*-(2-chlorophenyl)pentanamide (6). 4-Bromovaleryl chloride (0.7 mL, 5.23 mmol) was added dropwise to a solution of 2-chloroaniline (0.5 mL, 4.75 mmol) in benzene (10 mL) at 0 °C. Then the mixture was stirred at room temperature for 12 h. The resulting mixture was quenched by addition of water and extracted with dichloromethane. The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, EtOAc/*n*-Hex = 1:3) to give product **6** (0.82 g, 59.4%) as a white color solid: ^1H NMR (400 MHz, $CDCl_3$) $\delta = 1.86–2.04$ (4H, m, $2CH_2$), 2.49 (2H, t, $J = 16.64$ Hz, CH_2), 3.45 (2H, t, $J = 6.25$ Hz, CH_2), 7.63 (1H, br s, NH), 6.98–8.35 (4H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 23.95$, 31.95, 33.05, 36.69, 121.73, 122.68, 124.69, 127.75, 129.00, 134.48, 170.51.

3.1.3. 4-Bromo-*N*-(4-chlorophenyl)butyramide (8). 4-Bromobutyryl chloride (5.3 mL, 0.043 mol) was added dropwise to a solution of 4-chloroaniline (5.0 g, 0.039 mol) in benzene (100 mL) at 0 °C. Then the mixture was stirred at room temperature for 12 h. The resulting mixture was quenched by addition of water and extracted with dichloromethane. The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by recrystallization (EtOAc and hexane) to give product **8** (9.84 g, 90.8%) as a gray color solid: ^1H NMR (400 MHz, $CDCl_3$) $\delta = 2.27$ (2H, m, CH_2), 2.56 (2H, t, $J = 7.02$ Hz, CH_2), 3.52 (2H, t, $J = 6.18$ Hz, CH_2), 7.41 (1H, br s, NH), 7.26–7.47 (4H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta = 27.82$, 33.31, 35.26, 121.11, 129.02, 129.40, 136.17, 169.90.

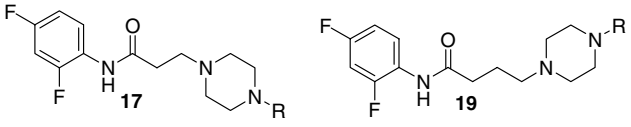
3.1.4. 4-Bromo-*N*-(2,4-dichlorophenyl)butyramide (10). 4-Bromobutyryl chloride (1.05 mL, 8.55 mmol) was added dropwise to a solution of 2,4-dichloroaniline (1.26 g, 7.78 mmol) in benzene (20 mL) at 0 °C. Then the mixture was stirred at room temperature for 12 h. The resulting mixture was quenched by addition of water and extracted with dichloromethane. The combined organic layer was dried over anhydrous $MgSO_4$,

Table 2. In vitro calcium channel blocking effects of derivatives with 5 features



Compound	X	*n*	R	FDSS HEK 293 cell (T-type $\alpha 1_G$) % inhibition (10 μM)	Patch–clamp HEK 293 cell (T-type $\alpha 1_G$)	
					% inhibition (10 μM)	IC_{50} (μM)
7a	2-Cl	3	3-Chlorophenyl	53.99	93.17 ± 1.48	1.91 ± 0.21
7b	2-Cl	3	3,4-Dichlorobenzyl	55.93	91.00 ± 1.86	**1.01 ± 0.10**
9a	4-Cl	2	Phenyl	30.38	75.1 ± 2.3	4.47 ± 0.36
9b	4-Cl	2	2-Fluorophenyl	36.06	79.2 ± 1.5	3.63 ± 0.21
9c	4-Cl	2	4-Fluorophenyl	32.50	76.1 ± 0.9	3.49 ± 0.27
9d	4-Cl	2	2-Chlorophenyl	37.34	60.5 ± 2.8	6.01 ± 0.89
9e	4-Cl	2	4-Chlorophenyl	48.27	94.6 ± 0.9	2.64 ± 0.59
9f	4-Cl	2	2-Methoxyphenyl	51.81	84.3 ± 2.3	2.61 ± 0.14
9g	4-Cl	2	3-Methoxyphenyl	40.61	84.1 ± 2.0	2.78 ± 0.18
9h	4-Cl	2	Benzyl	30.80	78.0 ± 0.4	3.52 ± 0.31
9i	4-Cl	2	3-Chlorobenzyl	38.33	92.4 ± 0.9	1.80 ± 0.09
9j	4-Cl	2	4-Chlorobenzyl	33.65	92.6 ± 3.2	**0.37 ± 0.04**
9k	4-Cl	2	2-Chloro-6-fluorobenzyl	39.84	72.8 ± 1.0	3.27 ± 0.36
9l	4-Cl	2	Piperonyl	33.36	81.9 ± 0.8	2.73 ± 0.13
11a	2,4-Cl	2	Phenyl	38.53	76.2 ± 1.2	4.21 ± 0.28
11b	2,4-Cl	2	3-Chlorophenyl	38.15	95.4 ± 1.4	**1.01 ± 0.10**
11c	2,4-Cl	2	2-Methoxyphenyl	42.70	79.1 ± 1.1	3.34 ± 0.10
11d	2,4-Cl	2	3-Methoxyphenyl	44.22	80.3 ± 0.8	3.81 ± 0.24
11e	2,4-Cl	2	Benzyl	44.87	79.3 ± 1.0	3.14 ± 0.40
11f	2,4-Cl	2	3-Chlorobenzyl	50.84	87.3 ± 1.1	1.48 ± 0.20
11g	2,4-Cl	2	4-Chlorobenzyl	61.26	91.1 ± 2.7	**0.28 ± 0.06**
11h	2,4-Cl	2	3,4-Dichlorobenzyl	47.61	94.4 ± 1.1	**0.75 ± 0.04**
11i	2,4-Cl	2	Piperonyl	49.80	88.1 ± 2.3	1.67 ± 0.12
11j	2,4-Cl	2	2-Chloro-6-fluorobenzyl	49.61	89.6 ± 0.3	2.40 ± 0.06
13a	2-F		3-Chlorobenzyl	48.82	87.13 ± 1.27	2.44 ± 0.24
13b	2-F		4-Chlorobenzyl	48.29	89.70 ± 1.53	1.81 ± 0.11
15a	H		4-Chlorobenzyl	50.38	79.70 ± 1.23	3.4 ± 0.18
15b	H		3,4-Dichlorobenzyl	56.23	73.80 ± 2.30	2.91 ± 0.25
			Mibefradil	74.29	95.9 ± 1.7	1.34 ± 0.49

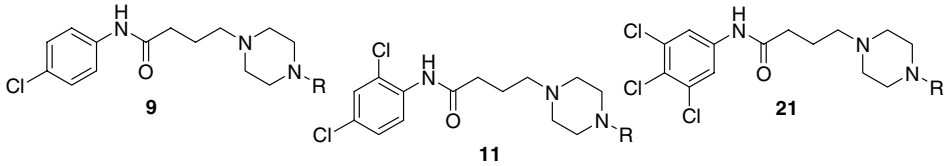
Table 3. In vitro calcium channel blocking effects of derivatives of 5 features with different chain lengths



Compound	R	FDSS[a]	Compound	FDSS[a]
17a	Phenyl	33.18	**19a**	36.80
17b	2-Fluorophenyl	30.29	**19b**	32.21
17c	4-Fluorophenyl	29.68	**19c**	38.14
17d	2-Chlorophenyl	28.36	**19d**	42.00
17e	3-Chlorophenyl	26.40	**19e**	51.04
17f	2-Methoxyphenyl	37.14	**19f**	33.00
17g	3-Methoxyphenyl	29.50	**19g**	40.57
17h	Benzyl	21.09	**19h**	23.22
17i	3-Chlorobenzyl	34.74	**19i**	30.24
17j	4-Chlorobenzyl	33.16	**19j**	48.04
17k	2-Chloro-6-fluorobenzyl	29.71	**19k**	38.93
17l	3,4-Dichlorobenzyl	34.60	**19l**	59.92
	Mibefradil	75.19		

[a] % inhibition value was obtained with T-type $\alpha 1_G$ (HEK 293 cell)and 10 μM of compounds.

Table 4. In vitro calcium channel blocking effects of derivatives of 5 features with different numbers of halogen



R	Compound	FDSS[a]	Compound	FDSS[a]	Compound	FDSS[a]
Phenyl	**9a**	30.38	**11a**	38.53	**21a**	38.31
2-Fluorophenyl	**9b**	36.06	**11k**	27.35	**21b**	20.08
4-Fluorophenyl	**9c**	32.50	**11l**	29.06	**21c**	20.68
2-Chlorophenyl	**9d**	37.34			**21d**	13.62
3-Chlorophenyl			**11b**	38.15	**21e**	16.78
2-Methoxyphenyl	**9f**	51.81	**11c**	42.70	**21f**	33.74
3-Methoxyphenyl	**9g**	40.61	**11d**	44.22	**21g**	21.65
Benzyl	**9h**	30.80	**11e**	44.87	**21h**	49.41
3-Chlorobenzyl	**9i**	38.33	**11f**	50.84	**21i**	36.98
4-Chlorobenzyl	**9j**	33.65	**11g**	61.26	**21j**	27.48
2-Chloro-6-fluorobenzyl	**9k**	39.84	**11j**	49.61	**21k**	35.15

[a] % Inhibition value was obtained with T-type $\alpha 1_G$ (HEK 293 cell) and 10 μM of compounds.

filtered, and concentrated under reduced pressure. The residue was purified by recrystallization (EtOAc and hexane) to give product **10** (2.17 g, 89.7%) as a pink color solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 2.29 (2H, m, CH_2), 2.65 (2H, t, J = 7.02 Hz, CH_2), 3.54 (2H, t, J = 6.23 Hz, CH_2), 7.24 (1H, J = 2.36 Hz, CH in Ph), 7.39 (1H, J = 2.36 Hz, CH in Ph), 7.60 (1H, br s, NH), 8.34 (1H, J = 8.89 Hz, CH in Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 27.76, 33.01, 35.53, 122.28, 123.13, 127.91, 128.73, 129.18, 133.14, 169.80.

3.1.5. 5-Bromo-1-[4-(2-fluorophenyl)piperazin-1-yl]pentan-1-one (12). 5-Bromovaleryl chloride (1.10 mL, 0.010 mol) was added dropwise to a solution of 2-fluorophenylpiperazine (1.5 mL, 0.009 mol) in benzene (30 mL) at 0 °C. Then the mixture was stirred at room temperature for 12 h. The resulting mixture was quenched by addition of water and extracted with dichloromethane. The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, EtOAc/*n*-Hex = 1:6) to give product **12** (2.02 g, 62.0%) as a white color solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.83 (2H, m, CH_2), 1.95 (2H, m, CH_2), 2.41 (2H, t, J = 7.24 Hz, CH_2), 3.07 (4H, m, $2CH_2$), 3.45 (2H, t, J = 6.47 Hz, CH_2), 3.64 (2H, s, CH_2), 3.80 (2H, s, CH_2), 6.91–7.09 (4H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 23.76, 32.22, 33.50, 41.69, 45.76, 50.69, 116.27, 119.24, 123.19, 124.55, 139.50, 170.92.

3.1.6. 5-Bromo-1-(4-phenylpiperazin-1-yl)pentan-1-one (14). 5-Bromovaleryl chloride (1.65 mL, 0.013 mol) was added dropwise to a solution of 1-phenylpiperazine (1.5 mL, 0.012 mol) in benzene (30 mL) at 0 °C. Then the mixture was stirred at room temperature for 12 h. The resulting mixture was quenched by addition of water and extracted with dichloromethane. The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by recrystallization (EtOAc and hexane) to give product **14** (2.64 g, 67.6%) as a pink color solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.83 (2H, m, CH_2), 1.93 (2H, m, CH_2), 2.41 (2H, t, J = 7.30 Hz, CH_2), 3.17 (4H, m, $2CH_2$), 3.45 (2H, t, J = 6.55 Hz, CH_2), 3.63 (2H, t, J = 4.92 Hz, CH_2), 3.78 (2H, t, J = 4.98 Hz, CH_2), 6.89–7.31 (5H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 23.68, 32.12, 32.18, 33.35, 41.46, 45.45, 49.40, 49.76, 116.64, 120.60, 129.21, 150.82, 170.87.

3.1.7. 3-Bromo-*N*-(2,4-difluorophenyl)propionamide (16). 3-Bromopropionyl chloride (2.7 mL, 0.027 mol) was added dropwise to a solution of 2,4-difluoroaniline (2.5 mL, 0.025 mol) in benzene (50 mL) at 0 °C. Then the mixture was stirred at room temperature for 12 h. The resulting mixture was quenched by addition of water and extracted with dichloromethane. The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, EtOAc/*n*-Hex = 1:8) to give product **16** (4.9 g, 75.6%) as a white color solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 2.99 (2H, t, J = 6.48 Hz, CH_2), 3.71 (2H, t, J = 6.50 Hz, CH_2), 7.33 (1H, br s, NH), 6.86–8.26 (3H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 26.53, 40.51, 103.36, 103.60, 103.86, 111.18, 111.40, 122.99, 167.86.

3.1.8. 4-Bromo-*N*-(2,4-difluorophenyl)butyramide (18). 4-Bromobutyryl chloride (8.4 mL, 0.069 mol) was added dropwise to a solution of 2,4-difluoroaniline (7.0 mL, 0.069 mol) in benzene (100 mL) at 0 °C. Then the mixture was stirred at room temperature for 12 h. The resulting mixture was quenched by addition of water and extracted with dichloromethane. The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, EtOAc/*n*-Hex = 1:8) to give product **18** (18.0 g, 94.1%) as a white color solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 2.29 (2H, m, CH_2), 2.61 (2H, t, J = 7.03 Hz, CH_2),

3.54 (2H, t, J = 6.22 Hz, CH_2), 7.30 (1H, br s, NH), 6.84–8.23 (3H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 27.79, 33.08, 35.20, 103.58, 111.13, 111.35, 122.43, 122.92, 169.80.

3.1.9. 4-Bromo-*N*-(3,4,5-trichlorophenyl)butyramide (20). 4-Bromobutyryl chloride (1.4 mL, 0.011 mol) was added dropwise to a solution of 3,4,5-trichloroaniline (2.0 g, 0.010 mol) in benzene (50 mL) at 0 °C. Then the mixture was stirred at room temperature for 12 h. The resulting mixture was quenched by addition of water and extracted with dichloromethane. The combined organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, EtOAc/*n*-Hex = 1:8) to give product **20** (2.36 g, 67.1%) as a white color solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 2.29 (2H, m, CH_2), 2.58 (2H, t, J = 6.95 Hz, CH_2), 3.53 (2H, t, J = 6.12 Hz, CH_2), 7.28 (1H, br s, NH), 7.64 (2H, s, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 27.64, 33.08, 35.25, 119.75, 126.58, 134.34, 136.76, 170.14.

3.1.10. General procedure of compounds 5a–o. To a solution of 1-substituted piperazine (0.2879 mmol) in CH_2Cl_2 (0.5 mL) was added triethylamine (0.05 mL, 0.3840 mmol) at room temperature. After 20 min, **4** (0.1920 mmol) in CH_2Cl_2 (1.5 mL) was added to the reaction mixture. The reaction mixture was stirred at room temperature for 24 h, and then the reaction mixture was quenched by addition of saturated NH_4Cl aqueous solution. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, CH_2Cl_2/MeOH = 9:1) to give product **5a**–**o** as a white solid.

Compound **5g**: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.10 (t, J = 7.1 Hz, 3H), 1.56–1.69 (m, 4H), 2.00–2.61 (m, 21H), 3.17 (s, 2H), 3.56 (br s, 2H), 3.71 (br s, 2H), 6.95 (d, J = 7.4 Hz, 1H), 7.23 (t, J = 7.7 Hz, 1H), 7.37 (m, 2H), 8.91 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 11.8, 21.5, 23.2, 26.4, 33.0, 41.6, 45.5, 52.3, 52.6, 53.0, 53.2, 53.6, 58.1, 62.0, 116.6, 120.1, 125.2, 128.9, 137.2, 139.1, 167.5, 171.4; MS (FAB) (*m*/*z*) 430 ([M+H]$^+$, 100), 428 (30), 154 (95), 136 (61); HRMS (FAB) calcd for $C_{24}H_{40}O_2N_5$: [M+H]$^+$ = 430.3104, found = 430.3177.

Compound **5h**: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.58–1.72 (m, 4H), 2.41 (m, 4H), 2.64 (m, 4H), 3.20 (d, J = 5.1 Hz, 4H), 3.22 (s, 2H), 3.59 (t, J = 4.6 Hz, 2H), 3.74 (br s, 2H), 6.85 (t, J = 7.3 Hz, 1H), 6.93 (d, J = 7.9 Hz, 2H), 7.09 (td, J = 1.5, 7.8 Hz, 1H), 7.28 (m, 3H), 7.38 (dd, J = 1.4, 9.4 Hz, 1H), 8.46 (dd, J = 1.5, 8.2 Hz, 1H), 9.87 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 23.1, 26.5, 30.0, 41.7, 45.7, 49.1, 53.1, 53.2, 53.5, 58.1, 62.0, 116.0, 119.7, 120.9, 122.6, 124.7, 127.9, 129.1, 134.3, 151.2, 167.9, 171.4; MS (FAB) (*m*/*z*) 498 ([M+H]$^+$, 99), 496 (34), 154 (100), 136 (68); HRMS (FAB) calcd for $C_{27}H_{37}O_2N_5Cl$: [M+H]$^+$ = 498.2558, found = 498.2635.

Compound **5i**: ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.56–1.74 (m, 4H), 2.38 (t, J = 7.2 Hz, 2H), 2.44 (t, J = 7.4 Hz, 2H), 2.64 (d, J = 2.7 Hz, 8H), 3.11 (t, J = 4.4 Hz, 4H), 3.22 (s, 2H), 3.59 (t, J = 4.4 Hz, 2H), 3.74 (br s, 2H), 6.90–7.07 (m, 5H), 7.29 (m, 1H), 7.38 (dd, J = 1.4, 8.0 Hz, 1H), 8.46 (dd, J = 1.4, 8.3 Hz, 1H), 9.87 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 23.1, 26.4, 33.0, 41.6, 45.6, 50.41, 50.43, 53.1, 53.2, 53.4, 58.1, 61.9, 115.9, 116.1, 118.8, 120.8, 122.3, 122.4, 122.6, 124.3, 124.4, 124.6, 127.8, 129.0, 134.2, 140.0, 140.1, 154.4, 156.8, 167.8, 171.4; MS (EI) (*m*/*z*) 352 (M$^+$, 100), 281 (23), 268 (27), 105 (21), 83 (18), 71 (26), 57 (34); MS (FAB) (*m*/*z*) 516 ([M+H]$^+$, 45), 647 (5), 328 (23), 176 (100), 154 (41); HRMS (FAB) calcd for $C_{27}H_{36}O_2N_5ClF$: [M+H]$^+$ = 516.2463, found = 516.2538.

3.1.11. General procedure of compounds 7a and 7b. Piperazine derivative (0.95 mmol) was added to a solution of 5-bromo-*N*-(2-chlorophenyl)pentanamide (**6**) (250 mg, 0.86 mmol) and Et_3N (0.18 mL, 1.29 mmol) in CH_2Cl_2 (5.0 mL) at room temperature. Then the mixture was stirred at reflux for overnight. The reaction mixture was cooled to room temperature and quenched by addition of water (10 mL). The mixture was extracted with dichloromethane (3× 10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, CH_2Cl_2/MeOH = 20:1) to give product **7a** and **7b**.

Compound **7a**: 54%; mp 102.9 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.63 (2H, m, CH_2), 1.81 (2H, m, CH_2), 2.43–2.50 (4H, m, $2CH_2$), 2.60 (4H, s, $2CH_2$), 3.20 (4H, t, J = 4.78 Hz, $2CH_2$), 7.66 (1H, br s, NH), 6.77–8.38 (8H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 23.40, 26.26, 37.67, 48.61, 53.04, 58.06, 113.83, 115.73, 119.24, 121.67, 124.60, 127.77, 128.99, 130.00, 134.57, 134.94, 152.32, 170.64; IR (KBr) 3282 (NH), 2939, 2822, 1660 (C=O), 1594, 1488 (amide), 1440, 1241, 767 cm^{-1}; HRMS (ES): *m*/*z* calcd for $C_{21}H_{25}Cl_2N_3NaO$: [M+Na]$^+$ = 428.1273, found = 428.1268.

Compound **7b**: 58%; mp 87.4 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.59 (2H, m, CH_2), 1.77 (2H, m, CH_2), 2.38–2.47 (12H, m, $6CH_2$), 3.44 (2H, s, CH_2(benzyl)), 7.65 (1H, br s, NH), 7.02–8.36 (7H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 23.45, 26.27, 37.69, 52.96, 53.14, 58.04, 61.73, 121.70, 124.57, 127.75, 128.31, 128.97, 130.15, 130.79, 132.29, 134.59, 138.75, 171.73; IR (KBr) 3271 (NH), 2944, 2810, 1656 (C=O), 1584, 1532 (amide), 1471, 1441, 1128, 756 cm^{-1}; HRMS (ES): *m*/*z* calcd for $C_{22}H_{26}Cl_3N_3NaO$: [M+Na]$^+$ = 476.1039, found = 476.1032.

3.1.12. General procedure of compounds 9a–l. Piperazine derivative (1.00 mmol) was added to a solution of 4-bromo-*N*-(4-chlorophenyl)butyramide (**8**) (250 mg, 0.90 mmol) and Et_3N (0.19 mL, 1.36 mmol) in CH_2Cl_2 (5.0 mL) at room temperature. Then the mixture was stirred at reflux for overnight. The reaction mixture was cooled to room temperature and quenched by addition of water (10 mL). The mixture was extracted with dichloromethane (3× 10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column

chromatography (SiO_2, CH_2Cl_2/MeOH = 20:1) to give product **9a–l**.

Compound **9f**: 80%; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.94 (2H, m, CH_2), 2.48 (2H, t, J = 5.15 Hz, CH_2), 2.54 (2H, t, J = 7.87 Hz, CH_2), 2.69 (4H, s, $2CH_2$), 3.12 (4H, s, $2CH_2$), 3.86 (3H, s, OCH_3), 6.86–7.49 (8H, m, Ph), 8.80 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.08, 35.77, 50.60, 53.24, 55.40, 56.95, 111.23, 111.31, 111.36, 118.18, 118.45, 121.00, 121.05, 128.83, 128.96, 152.28, 171.47; IR (KBr) 3257 (NH), 2939, 2815, 1665 (C=O), 1595, 1500 (amide), 1239, 1027, 748 cm^{-1}; HRMS (ES): *m/z* calcd for $C_{21}H_{26}ClN_3NaO_2$: $[M+Na]^+$ = 410.1611, found = 410.1611.

3.1.13. General procedure of compounds 11a–j. Piperazine derivative (0.88 mmol) was added to a solution of 4-bromo-*N*-(2,4-dichlorophenyl)butyramide (**10**) (250 mg, 0.80 mmol) and Et_3N (0.22 mL, 1.61 mmol) in CH_2Cl_2 (5.0 mL) at room temperature. Then the mixture was stirred at reflux for overnight. The reaction mixture was cooled to room temperature and quenched by addition of water (10 mL). The mixture was extracted with dichloromethane (3× 10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, CH_2Cl_2/MeOH = 20:1) to give product **11a–j**.

Compound **11c**: 44%; mp 114.4 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.96 (2H, m, CH_2), 2.50–2.53 (4H, m, $2CH_2$), 2.66 (4H, s, $2CH_2$), 3.07 (4H, s, $2CH_2$), 3.86 (3H, s, OCH_3), 8.05 (1H, br s, NH), 6.85–8.34 (7H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.35, 35.71, 50.59, 53.32, 55.35, 57.11, 111.20, 118.17, 120.99, 122.70, 122.96, 123.38, 127.87, 128.73, 129.04, 133.55, 141.25, 152.28, 171.40; IR (KBr) 3246 (NH), 2959, 2935, 2811, 1660 (C=O), 1580, 1521 (amide), 1243, 742 cm^{-1}; HRMS (ES): *m/z* calcd for $C_{21}H_{25}Cl_2N_3NaO_2$: $[M+Na]^+$ = 444.1221, found = 444.1227.

Compound **11d**: 51%; mp 63.8 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.97 (2H, m, CH_2), 2.49 (2H, t, J = 4.83 Hz, CH_2), 2.51 (2H, t, J = 5.14 Hz, CH_2), 2.60 (4H, t, J = 4.96 Hz, $2CH_2$), 3.18 (4H, t, J = 4.97 Hz, $2CH_2$), 3.79 (3H, s, OCH_3), 7.94 (1H, br s, NH), 6.41–8.35 (7H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.31, 35.58, 49.03, 53.07, 55.19, 57.02, 102.51, 104.46, 108.86, 122.57, 127.90, 128.74, 129.78, 133.48, 152.64, 160.58, 171.29; IR (KBr) 3307 (NH), 2954, 2826, 2807, 1665 (C=O), 1586, 1511 (amide), 1210, 1181, 971, 825 cm^{-1}; HRMS (ES): *m/z* calcd for $C_{21}H_{25}Cl_2N_3NaO_2$: $[M+Na]^+$ = 444.1221, found = 444.1225.

Compound **11e**: 43%; mp 81.0 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.91 (2H, m, CH_2), 2.41–2.49 (12H, m, $6CH_2$), 3.48 (2H, s, CH_2(benzyl)), 8.06 (1H, br s, NH), 7.22–8.30 (8H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.36, 35.71, 52.96, 53.06, 57.05, 63.05, 122.82, 127.05, 127.84, 128.20, 128.73, 129.05, 129.20, 133.56, 137.98, 171.40; IR (KBr) 3313 (NH), 2816, 1660 (C=O), 1578, 1509 (amide), 743 cm^{-1}; HRMS (ES): *m/z* calcd for $C_{21}H_{25}Cl_2N_3NaO$: $[M+Na]^+$ = 428.1273, found = 428.1278.

Compound **11f**: 40%; mp 82.2 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.94 (2H, m, CH_2), 2.42–2.49 (12H, m, $6CH_2$), 3.44 (2H, s, CH_2(benzyl)), 8.02 (1H, br s, NH), 7.16–8.31 (7H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.35, 35.70, 52.95, 53.02, 57.04, 63.37, 122.75, 127.17, 127.22, 127.86, 128.73, 129.03, 129.45, 133.54, 134.15, 140.31, 171.36; IR (KBr) 3300 (NH), 2939, 2820, 1656 (C=O), 1578, 1519 (amide), 777 cm^{-1}; HRMS (ES): *m/z* calcd for $C_{21}H_{24}Cl_3N_3NaO$: $[M+Na]^+$ = 462.0883, found = 462.0891.

3.1.14. General procedure of compounds 13a and b. Piperazine derivative (0.801 mmol) was added to a solution of 5-bromo-1-[4-(2-fluorophenyl)piperazin-1-yl]-pentan-1-one (**12**) (250 mg, 0.728 mmol) and Et_3N (0.15 mL, 1.09 mmol) in CH_2Cl_2 (5.0 mL) at room temperature. Then the mixture was stirred at reflux for overnight. The reaction mixture was cooled to room temperature and quenched by addition of water (10 mL). The mixture was extracted with dichloromethane (3× 10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, CH_2Cl_2/MeOH = 20:1) to give product **13a** and **13b**.

Compound **13a**: 48%; mp 85.0 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.56 (2H, m, CH_2), 1.68 (2H, m, CH_2), 1.80 (4H, s, $2CH_2$), 2.38 (4H, t, J = 7.40 Hz, $2CH_2$), 2.48 (4H, s, $2CH_2$), 3.05 (4H, m, $2CH_2$), 3.47 (2H, s, CH_2(benzyl)), 3.64 (2H, t, J = 4.84 Hz, CH_2), 3.79 (2H, t, J = 4.91 Hz, CH_2), 6.90–7.33 (8H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 23.27, 26.54, 33.10, 41.61, 45.74, 50.72, 53.07, 58.11, 62.39, 116.17, 116.38, 119.19, 123.12, 124.54, 127.20, 129.06, 129.45, 134.14, 140.39, 171.45; IR (KBr) 2940, 2815, 1641 (C=O), 1505, 1436, 1234, 1207, 758 cm^{-1}; HRMS (ES): *m/z* calcd for $C_{26}H_{34}ClFN_4NaO$: $[M+Na]^+$ = 495.2303, found = 495.2304.

Compound **13b**: 43%; mp 86.1 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.55 (2H, m, CH_2), 1.67 (2H, m, CH_2), 1.75 (4H, s, $2CH_2$), 2.38 (4H, t, J = 7.46 Hz, $2CH_2$), 2.47 (4H, s, $2CH_2$), 3.04 (2H, t, J = 5.13 Hz, CH_2), 3.07 (2H, t, J = 5.05 Hz, CH_2), 3.50 (2H, s, CH_2(benzyl)), 3.63 (2H, t, J = 4.90 Hz, CH_2), 3.79 (2H, t, J = 4.99 Hz, CH_2), 6.92–7.29 (8H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 23.26, 26.51, 33.08, 41.62, 45.77, 50.74, 53.05, 58.12, 62.24, 116.17, 116.38, 119.22, 123.12, 124.58, 128.35, 130.43, 132.74, 136.70, 171.44; IR (KBr) 2942, 2818, 1638 (C=O), 1505, 1438, 1237, 1208, 754 cm^{-1}; HRMS (ES): *m/z* calcd for $C_{26}H_{34}ClFN_4NaO$: $[M+Na]^+$ = 495.2303, found = 495.2301.

3.1.15. General procedure of compounds 15a and b. Piperazine derivative (0.85 mmol) was added to a solution of 5-bromo-1-(4-phenylpiperazin-1-yl)pentan-1-one (**14**) (250 mg, 0.77 mmol) and Et_3N (0.16 mL, 1.15 mmol) in CH_2Cl_2 (5.0 mL) at room temperature. Then the mixture was stirred at reflux for overnight.

The reaction mixture was cooled to room temperature and quenched by addition of water (10 mL). The mixture was extracted with dichloromethane (3× 10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, CH_2Cl_2/MeOH = 20:1) to give product **15a** and **15b**.

Compound **15a**: 38%; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.54 (2H, m, CH_2), 1.67 (2H, m, CH_2), 2.35–2.46 (8H, m, $4CH_2$), 3.16 (4H, m, $2CH_2$), 3.45 (2H, s, CH_2(benzyl)), 3.62 (2H, t, J = 4.90 Hz, CH_2), 3.77 (2H, t, J = 5.00 Hz, CH_2), 6.89–7.30 (9H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 23.28, 26.58, 33.11, 41.48, 45.55, 49.65, 53.10, 58.15, 62.26, 116.02, 116.64, 120.56, 128.34, 129.25, 130.42, 132.71, 136.73, 150.97, 171.42; HRMS (ES): m/z calcd for $C_{26}H_{35}ClN_4NaO$: $[M+Na]^+$ = 477.2397, found = 477.2391.

3.1.16. General procedure of compounds 17a–l. Piperazine derivative (1.04 mmol) was added to a solution of 3-bromo-*N*-(2,4-difluorophenyl)propionamide (**16**) (250 mg, 0.95 mmol) and Et_3N (0.20 mL, 1.42 mmol) in CH_2Cl_2 (5.0 mL) at room temperature. Then the mixture was stirred at reflux for overnight. The reaction mixture was cooled to room temperature and quenched by addition of water (10 mL). The mixture was extracted with dichloromethane (3× 10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, only EtOAc) to give product **17a**–**l**.

Compound **17a**: 96%; mp 91.7 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 2.61 (2H, t, J = 5.74 Hz, CH_2), 2.77 (6H, s, $3CH_2$), 3.30 (4H, s, $2CH_2$), 6.78–8.39 (8H, m, Ph), 11.22 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 32.57, 48.91, 52.44, 53.65, 103.30, 110.99, 116.21, 120.01, 122.32, 123.48, 129.18, 170.64; IR (KBr) 3113 (NH), 2827, 1674 (C=O), 1599, 1555, 1503 (amide), 1248, 1096, 959, 753 cm^{-1}; HRMS (ES): m/z calcd for $C_{19}H_{21}F_2N_3NaO$: $[M+Na]^+$ = 368.1550, found = 368.1548.

Compound **17b**: 98%; mp 102.3 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 2.61 (2H, t, J = 5.76 Hz, CH_2), 2.79 (6H, s, $3CH_2$), 3.21 (4H, s, $2CH_2$), 6.83–8.42 (7H, m, Ph), 11.25 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 32.53, 50.15, 52.49, 53.66, 103.28, 111.01, 116.16, 122.37, 122.75, 123.52, 139.75, 170.69; IR (KBr) 3124 (NH), 2929, 2830, 1687 (C=O), 1610, 1561, 1500 (amide), 1244, 1137, 923, 758 cm^{-1}; HRMS (ES): m/z calcd for $C_{19}H_{20}F_3N_3NaO$: $[M+Na]^+$ = 386.1456, found = 386.1450.

Compound **17i**: 89%; ^1H NMR (400 MHz, $CDCl_3$) δ = 2.54–2.78 (12H, m, $6CH_2$), 3.52 (2H, s, CH_2(benzyl)), 6.83–8.41 (7H, m, Ph), 11.28 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 32.56, 52.36, 52.65, 53.56, 62.24, 103.28, 110.94, 122.46, 123.50, 127.21, 128.99, 129.51, 134.21, 140.32, 170.81; HRMS (ES): m/z calcd for $C_{20}H_{22}ClF_2N_3NaO$: $[M+Na]^+$ = 416.1317, found = 416.1321.

Compound **17j**: 49%; mp 111.2 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 2.55–2.72 (12H, m, $6CH_2$), 3.51 (2H, s, CH_2(benzyl)), 6.82–8.38 (7H, m, Ph), 11.27 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 32.54, 52.36, 52.59, 53.54, 62.07, 103.29, 110.94, 122.44, 123.63, 128.40, 130.36, 132.82, 136.57, 170.80; IR (KBr) 3118 (NH), 3049, 2939, 2819, 1680 (C=O), 1613, 1555, 1506 (amide), 1491, 1432, 1234, 1132, 1010, 961, 807 cm^{-1}; HRMS (ES): m/z calcd for $C_{20}H_{22}ClF_2N_3NaO$: $[M+Na]^+$ = 416.1317, found = 416.1313.

Compound **17l**: 69%; mp 104.3 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 2.56–2.72 (12H, m, $6CH_2$), 3.50 (2H, s, CH_2(benzyl)), 6.83–8.39 (6H, m, Ph), 11.24 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 32.53, 52.31, 52.58, 53.51, 61.55, 103.27, 110.96, 122.40, 123.60, 128.23, 130.19, 130.71, 130.97, 132.34, 138.56, 170.74; IR (KBr) 3182 (NH), 2940, 2823, 1678 (C=O), 1612, 1568, 1504 (amide), 1468, 1435, 1254, 1136, 960, 840, 809 cm^{-1}; HRMS (ES): m/z calcd for $C_{20}H_{21}Cl_2F_2N_3NaO$: $[M+Na]^+$ = 450.0927, found = 450.0925.

3.1.17. General procedure of compounds 19a–l. Piperazine derivative (1.80 mmol) was added to a solution of 4-bromo-*N*-(2,4-difluorophenyl)butyramide (**18**) (500 mg, 1.80 mmol) and Et_3N (0.30 mL, 2.16 mmol) in CH_2Cl_2 (10.0 mL) at room temperature. Then the mixture was stirred at reflux for overnight. The reaction mixture was cooled to room temperature and quenched by addition of water (20 mL). The mixture was extracted with dichloromethane (3× 15 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, only EtOAc) to give product **19a-l**.

Compound **19b**: 46%; mp 105.2 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.96 (2H, m, CH_2), 2.52 (4H, m, $2CH_2$), 2.60 (4H, s, $2CH_2$), 3.12 (4H, s, $2CH_2$), 6.82–8.26 (7H, m, Ph), 8.29 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.10, 35.38, 50.35, 53.08, 56.67, 103.50, 111.19, 116.09, 118.87, 122.51, 122.81, 123.21, 124.45, 139.97, 171.42; IR (KBr) 3274 (NH), 2756, 1654 (C=O), 1613, 1535, 1502 (amide), 1429, 1354, 1251, 1207, 1139, 1099, 959, 844, 747 cm^{-1}; HRMS (ES): m/z calcd for $C_{20}H_{22}F_3N_3NaO$: $[M+Na]^+$ = 400.1613, found = 400.1617.

Compound **19c**: 43%; mp 86.7 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.96 (2H, m, CH_2), 2.52 (4H, m, $2CH_2$), 2.62 (4H, t, J = 4.95 Hz, $2CH_2$), 3.12 (4H, t, J = 4.95 Hz, $2CH_2$), 8.18 (1H, br s, NH), 6.82–8.25 (7H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.14, 35.35, 50.07, 53.03, 56.58, 103.49, 111.20, 115.51, 117.80, 123.07, 147.86, 171.34; IR (KBr) 3288 (NH), 2945, 2820, 1667 (C=O), 1531, 1509 (amide), 1267, 1239, 1229, 1153, 825, 816 cm^{-1}; HRMS (ES): m/z calcd for $C_{20}H_{22}F_3N_3NaO$: $[M+Na]^+$ = 400.1613, found = 400.1618.

Compound **19d**: 33%; mp 123.1 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.97 (2H, m, CH_2), 2.51 (2H, t,

J = 6.93 Hz, CH_2), 2.55 (2H, t, J = 6.66 Hz, CH_2), 2.67 (4H, s, $2CH_2$), 3.08 (4H, s, $2CH_2$), 6.85–8.28 (7H, m, Ph), 8.34 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.12, 35.43, 51.03, 53.16, 56.65, 103.49, 111.19, 120.27, 123.20, 123.72, 127.56, 128.75, 130.62, 149.10, 171.43; IR (KBr) 3276 (NH), 2961, 2820, 1665 (C=O), 1614, 1504 (amide), 1480, 1261, 1142, 854, 756, 690 cm^{-1}; HRMS (ES): m/z calcd for $C_{20}H_{22}ClF_2N_3NaO$: $[M+Na]^+$ = 416.1317, found = 416.1313.

Compound **19e**: 30%; mp 90.3 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.98 (2H, m, CH_2), 2.52 (4H, t, J = 6.66 Hz, $2CH_2$), 2.64 (4H, s, $2CH_2$), 3.22 (4H, s, $2CH_2$), 8.13 (1H, br s, NH), 6.76–8.24 (7H, m, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 21.97, 35.18, 48.43, 52.77, 56.58, 103.27, 111.21, 113.90, 115.80, 119.45, 123.15, 130.03, 134.94, 152.12, 171.22; IR (KBr) 3245 (NH), 2962, 2816, 1666 (C=O), 1593, 1533 (amide), 1485, 1258, 1242, 1143, 1099, 943, 849, 680 cm^{-1}; HRMS (ES): m/z calcd for $C_{20}H_{22}ClF_2N_3NaO$: $[M+Na]^+$ = 416.1317, found = 416.1315.

Compound **19f**: 53%; mp 83.9 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.96 (2H, m, CH_2), 2.52 (4H, m, $2CH_2$), 2.67 (4H, s, $2CH_2$), 3.09 (4H, s, $2CH_2$), 3.87 (3H, s, OCH_3), 6.82–8.23 (7H, m, Ph), 8.43 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.13, 35.48, 50.49, 53.23, 55.32, 56.70, 103.49, 111.18, 118.11, 120.95, 122.97, 123.26, 141.15, 152.22, 171.52; IR (KBr) 3289 (NH), 2941, 2818, 1660 (C=O), 1595, 1531, 1505 (amide), 1430, 1243, 1130, 960, 743 cm^{-1}; HRMS (ES): m/z calcd for $C_{21}H_{25}F_2N_3NaO_2$: $[M+Na]^+$ = 412.1812, found = 412.1817.

Compound **19g**: 53%; mp 55.3 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.96 (2H, m, CH_2), 2.51 (4H, m, $2CH_2$), 2.61 (4H, t, J = 4.78 Hz, $2CH_2$), 3.20 (4H, t, J = 4.78 Hz, $2CH_2$), 3.80 (3H, s, OCH_3), 6.41–8.26 (8H, m, Ph and NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.11, 35.34, 48.94, 52.98, 55.16, 56.62, 102.50, 103.52, 104.44, 108.83, 111.28, 123.17, 129.77, 152.58, 160.55, 171.38; IR (KBr) 3294 (NH), 2944, 2822, 1670 (C=O), 1611, 1580, 1524, 1501 (amide), 1465, 1258, 1203, 1139, 1010, 844, 759 cm^{-1}; HRMS (ES): m/z calcd for $C_{21}H_{25}F_2N_3NaO_2$: $[M+Na]^+$ = 412.1812, found = 412.1818.

Compound **19j**: 59%; mp 79.0 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.90 (2H, m, CH_2), 2.45 (12H, m, $6CH_2$), 3.44 (2H, s, CH_2(benzyl)), 6.85–8.19 (7H, m, Ph), 8.44 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.09, 35.40, 52.80, 52.91, 56.60, 62.14, 103.46, 111.10, 123.38, 128.30, 130.32, 132.67, 136.57, 171.48; IR (KBr) 3328 (NH), 2953, 2821, 1792, 1671 (C=O), 1610, 1535, 1520, 1500 (amide), 1431, 1259, 1137, 1097, 1009, 849 cm^{-1}; HRMS (ES): m/z calcd for $C_{21}H_{24}ClF_2N_3NaO$: $[M+Na]^+$ = 430.1474, found = 430.1480.

Compound **19k**: 65%; mp 69.3 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.89 (2H, m, CH_2), 2.46 (12H, m, $6CH_2$), 3.70 (2H, s, CH_2(benzyl)), 6.82–8.19 (6H, m, Ph), 8.49 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.12, 35.57, 52.29, 52.50, 52.95, 56.64, 103.53, 111.11, 113.92, 123.76, 125.40, 129.09, 136.60, 171.56; IR (KBr) 3303 (NH), 2943, 2821, 1680 (C=O), 1661, 1610, 1556, 1538, 1500 (amide), 1453, 1430, 1141, 1007, 854 cm^{-1}; HRMS (ES): m/z calcd for $C_{21}H_{23}ClF_3N_3NaO$: $[M+Na]^+$ = 4448.1379, found = 448.1373.

Compound **19l**: 50%; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.91 (2H, m, CH_2), 2.44 (12H, m, $6CH_2$), 3.42 (2H, s, CH_2(benzyl)), 6.83–8.24 (6H, m, Ph), 8.36 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 22.13, 35.45, 52.88, 52.92, 56.59, 61.70, 103.50, 111.15, 123.35, 128.24, 130.15, 130.72, 132.29, 138.65, 171.46; HRMS (ES): m/z calcd for $C_{21}H_{23}Cl_2F_2N_3NaO$: $[M+Na]^+$ = 464.1084, found = 464.1087.

3.1.18. General procedure of compounds 21a–l. Piperazine derivative (0.80 mmol) was added to a solution of 4-bromo-*N*-(3,4,5-trichlorophenyl)butyramide (**20**) (250 mg, 0.72 mmol) and Et_3N (0.15 mL, 1.09 mmol) in CH_2Cl_2 (5.0 mL) at room temperature. Then the mixture was stirred at reflux for overnight. The reaction mixture was cooled to room temperature and quenched by addition of water (10 mL). The mixture was extracted with dichloromethane (3× 10 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, CH_2Cl_2/MeOH = 20:1) to give product **21a–l**.

Compound **21f**: 47%; mp 178.0 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.94 (2H, m, CH_2), 2.50 (2H, t, J = 6.61 Hz, CH_2), 2.56 (2H, t, J = 6.22 Hz, CH_2), 2.72 (4H, s, $2CH_2$), 3.15 (4H, s, $2CH_2$), 3.87 (3H, s, OCH_3), 6.87–7.68 (6H, m, Ph), 9.34 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 21.69, 36.25, 50.54, 53.31, 55.41, 57.17, 111.24, 118.27, 119.74, 121.09, 123.37, 125.81, 134.20, 137.64, 140.80, 152.27, 171.69; IR (KBr) 3247 (NH), 2941, 2820, 1673 (C=O), 1588, 1522, 1498 (amide), 1443, 1240, 1186, 1150, 754 cm^{-1}; HRMS (ES): m/z calcd for $C_{21}H_{24}Cl_3N_3NaO_2$: $[M+Na]^+$ = 478.0832, found = 478.0836.

Compound **21i**: 54%; mp 115.8 °C; ^1H NMR (400 MHz, $CDCl_3$) δ = 1.90 (2H, m, CH_2), 2.49 (12H, m, $6CH_2$), 3.51 (2H, s, CH_2(benzyl)), 7.00–7.73 (6H, m, Ph), 9.43 (1H, br s, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 21.65, 36.38, 52.83, 53.05, 57.28, 62.35, 119.84, 127.13, 127.04, 128.98, 129.57, 134.15, 134.27, 137.65, 140.10, 171.67; IR (KBr) 3307 (NH), 2951, 2809, 1663 (C=O), 1577, 1507 (amide), 1439, 1383, 1148, 857, 778 cm^{-1}; HRMS (ES): m/z calcd for $C_{21}H_{23}Cl_4N_3NaO$: $[M+Na]^+$ = 496.0493, found = 496.0486.

3.2. Biological assay

3.2.1. FDSS 6000 assay (calcium imaging plate reader). HEK293 cells which stably express both α_{1G} and Kir2.1 subunits[23] were grown in Dulbecco's modified Eagle's

medium supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), geneticin (500 μg/ml), and puromycin (1 μg/ml) at 37 °C in a humid atmosphere of 5% CO_2 and 95% air. Cells were seeded into 96-well black wall clear-bottomrf plates at a density of 40,000 cells/well and were used on the next day for high-throughput screening (HTS) FDSS 6000 assay.[24] For FDSS6000 assay, cells were incubated for 60 min at room temperature with 5 μM fluo3/AM and 0.001% Pluronic F-127 in a HEPES-buffered solution composed of (in mM): 115 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 20 HEPES, and 13.8 glucose (pH 7.4). During fluorescence-based FDSS6000 assay, α_{1G} T-type Ca^{2+} channels were activated using a high concentration of KCl (70 mM) in 10 mM $CaCl_2$ containing HEPES-buffered solution, and the increase in $[Ca^{2+}]_i$ by KCl-induced depolarization was detected. All data were collected and analyzed using FDSS6000 and related software (Hamamatsu, Japan).

3.2.2. Electrophysiological recordings. For the recordings of α_{1G} T-type Ca^{2+} currents, the standard whole-cell patch–clamp method was utilized as previously described.[25] Briefly, borosilicate glass electrodes with a resistance of 3–4 MΩ were pulled and filled with the internal solution containing (in mM): 130 KCl, 11 EGTA, 5 Mg-ATP, and 10 HEPES (pH 7.4). The external solution contained (in mM): 140 NaCl, 2 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4). α_{1G} T-type Ca^{2+} currents were evoked every 15 s by a 50 ms depolarizing voltage step from −100 mV to −30 mV. The molar concentrations of test compounds required to produce 50% inhibition of peak currents (IC_{50}) were determined from fitting raw data into dose–response curves. The current recordings were obtained using an EPC-9 amplifier and Pulse/Pulsefit software program (HEKA, Germany).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.11.004.

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