Discovery of Potential Novel TRPC 4 Inhibitor by Combination of Virtual Screening and 3D-QSAR Modeling

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Abstract The modulation of canonical transient receptor potential 4 (TRPC4) may be pivotal in diverse physiological and physiopathological functions in human. To investigate the potential therapeutical roles of novel TRPC4 ligands in diabetes mellitus, a series of compounds with the top rank of Hiphop pharmacophore model and with good fitness were chosen through computer-aided drug design and a series of virtual screening methods. Then, the effects of these compounds were evaluated by bioassay studies on the growth of rat islet cells. The novel compound 7 amoung these compounds promoted the growth of rat islet cells with an EC_{50} value at 3.58 μmol/L. However, compound 7 failed to show any effect on rat islet cells with TRPC4 knocked down. Meanwhile, the expressions of TRPC4 in mRNA level were significantly decreased after the administration of compound 7 in rat islet cells. Therefore, the therapeutical potential of compound 7 in diabetes mellitus may function through inhibiting TRPC4.

Keywords computer-aided drug design; canonical transient receptor potential; inhibitor; virtual screening; diabetes mellitus

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综合运用虚拟筛选三维定量构效关系模型发现潜在 TRPC4 配体

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摘 要 瞬时感受器电位通道 4(TRPC4)可能是一种调节人体生理和病理生理功能的关键靶点. 通过计算机辅助药物设计和一系列虚拟筛选方法, 结合 Hiphop 药效学团模型, 筛选出一系列可能具有良好活性的的 TRPC4 配体化合物, 并研究了这些化合物对大鼠胰岛细胞生长的影响. 结果显示, 其中新化合物 7 促进了大鼠胰岛细胞的生长, EC_{50} 值为 3.58 μmol/L. 然而, 化合物 7 对被干扰了 TRPC4 表达的大鼠胰岛细胞的生长无显著性作用. 与此同时, 给予化合物 7 后, 大鼠胰岛细胞 TRPC4 mRNA 的表达显著降低. 因此, 化合物 7 可能是一个潜在的 TRPC4 抑制剂, 从而可能对糖尿病的治疗起到一定的作用.

关键词 计算机辅助药物设计; 瞬时感受器电位通道 4; 抑制剂; 虚拟筛选; 糖尿病

Canonical transient receptor potential 4 (TRPC4) can regulate Ca^{2+} influx and Ca^{2+} store-operated

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mechanisms, and involve in diverse physiological processes[1,2]. In recent years, increasing studies have indicated the relationship between TRPC4 and diabetes mellitus. For example, the down-regulation of TRPC4 can restore the erectile function of diabetic rats[3]. Another study found that dexamethasone, Rasd1 and TRPC4 may be related to the secretion of insulin in pancreatic β cells[4]. All these findings may provide a new research direction for the treatment of diabetes through inhibiting TRPC4.

It's publicly known TRPC4 inhibitors can be a tool for further study of TRPC4 function. Although many TRPC4 inhibitors such as SKF96365, 2-APB, Fenamic acid and its analogues, ML204 and Benzimidazole derivatives have been discovered[5-7], there are *hitherto* no known specific inhibitors for this channel. Meanwhile, other TRP channel antagonists and their analogs, including compound groups such as ureas, cinnamides, carboxamides, and imidazoles, are reasonable candidates for inhibiting the function of the TRPC4 protein. For example, several studies have shown that TRPC5 inhibitors are likely to show more potent inhibition of TRPC4[8]. To find more effective and specific TRPC4 inhibitors, candidate molecules from large databases were screened by computer-aided drug design (CADD). The screening results were ultimately validated through bioassays. The related workflow is shown in Fig.1.

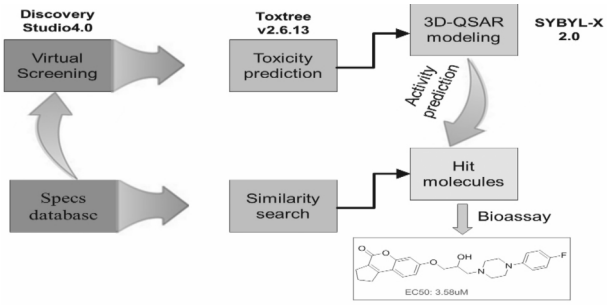


Fig.1 Flowchart for screening of TRPC4 ligand

图 1 筛选 TRPC4 抑制剂的流程图

1 Material and Methods

1.1 Chemicals

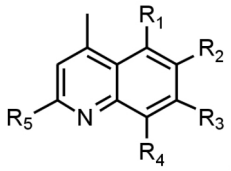
MTT and glibenclamid were obtained from Solarbio (Beijing, China), DMSO and other commonly used chemicals were purchased from Xilong Scientific (Guangdong, China).

1.2 Data-set preparation

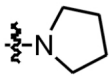
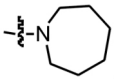
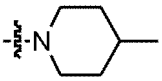
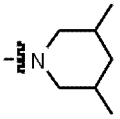
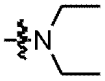
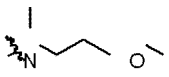
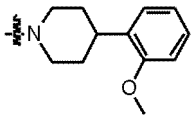
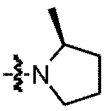
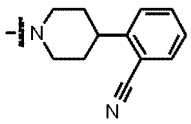
Twenty-five TRPC4 inhibitors were collected from publications[7], as shown in Tab. 1. The training set contained 18 active molecules and 7 was selected as test set to build 3D-QSAR model. In addition, the specification data from Specs Database were screened by our laboratory through drug-five rules and Veber rules.

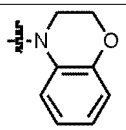
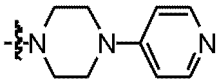
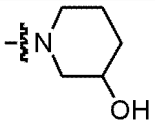
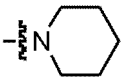
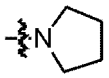
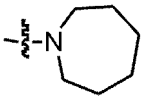
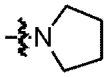
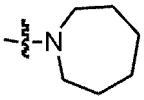
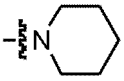
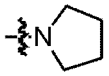
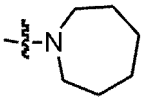
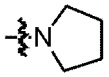
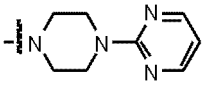
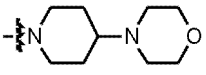
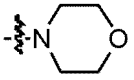
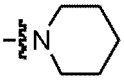
Tab.1 Prublished TRPC4 inhibitors

表 1 已报导的 TRPC4 抑制剂的活性分子



1-25

No.	R_1	R_2	R_3	R_4	R_5
1	H	H	H	H	
2	H	H	H	H	
3	H	H	H	H	
4	H	H	H	H	
5	H	H	H	H	
6	H	H	H	H	
7	H	H	H	H	
8	H	H	H	H	
9	H	H	H	H	

10	H	H	H	H	
11	H	H	H	H	
12	H	H	H	H	
13	H	CH_3	H	H	
14	H	CH_3	H	H	
15	H	CH_3	H	H	
16	H	CH_2 CH_3	H	H	
17	H	CH_2 CH_3	H	H	
18	H	F	H	H	
19	H	F	H	H	
20	H	F	H	H	
21	H	H	H	CH_3	
22	H	H	H	H	
23	H	H	H	H	
24	H	H	H	H	
25	H	H	H	H	

1.3　3D-QSAR modeling

The CoMFA model as constructed by using the molecular alignment method[9] (Fig.2). In this study, the grid spacing was set to 2 Å in the X, Y, and Z directions and automatically generated as a three-dimensional cubic lattice in the grid area. Lennard-Jones potential and Coulomb potential were employed to calculate steric and electrostatic energies of each molecule, respectively, and a sp^3-hybridized carbon atom with a + 1 charge was used as the probe atom to determine the magnitude of the field values[10]. To improve the signal-to-noise ratio, the column filtration and cut-off values were set at 2.0 kcal/mol and 30 kcal/mol, respectively, and the domination was reduced by the large steric and electrostatic energies. Finally, regression analysis was performed by partial least squares (PLS), and the final model was obtained. A satisfactory result of CoMFA model was obtained as shown in Tab. 2.

Toxic hazard of carcinogenicity and genotoxicity was predicted by Toxtree(version, v2.6.13).

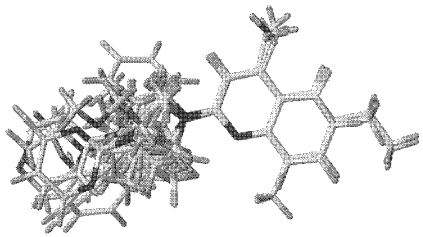


Fig.2　The alignment of small molecules

图 2　小分子的空间结构排列

Tab. 2　The experimental inhibitory activity, the predicted values and residuals

表 2　化合物抑制率、预测值和残差值

No.	pIC_{50}	Pred.	Res.
1*	5.5607	5.247	0.3137
2	5.8239	5.296	0.5279
3	5.2749	5.251	0.0239
4	4.8986	5.349	−0.4504
5	5.2807	5.152	0.1287
6	4.6815	5.213	−0.5315
7	5.1938	5.206	−0.0122
8	5.6038	5.221	0.3828
9	4.9598	4.984	−0.0242
10*	4.9219	5.211	−0.2891
11	4.7007	4.735	−0.0343
12	5.1355	4.955	0.1805
13*	5.2351	5.333	−0.0979
14*	5.8239	5.48	0.3439

15*	5.8861	5.448	0.4381
16	6.2366	5.511	0.7256
17	5.1325	5.6	−0.4675
18	4.9788	5.127	−0.1482
19	5.9788	5.237	0.7418
20	5.399	5.124	0.275
21	4.9722	5.316	−0.3438
22*	4.6345	5.294	−0.6595
23*	4.4225	4.64	−0.2175
25*	4.4045	4.996	−0.5915
25	6.0177	5.268	0.7497

Notes: * Test samples for 3D-QSAR model validation.

Abbreviations: No., compound number; IC_{50}, half-maximal inhibitory concentration; pIC_{50}, negative logarithm of IC_{50}; Pred., predicted activity; Res., residue.

1.4 Cells culture

Rat pancreatic islet cell line (INS-1) was purchased from China infrastructure of cells line resources in Kunming. Cells were routinely cultured in RPMI 1640 supplied with 10% fetal bovine serum (FBS), 100 U/mL G-penicillin and 100 μg/mL streptomycin in a humidified environment of 5% CO_2 and air at 37℃. Cells were passaged every 3-4 days.

1.5 MTT assay

MTT assays were used for determining cell viability and proliferation. MTT stock solution was prepared by solving MTT at a concentration of 5 mg/mL in phosphate-buffered saline (PBS), sterilized with a 0.2 μm filter and then stored in a capped lightproof container at 4 ℃. For this study, islet cells were plated onto 96-well plates. After being cultured for 24 h, the culture medium was removed. Then compound 7 was added into the medium at three different concentrations (3.14 μmol/L, 0.314 μmol/L, 0.0314 μmol/L). RPMI 1640 medium with 10% FBS was used for control group. Glibenclamide was used as the positive control group at the concentration of 1 μg/L. After another 24 h, MTT was added 100 μL each well and incubated for 4 h. Next, DMSO was added 200 μL each well. After mixed on a shaking table for 10 min, absorbance was detected at 490 nm with a Multimode Reader (Thermo Fisher).

1.6 RNA interference

Small interfering RNAs (siRNAs) for rat TRPC4 and TRPC5 were obtained from Qiagen (Valencia, USA). The cultured islet cells were seeded onto six well plates (2×10^5 cells per well) in complete medium. After 24 h, the cells were serum-deprived and 10 μL of 20 μmol · L^{-1} siRNA mixture, or siRNA control was transfected into the cells using HiPerFect transfection reagent (Qiagen). After 72 h of transfection, RT-PCR was carried out to confirm that the mRNA expressions of TRPC4 or TRPC5 had been knocked down in the cells.

1.7 RNA isolation and real-time quantitative PCR

Total RNA was extracted from MAECs using TRIzol reagent (CWbio. Beijing) following the manufacturer's protocol. Equal amounts of RNA were reverse-transcribed into cDNA. Primers for TRPC4 (sense: 5′-ACCATCGTGGAGTGGATGA-3′; antisense: 5′-TGTCGCCAGATACAAGGAGT-3′) and β-actin (sense: 5′-CGGCCATCACGCCACAGGCTT-3′; antisense: 5′-CGTCTTCACCACCATGGAGA-3′) were used to generate a 147 bp and a 197 bp product, respectively. Quantitative real-time PCR was performed using the UltraSYBR (with ROX, CWbio). The PCR amplification included an initial denaturation at 95 ℃ for 3 min, 40 cycles of denaturation at 94 ℃ for 40 s, annealing at 60 ℃ for 2 min, and extension for 30 s, at 72 ℃. Results of the log-linear phase of the growth curve were analyzed and relative quantification was performed using the $2^{-\Delta\Delta CT}$ method with β-actin as a house-keeping gene.

1.8 Statistical analysis

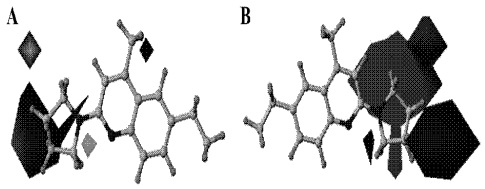
Statistical analyses were performed using SPSS22.0. Data were presented as means ± S.E.M. Comparison between two groups was analyzed using Student's *t*-test.

2 Results

2.1 Analysis of 3D-QSAR modeling

After building 3D-QSAR model, we got a q^2 value of 0.51 and the r^2 value of 0.979 as well as the optimum number of components of 6 and the r^2_{pred} value of 0.605, which confirms the reliability and predictability of the CoMFA model. The 3D-QSAR models graphically displays the distribution of various fields in a different color pattern, so that the effects of the various field distributions on the activity of the compounds can be clearly seen, and the new and effective molecules can be further designed by modifying or altering the existing compound structures. 3D contour maps of CoMFA model is shown in Fig.3.

According to the results of activity prediction by 3D-QSAR model, the activity of candidate molecules at



1) Steric fields; B) Electrostatic fields

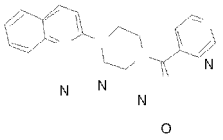
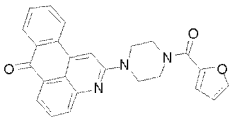
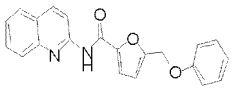
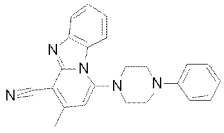
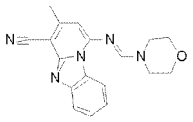
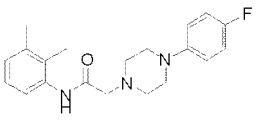
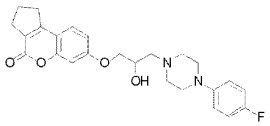
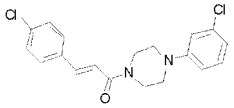
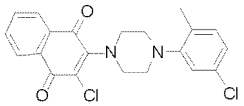
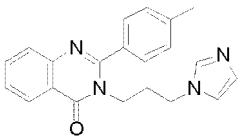
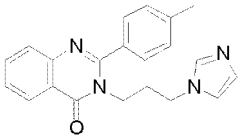
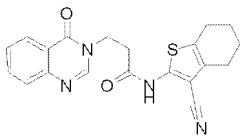
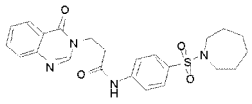
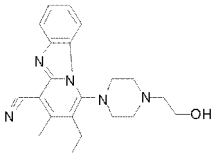
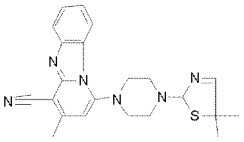
Fig.3　The 3D contour maps of CoMFA model

图 3　CoMFA 模型的三维轮廓图

themicro mole level remained (Tab. 3). However, there were several new skeletons appearing in the hit molecules, which were different from quinoline compounds. It deserves further study to discover more effective and novel skeleton TRPC4 inhibitors. Furthermore, the effectiveness of these hit molecules would be tested by bioassay.

Tab.3　The results of PLS analysis for the CoMFA

表 3　CoMFA 模型的偏最小二乘法的分析结果

No.	Specs ID.	Structures	Pred CoMFA (pIC50)
1	AG-664/32340051		4.954
2	AO-022-43452489		5.013
3	AO-080-43441654		5.23
4	AO-476-14976024		5.221
5	AO-476-40672264		5.231
6	AA-768-37210004		4.805
7	AP-124-43383641		5.042
8	AA-768-37237005		5.139
9	AA-768-37237006		5.07
10	AE-848-37390018		5.007
11	AO-022-43453045		5.203
12	AM-879-42000757		4.855
13	AN-153-43092958		5.251
14	AE-641-15086199		5.211
15	AF-399-34464049		5.207

Abbreviations: No., compound number; Pred CoMFA, predicted activity by CoMFA model

2.2　Effects of compound 7 on viability of rat islet cells (INS-1 cell line)

The EC_{50} values of this compound and glibenclamide were shown in Tab.4. As demonstrated in Fig.4, the administration of compound 7 significantly decreased the expressions of TRPC4 in INS-1 cell line.

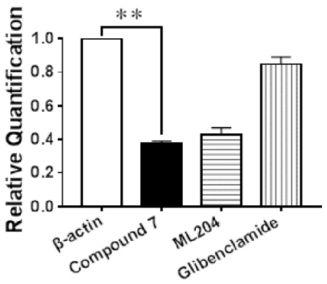
The inhibitory effect of compound 7 was similar to that of ML204[7]. Glibenclamide showed no effect on the expression of TRPC4 in INS-1 cells.

Tab.4 EC_{50} values of compound 7 and glibenclamide obtained by MTT

表4 MTT法测得的化合物7与格列本脲的EC_{50}值

Compounds	Glibenclamide /(μmol/L)	Compound 7 /(μmol/L)	Compound 7 (INS-1 with TRPC4 knocked down) /(μmol/L)	Compound 7 (INS-1 with TRPC5 knocked down) /(μmol/L)
EC_{50}	0.162±0.042	3.581±0.214	1.469±0.092	5.002±1.024

Values are means ± SEM. Data were obtained from five separate experiments ($n=5$). Significance of the difference between groups is indicated as follows: $^{*}P<0.05$, compared with Compound 7 group.



Values are means ± SEM; data were obtained from five separate experiments ($n=5$); significance of the difference between groups was indicated as follows: $^{**}P<0.01$

Fig.4 Effect of the Compound 7 on the expressions of TRPC4 in INS-1 (relative to β-actin)

图4 化合物7对INS-1中TRPC4表达的影响(相对于β-actin).

3 Discussion

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both[11]. Therefore, chemicals with stimulative function on the growth of islet cells may show potential therapeutic effect on diabetes mellitus. Studies have shown that the expressions of various TRPC subtypes (such as TRPC1 and TRPC4) are significantly changed in type 1 and 2 diabetes patients and diabetic animal tissues (such as kidney, blood vessels, and inflammatory cells)[12]. By performing MTT assay in the present study, EC_{50} value of compound 7 was not significantly different from that of glibenclamide. As type I diabetes has the feature of low secretion of insulin, increasing the amount of islet cells may be helpful for increasing the release of insulin in patients with type I diabetes. Moreover, compound 7 shows significant inhibitory effect on the expression of TRPC4 in islet cells. The results suggested that the inhibitor of TRPC4, compound 7, may have the potential positive effect on type 1 diabetes and related diseases. More importantly, since the close homology of TRPC4 and TRPC5 is particularly evident in the N-terminus and the transmembrane domains with about 80% identity in primary sequence[13], it is imperative to distinguish effects of compound 7 on TRPC4 and TRPC5. By performing RNA interference, compound 7 still markedly influenced the growth of INS-1 with TRPC5 knocked down but did not show effect on that of INS-1 with TRPC4 knocked down. This finding indicates compound 7 may play selective role between TRPC4 and TRPC5. Of course, further studies such as patch clamp analysis and compound affinity detection should be performed to verify the specificity and potency of the inhibitory effect of compound 7 on TRPC4, especially in islet cells.

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