Original Article

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Synthesis, Evaluation of Vasorelaxant Activity, and Molecular Docking of Pyranopyrazole Derivatives as Calcium Channel Blockers

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Abstract

Pyranopyrazole analogs are novel synthetic compounds with many biological activities. In this research,we synthesized six new pyranopyrazole derivatives using a biocompatible catalyst and evaluated their vasorelaxant and calcium channel binding properties in isolated rat thoracic aorta. Male Sprague-Dawley rats (n=42) were used. The thoracic aorta was isolated and divided into four 4 mm rings. Each ring was connected to a pressure transducer and a hook in an organ bath. The rings were treated with KCl (40 mM) solution and the increased contractions were recorded. After washing out and maintaining the baseline tension, the tissues were pre-incubated with different concentrations of nifedipine (10^{-10} to 10^{-6} M) or each of the synthetic compounds (10^{-9} to 10^{-5} M) for 20 minutes, and exposed once again with KCl (40 mM). The concentration-response curves were plotted and their pIC_{50} (negative logarithm of the required concentrations of compounds to achieve half-maximal relaxation) and R_{max} (percent of compounds-evoked maximum relaxation) were calculated. Molecular docking studies were carried out using AutoDock software. Homology modeling was done to make the human $Ca_v1.2$ ($hCa_v1.2$) protein pdb file. The results showed that all compounds sat efficiently in the calcium channel active site. Also, we found that all compounds (except compound 6) significantly attenuated the KCl-induced contractions of isolated aorta rings in a concentration-dependent manner, although not as potent as nifedipine. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. In conclusion, most of our new pyranopyrazole analogs showed vasorelaxant and calcium channel blocking activities and could be good candidates for further investigations to develop new antihypertensive drugs.

Keywords: Calcium Channel blockers, Heterogeneous Catalyst, Molecular Docking, Pyranopyrazoles, Vasorelaxant.

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1. Introduction

Pyranopyrazole scaffold is one of the most attractive pharmacophores (1). A large number of pyranopyrazoles have been tested for many biological properties and have shown good activity as fungicidal (2), bactericidal (3), antiplatelet (4), and anticancer (5, 6). The first goal of this research was to synthesize six new 6-Amino-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile derivatives. The molecular structures of all six synthesized compounds were the same except in the substit-

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uent at C4 position of the pyran ring. There are several methods for the chemical synthesis of pyranopyrazoles (7-9). In this research, we synthesized our new pyranopyrazole derivatives using the reaction of aromatic or aliphatic aldehydes, hydrazine hydrate, malonitrile and ethyl acetoacetate in the presence of a nanocrystalline catalyst (TiO_2/Hydroxylapatite on magnetic bovine serum albumin) (NCTHMB)without any toxic residues. The second aim of this study was to investigate the vasorelaxant activity of these molecules in isolated rat thoracic aorta. As the third goal, we were interested in evaluating the calcium channel blocking activity of the compounds using molecular docking studies.

2. Materials and methods

2.1. Animals

Forty-two male Sprague-Dawley rats (200-220 g) were obtained from Animal Breeding Center, Shiraz University of Medical Sciences, Shiraz, Iran. The animals were housed in groups of 6-7 under standard conditions (12 h light/dark cycle, temperature: 20-25 °C, and humidity: 25-35%) with standard rat chow and drinking water ad libitum. All animal procedures were approved by the Institution of Animal Care and Use Committee ofShiraz University of Medical Sciences. (Approval number; 975317)

2.2. Materials

The chemical materials used in this study were as follow: aromatic aldehyde (1 mM), β-ketoesters (1 mM), hydrazinehydrate 96% (1 mM), malonitrile (1 mM), the catalyst (5 mol%), NaCl (118 mM) KCl (4.8mM), $CaCl_2$ (2.5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $NaHCO_3$(25 mM), D-glucose (11mM) and nifedipine. All of the chemical compounds were of analytical grade and purchased from Sigma.

The reaction products were characterized by thin layer choromatography (TLC).

Nifedipine and the synthetic compounds were dissolved in distilled water.

2.3. Methods

2.3.1. Synthesis of pyranopyrazole derivatives

Aromatic or aliphatic aldehyde (1mM), β-ketoesters (1 mM), hydrazinehydrate 96% (1 mM), malonitrile (1 mM), the catalyst (5 mol%), and H_2O (5 mL) were mixed together at room temperature while stirring vigorously. The precipitated solid was filtered, washed out with water and ethanol and purified by recrystallization method (10).

2.4. Pharmacological analyses

2.4.1. Tissue preperation

Rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and their thoraxes were opened by midline incisions. Thoracic aorta of each animal was rapidly excised, and divided into four 4-mm rings after removing the connective tissue. The endothelium was removed using a small platinium rod. Each ring was connected to a pressure transducer (K 30, Hugo Sachs Elektronik, Germany) and a triangular-shaped hook in an organ bath filled with Bicarbonate-Buffered Physiological Saline Solutions (PSS buffer). Vascular tension was recorded by a PC software (HSE-ACAD, Hugo Sachs Elektronik, Germany). The PSS solution was continuously bubbled with O_2 (95%) and CO_2 (5%) and thermostated at 37 °C. The aorta rings were allowed to stabilize for an hour with a resting tension of 1 g. The PSS solution was changed every 20 minutes.

2.4.2. Measurement of aortic response

Initially, the isolated aortic rings were treated with KCl solution (40 mM) and the increased contractions were recorded. After washing out and maintaining the baseline tension, the tissues were pre-incubated with different concentrations of nifedipine (10^{-10} to 10^{-6} M) or each of the synthetic compounds (10^{-9} to 10^{-5} M) for 20 minutes, and exposed once again with KCl (40 mM) solution. Finally, the concentration-response curves for nifedipine and synthetic compounds were plotted, and their pIC_{50} (negative logarithm of the required concentration of compounds to achieve half-maximal relaxation) and R_{max} (percent of compounds-evoked maximal relaxation at the studied concentrations) were calculated.

2.5. Homology Modeling and Molecular Docking Studies

2.5.1. Homology Modeling

As the X-ray crystal structure of the human $Ca_v1.2$ ($hCa_v1.2$) protein is not available, homology modeling method was employed to investigate the ligand-protein interactions. At first, the sequence target of $hCa_v1.2$ protein was chosen (ID: Q13936), and homology modeling was performed using SWISS-MODEL (http://swissmodel.expasy.org/). Then, the rabbit $Ca_v1.1$ ($rCa_v1.1$) channel with the PDB ID of 5GJW was selected as a template structure and, three dimensional (3D) structure of the protein was designed. Finally, the designed 3D structure was optimized by energy minimization using the steepest descent and conjugated gradient algorithm for 500 ps.

2.5.2. Molecular Docking

Docking validation was performed between the optimized hCa_v 1.2 model and nifedipine by the Autodock 4.2 program (11). The docking procedure was carried out in three steps: protein structure preparation, ligands preparation and docking procedure.

2.5.2.1. Protein structure preparation

Initially, all of the water molecules were removed from the protein structure, and then; all missing hydrogen atoms were added, and the Kolman atom charge was calculated. Non-polar hydrogens were merged using AutoDock Tools 1.5.6 package (12).

2.5.2.2. Ligands preparation

All three-dimensional structures of compounds were drawn using Marvin Sketch Ver. 5.7 ChemAxon (13). The Gasteiger-Marsili charge was calculated using the AutoDock Tools, and then, non-polar hydrogens of compounds were merged.

2.5.2.3. Docking procedure

A box with(60 × 60 × 60) was used,and the center of the grid box was determined as ±148.378, ±184.750, and ±174.310 in X, Y, and Z, respectively (14), the map files were obtained. Docking parameter files were made with keeping default settings and the docking process with 100 runs and a maximum number of energy evaluations 2500000 was done. The obtained .dlg files were studied, and the ligand-protein interactions were analyzed and visualized by PyMOL software (15). Drug likeness and ADMET properties of ligands were performed by Maestro 10.2 software (Maestro, Schrödinger, LLC, NewYork, NY, 2017).

2.6. Statistical analysis

Data were represented as means±S.E.M. and analyzed using SPSS (version 18.0). One way analyses of variance (ANOVA) and post hock Tukey's were used to analyze the dose response. A p value <0.05 was considered statistically significant.

3. Results

3.1. Synthesis of pyranopyrazole derivatives

As shown in table 1, we synthesized 6 new pyranopyrazole derivativesusing the NCTHMB-catalyst with high efficiency.

3.2. Effects of pyranopyrazole derivatives on aortic tension

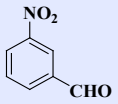
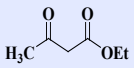
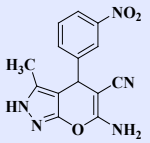
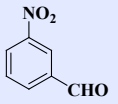
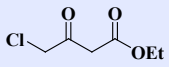
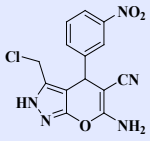
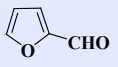
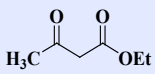
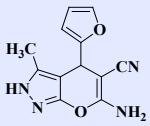
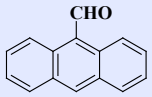
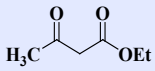
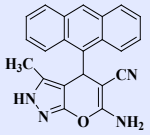
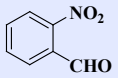
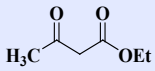
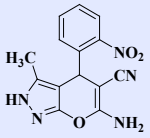
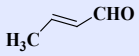
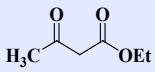
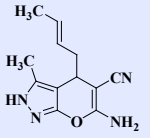
Figure 1. shows that KCl-induced contractions of isolated rat aorta were reduced in a concentration-dependent manner after pre-incubation with nifedipine or synthetic compounds although pIC_{50} value of nifedipine to inhibit KCl-induced contraction was significantly greater than those of newly synthesized compounds. The maximal efficacy (R_{max} value) of nifedipine at the studied concentrations was also higher compared to the synthetic compounds (Table 2).

3.3. Homology modeling

Three-dimensional (3D) structure of $hCa_v1.2$ was generated from $rCa_v1.1$ (5gjw PDB) as a calcium channel template with 72.35% similarity. Superposition of $hCa_v1.2$ and $rCa_v1.1$ are visualized using PyMOL software (Figure 2), and sequence alignment between $hCa_v1.2$ and $rCa_v1.1$ are shown in Figure 3. The key amino acids in the binding site of $hCa_v1.2$ were introduced Gln1060, Phe1129, Ser1132, and Ile1173.

Cyan cartoon indicates 3D $rCa_v1.1$ structure. The yellow cartoon is hCa_v 1.2 model, and the magenta stick is the nifedipine structure.

Table 1. Results of the synthesis of pyranopyrazoles

Product number	Aldehyde	β-ketoester	Product structure	Time (min)	Yield (%)
1				6	96
2				10	72
3				6	91
4				11	83
5				12	87
6				11	70

Reagents and conditions: Aldehyde, β-ketoesters, hydrazine hydrate 96%, malonitrile, catalyst, H_2O at room temperature. All yields are isolated yield.

The binding site residues of $hCa_v1.2$ and $rCa_v1.1$ at Gln (1060 and 939), Phe (1129 and 1008), Ser (1132 and 1011), and Ile (1173 and 1052) are seen respectively. Dash: residue deletion, Vertical bar: identical amino acids, Colon:amino acids with similar properties, and White dot:different amino acids.

3.4. Docking simulation analysis

The validation process was carried out by the docking of nifedipine with $hCa_v1.2$, and its main interactions were investigated. In Figure 3A, the hydrogen bond is observed between Gln1060 and NH moiety of nifedipine, and hydrophobic residues are Met1125, Met1126, Phe1129,

Table 2. The potencies and maximum efficacies of nifedipine and pyranopyrazole compounds on KCl-induced contraction of isolated rat aorta.

Compounds	pIC_{50}	R_{max} (%)	Compounds	pIC_{50}	R_{max} (%)
Nifedipine	8.38 ± 0.35	85.11 ± 5.58			
Compound 1	7.42 ± 0.21*	46.46 ± 7.31*	Compound 4	6.56 ± 0.26*	47.65 ± 8.72*
Compound 2	6.73 ± 0.17*	79.36 ± 6.48	Compound 5	6.74 ± 0.18*	71.03 ± 9.21
Compound 3	6.74 ± 0.15*	72.62 ± 9.53	Compound 6	NC	19.71 ± 4.05*

NC; not calculated,pIC_{50}; negative logarithm of the required concentration of nifedipine or synthetic compounds to achieve half-maximal relaxation, R_{max}(%); percent of maximal relaxation. Data are expressed as means ± SEM.*$p<0.05$ versus nifedipine group.

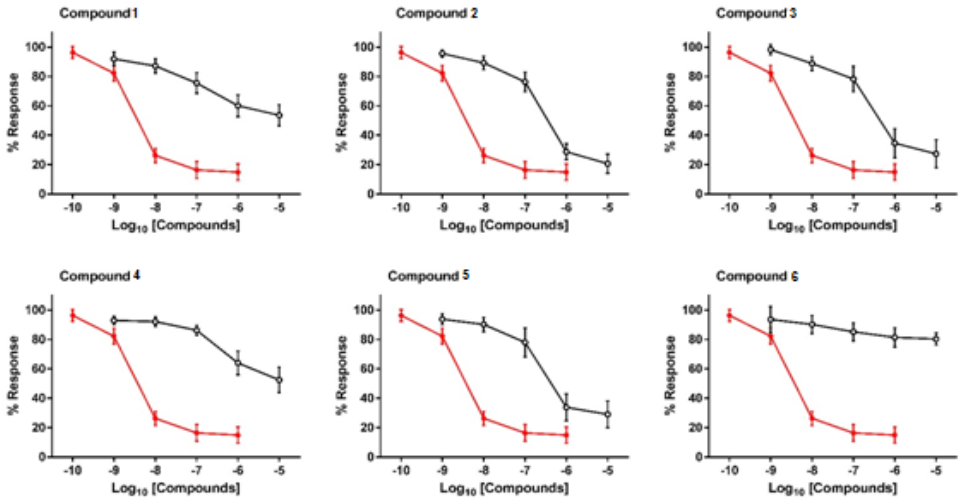


Figure 1. The inhibitory effect of nifedipine (red lines) and pyranopyrazolec ompounds (black lines) on KCl-induced contraction of isolated rat aorta. Data are represented as means ± SEM.

Ser1132, and Ile1173 in the active site. All ligands were docked with the hCa_v 1.2 protein to determine these ligands as calcium channel blocker. 100 docking modes were created, and all the docking poses were investigated in term of the binding energy and the orientation of the ligands in the active site. The best interactions between ligands and protein are shown in Figure 4. Drug-likeness and ADMET properties of ligands were reported in Table 3.

4. Discussion

Pyranopyrazoles are widely used as an important medicinal scaffold to design new drug molecules. The vasorelaxant activities of some types of pyranopyrazole derivatives have been shown by some researchers. Etman *et al*.(16)synthesized some 5-aminoethylpyranopyrazole derivatives and showed their hypotensive and vasodilator activities. Yu *et al* (17)investigated the vasodilator effects of (2-(4′methoxyphenylmethyl)-3,4-dimethylpyrano[2,3- c]pyrazol-6 (2H)-one) (18) and (2-(2′-thienylmethyl)-3,4-dimethylpyrano [2,3-c]pyrazol-6(2H)-one) (3) on rat isolated thoracic aorta and showed that these compounds have vasorelaxant and voltage-gated calcium channel blocker activities.In this study, we synthesized six new pyranopyrazole derivatives using Nanocrystalline TiO_2/Hydroxylapatite (HA) on Magnetic BSA (Bovine Serum Albumin) (NCTHMB) cata-

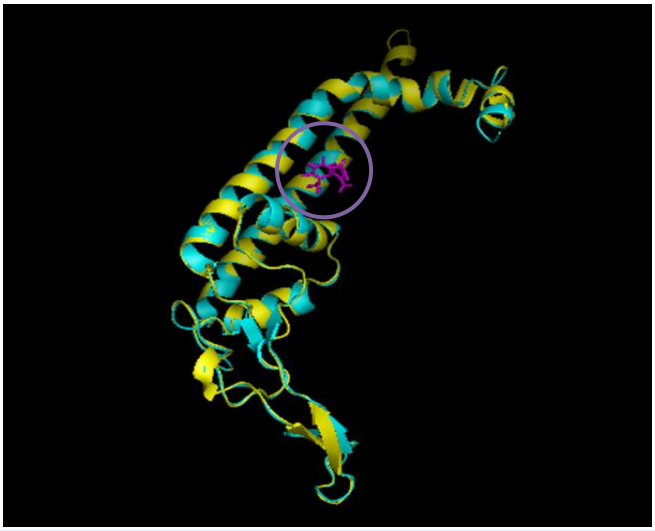


Figure 2. Superposition of 3D structure between hCa_v 1.2 and rCa_v1.1.

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5GJW    900  --------------------RVLRPLRAINRAKGLKHVVQCVFVAIRTIG   929
                                  ||||||||||||||||||||||||||||
Q13936 1001  LISFGIQSSAINVVKILRVLRVLRPLRAINRAKGLKHVVQCVFVAIRTIG  1050

5GJW    930  NIVLVTTLLQFMFACIGVQLFKGKFFSCNDLSKMTEEECRGYYYVYKDGD   979
             |||:|||||||||||||||||||.::|:|.||.||.||:|.|..||||:
Q13936 1051  NIVIVTTLLQFMFACIGVQLFKGKLYTCSDSSKQTEAECKGNYITYKDGE  1100

5GJW    980  PTQMELRPRQWIHNDFHFDNVLSAMMSLFTVSTFEGWPQLLYRAIDSNEE  1029
             .....::||.|.::.|.|||||:|||:||||||||||:||||:|||:.|
Q13936 1101  VDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSIDSHTE  1150

5GJW    1030 DMGPVYNNRVEMAIFFIIYIILIAFFMMNIFVGFVIVTFQE---------  1069
             |.||:||.|||::||||||||:|||||||||||||||||||
Q13936 1151  DKGPIYNYRVEISIFFIIYIIIIAFFMMNIFVGFVIVTFQEQGEQEYKNC  1200
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Figure 3. Sequence alignment between hCa$_V$1.2 (Q13936) and rCa$_V$1.1 (5GJW).

lyst and evaluated their vasorelaxant effects in isolated rat thoracic aorta. According to the results, most of our newly synthesized derivatives (except compound 6), did have significant vasorelaxant activities with maximal efficacies as great as that for nifedipine which is a well known peripheral arterial vasodilator. As we know about the structure activity relationship (SAR) of nifedipine, the presence of N1 atom on pyridine ring is necessary for hydrogen bonding to glycine 1061 residue in calcium channel (hCa$_V$1.2) (19, 20). The molecular structure of pyranopyrazole scaffold is similar to nifedipine in having an aromatic heterocyclic ring with strong nucleophilicity like pyridine. Therefore, we expected that our newly synthesized compounds show calcium channel blocker effect. The results of our molecular docking studies confirm this expectation.It was found that all compounds at efficiently in the calcium channel active site. The ranking order of the binding energies was consistent with the ranking orders of pharmacological potencies and maximal efficacies of their vasorelaxant effects. Comparing with the binding energy of nifedipine (-8.312 kcal/mol), compound 2 by having the highest potency and maximal efficacy in the list, had a binding energy

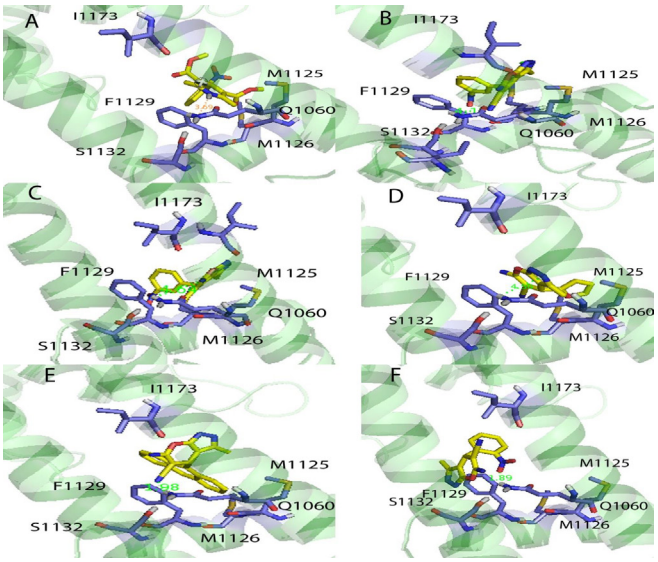

Figure 4. The main hydrophobic and hydrophilic interactions

A: Residues involved in the interaction of nifedipine with hCa$_V$1.2.B: The interaction between ligand1and hCa$_V$1.2. C: The interaction between ligand 2 and hCa$_V$1.2. D: The interaction between ligand 3 and hCa$_V$1.2. E: The interaction between ligand4and hCa$_V$1.2. F: The interaction between ligand 5 and hCa$_V$1.2.

Table 3. Physicochemical properties of predicted compounds using Maestro software.

Compound	MW	SASA	MV	NRB	DHB	AHB	Logp o/w	Logk	LogBB	NPM	PCA
1	331.72	520.34	911.23	3	3	4.5	1.34	0.027	-1.84	4	-
2	297.27	493.8	863	3	3	4.5	0.76	-0.08	-1.98	4	-
3	242.24	430	732.8	2	3	4	0.86	-0.26	-1.02	3	-
4	352.4	565.59	1033.6	2	3	3.5	3.04	0.51	-1.12	2	-
5	297.27	480	849.46	3	3	4.5	0.96	-0.1	-1.62	4	-

MW= Molecular weight; SASA= Total Solvent-accessible Surface Area; MV= Molecular Volume; NRB= No. of Rotatable Bonds; DHB=Donor - Hydrogen Bonds; AHB= Acceptor - Hydrogen Bonds; log p o/w= log P for octanol/water; logk= log K hsa Serum Protein Binding; NPM= No. of Primary Metabolites; PCA= Predicted CNS Activity.

of -7.945 kcal/mol,and was the nearest compound to nifedipine. The compounds 3 and 5 with binding energies of -7.65 and -7.59 kcal/mol respectively, were ranked next. Looking at the molecular structures of the compounds, it seems that placing an aliphatic side chain (compound number 6) or a bulky aromatic group (compound 4) causes steric hindrance and prevents the binding of the molecule to the calcium channel binding site. In addition, it was found that placing a small aromatic ring like furan at the C4 of the pyran ring strengthens the binding of the compound to $hCa_v1.2$. On the other hand, it was interestingly observed that the placement of 3-nitrophenyl group atC4 of the pyran ring in compound 5 resulted in very good binding of the compound to $hCa_v1.2$, while replacing it with 3-nitrophenyl group in compound 1, resulted in a dramatic reduction in the binding of the compound to $hCa_v1.2$ and almost eliminated its vasodilating effect. It seems that the spatial position of the nitro group on the benzene ring is of key importance in this matter.In other words,the presence of nitro group in the meta position of the benzene ring causes a steric hindrance for binding of the compound to the calcium channel. On the contrary, changing the position of the phenyl group to the ortho position facilitates this binding. The best efficacy and the best binding to the calcium channel was observed in compound number 2 which has a 3-nitrophenyl group at C4 position of the pyran ring.The only difference between the molecular structure of compound 2 (with the highest maximal efficacy and the best binding affinity to calcium channel) and compound 1 (with low efficacy and low binding affinity to calcium channel) is in having an extra chlorine atom on the methyl group of the pyrazole ring. So, we found that the presence of chlorine atom at pyrazole ring enhances the binding of pyranopyrazole derivatives to $hCa_v1.2$ and potentiates the vasodilating effects of these compounds.

5. Conclusion

In this research, we used a high performance and ecofriendly method for the preparation of some new pyranopyrazole derivatives and showed that most of these compounds have remarkable vasorelaxant activities. Regarding the results of our docking studies, these newly synthesized pyranopyrazole derivatives showed calcium channel ($hCa_v1.2$) binding properties and maybe regarded as good candidates for further investigations to develop new calcium channel blockers.

Conflict of Interest

None declared.

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