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New homoisoflavonoid analogues protect cells by regulating autophagy

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ABSTRACT

As a special group of naturally occurring flavonoids, homoisoflavonoids have been discovered as active components of several traditional Chinese medicines for nourishing heart and mind. In this study, twenty homoisoflavonoid analogues, including different substitution groups on rings A and B, as well as heteroaromatic B ring, were synthesized and evaluated for their cardioprotective and neuroprotective activities. In a H_2O_2-induced H9c2 cardiomyocytes injury assay, nine homoisoflavonoid analogues showed promising activities in the same level as the positive control, diazoxide. Six cardioprotective compounds with representative structure diversities were then evaluated for their neuroprotective effects on MPP+ induced SH-SY5Y cell injury model. Furthermore, autophagy inducing monodansylcadaverine (MDC) fluorescence staining methods and molecular docking studies indicated the action mechanism of these compounds may involve autophagy regulating via class I PI3K signaling pathway.

Homoisoflavonoids are a special group of naturally occurring flavonoids with an additional carbon between the B and C rings on the isoflavonoid skeleton.[1] Up to date, over 200 homoisoflavonoids have been isolated from plant genus of *Ophiopogon*, *Polygonatum*, *Caesalpinia*, *Muscari*, *Eucomis*, etc.[2,3] and some of these plants, such as *O. japonicas*, *P. odoratum* and *P. cyrtonema*, were frequently used in TCM for nourishing heart and mind. Accordingly, these structures have demonstrated biological activities including anti-inflammation,[4] antioxidation,[5] anti-pathogen,[6] antitumor,[7] and cardiovascular protection.[8] In our previous studies, a series of homoisoflavonoids were isolated from *O. japonicas*[9] and *P. cyrtonema*[10] and their cytotoxic, myocardial protective, and antioxidative activities were evaluated. These minor components have demonstrated promising bioactivity while their contents in plants are very low. Therefore, synthetic approaches have been tried for homoisoflavonoid skeletons by several groups since 1980s and more than twenty homoisoflavonoid derivatives have been reported.[11–14]

In the current study, twenty homonisoflavonoid derivatives Fig. 1, including different substitution groups on rings A and B, as well as heteroaromatic B ring analogues, were synthesized respectively from phloroglucinol, 2,4-dihydroxyacetophenone, and 2,6-dihydroxyacetophenone. Myocardial protective activities of all compounds were evaluated by H_2O_2-induced H9c2 cardiomyocytes injury model. Furthermore, six compounds with promising activities and representative structure diversities were evaluated for their neuroprotective effects by MPP+ induced SH-SY5Y cell injury model. The action mechanism of these compounds was studied by autophagy inducing monodansylcadaverine (MDC) fluorescence staining methods together with molecular docking modeling. Regulating effects of these compounds on autophagy via class I PI3K signaling pathway have been revealed.

Schemes 1–3 summarized procedures for preparing three series of homoisoflavonoid analogues. Synthesis of homoisoflavonoid derivatives **6a–f** were depicted in Scheme 1A. Friedel-Craft's acylation of phloroglucinol with acetonitrile in the presence of phosphorus oxychloride and boron trifluoride gave ketone **1**.[15] Ketone **1** was then protected as benzyl ether **2** by using standard conditions.[16] Subsequently, condensation of compound **2** with substituted benzaldehyde **3a–f** resulted in chalcones **4a–f**, which were then hydrogenated under the catalysis of palladium/carbon to afford **5a–f** and the benzyl protecting groups were removed simultaneously.[17,18] Finally, cyclization of **5a–f** with methanesulfonyl chloride in the presence of the catalytic boron trifluoride provided homoisoflavonoids **6a–f**.[19] For the syntheses of homoisoflavonoid **10a–d**, compounds **7a** and **7b** were chosen as the starting materials (Scheme 1B). After protection of the hydroxy group of **7** with benzyl group, chalcones **9a–d** were obtained in high yields (80–83% in two steps) by condensation with *p*-anisicaldehyde and *o*-anisaldehyde, respectively. With the key intermediates **9a–d** in hand, homoisoflavonoid derivatives **10a–d** were prepared by applying the same reduction/cyclization sequence as **6** series.

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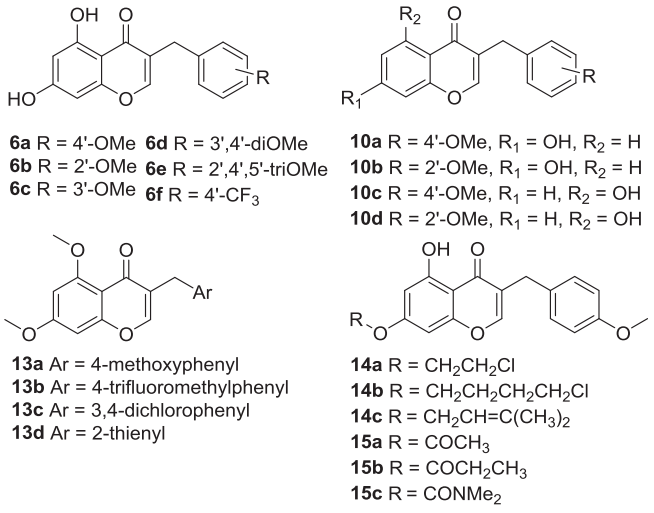


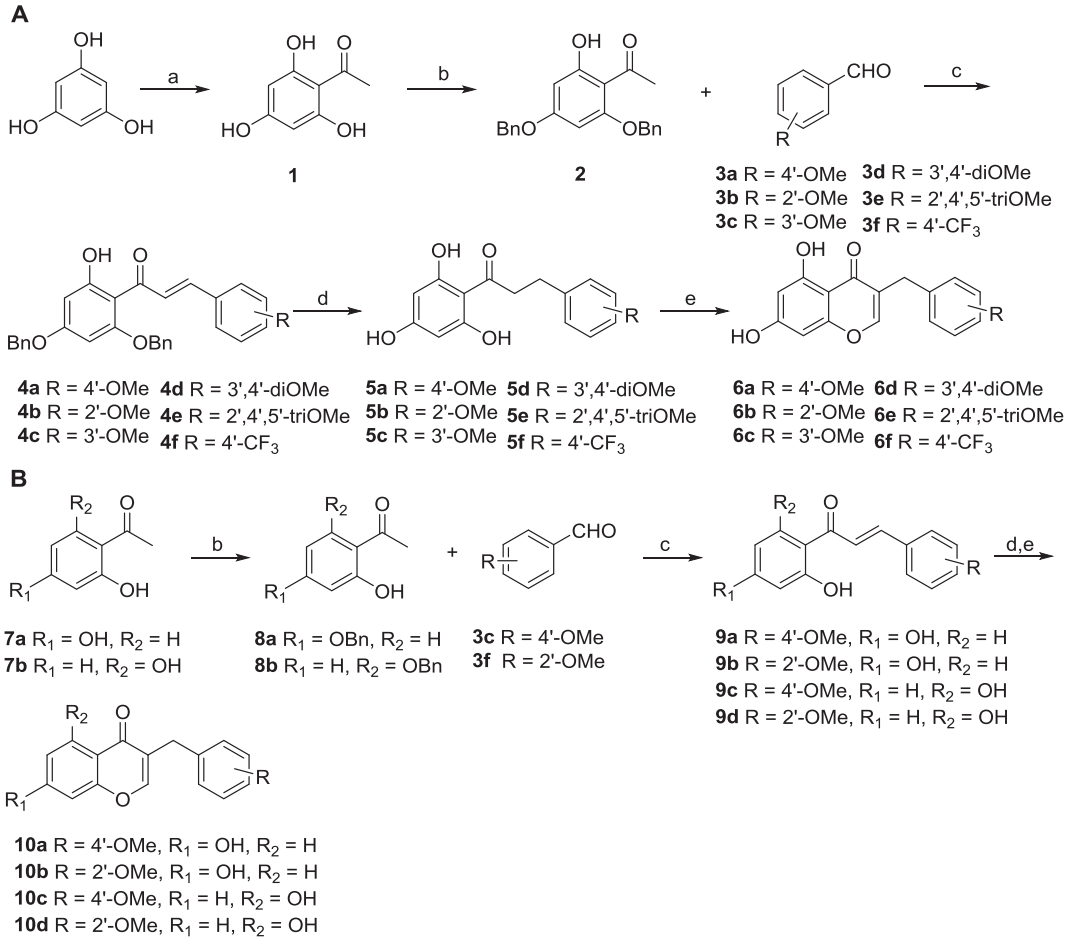
Fig. 1. Synthesized homonisoflavonoid analogues.

The 6,8-dimethoxy homoisoflavonoid analogues were prepared as shown in Scheme 2. Methylation of ketone **1** with dimethyl sulfate led to 4′,6′-dimethoxy-2′-hydroxyacetophenone **11** with 84% yield. Intermediate **11** was then condensed with a substituted benzaldehyde provided **12a–c**. Finally, conversion of compounds **12a–c** to homoisoflavonoids **13a–c** was achieved smoothly by above-described reduction/cyclization sequence. Additionally, compound **13d** was prepared in a similar fashion by condensation of **11** with 2-thenaldehyde, followed by means of a successive three-step sequence (for details of experimental procedures and conditions, see supplementary data).

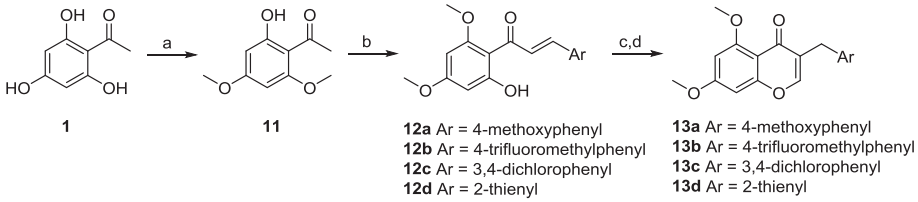
The third 7-*O*-substitution analogue series were derived directly from compound **6a**. Compounds **14a–c** were prepared by alkylating of 7-OH of **6a** with several alkyl halides in the presence of base. In turn, **15a–c** could be obtained by acylation of the same functional group.

As summarized in Table 1, all the homoisoflavonoid analogues were tested against H_2O_2-induced H9c2 cardiomyocytes injury. Compounds **6d**, **6e**, **6f**, **10a**, **10b**, **13c**, **13d**, and **15c** showed protective effects with EC_{50} in the range of 12.1–74.3 μM. The positive control, diazoxide,[20,21] exhibited cardiomyocyte protective activity in the same level with an EC_{50} of 12.6 μM. The results indicated the following primary structure activity relationship: (1) more than two OMe groups on ring B showed more active properties. (2) 4′-CF_3 or other electron withdrawing groups on ring B will increase activity. (3) 7-OH or polar groups at C-7 is very important to maintain the biological activity.

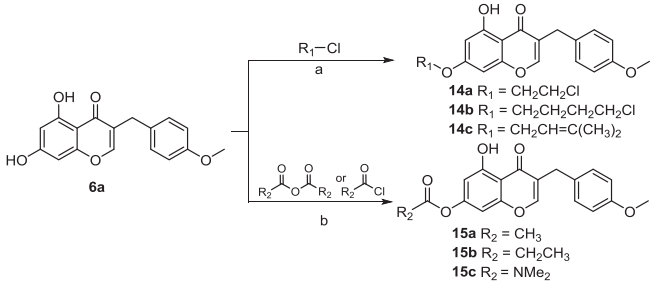
According to clinic usage of homoisoflavonoid-containing natural medicine on heart and mind, more therapeutic potential of homoisoflavonoid analogues on neuron cell protection was studied. Six active compounds in the first assay with representative structure diversity, **6d**, **6e**, **10b**, **13c**, **13d**, and **15c**, were selected for further neuroprotective capacity tests using human neuroblastoma cell line SH-SY5Y. As summarized in Table 2, all of the



Scheme 1. (a) CH_3CN, $POCl_3$, 0 °C, 6 h; H_2O, reflux, 2 h; (b) BnCl, K_2CO_3, DMF, 70 °C, 16 h; (c) NaH, DMF, 0 °C, 30 min; (d) Pd/C, H_2, THF, rt, 16 h; (e) $MeSO_2Cl$, DMF, BF_3-E_2O, 80 °C, 2 h.



Scheme 2. (a) $(CH_3)_2SO_4$, K_2CO_3, acetone, rt, 3 h; (b) NaH, DMF, aldehyde, 0 °C, 30 min; (c) Pd/C, H_2, THF, rt, 16 h; (d) $MeSO_2Cl$, DMF, BF_3-E_2O, 80 °C, 2 h.



Scheme 3. (a) K_2CO_3, DMF, rt; (b) DMAP, THF, rt.

compounds showed neuroprotective capacity at 5 μM level and compounds **6d** and **13c** showed better protective activity than other analogues. These observations indicated that these new derivatives had the potential to be efficient multifunctional agents, including antioxidant activity, for the treatment of neurodegenerative diseases. Meanwhile, the colorimetric MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] assay on MCF-7 cell line was performed to examine the potential cytotoxic effects of each compound. As indicated in Table 2, these compounds did not show significant effect on cell viability at 50 μM after incubation for 24 h.

To better understand the action mechanism of these compounds on neuroprotection, monodansylcadaverine (MDC) fluorescence staining analysis was performed to evaluate their effects on autophagy, which will be activated during neuronal cell injury and plays a cell-protective role in neurodegenerative diseases. As shown in Fig. 2, compound **6d** and **13c** again showed better autophagy-modulating activity than others, which indicated these homoisoflavonoid analogues may protect cells by inducing autophagy.

Autophagy inducing effects of these compounds were then analyzed by docking and reverse docking modeling. Firstly, a pool of potential targets was constructed with reverse docking procedure (Sea-docking). As we can see from Table 3, poly phosphoinositide 3-kinase (PK3CA_HUMAN), a classic autophagy biomarker, frequently appeared in the predicted targets of these compounds. The binding conformations of the most active compound **6d** to Phosphoinositide 3-Kinase, a key enzyme in autophagy negative regulation PI3K-I/PKB pathway, was further predicted by molecular docking modeling using the Libdock method. The result showed that **6d** interacted with the active site of Phosphoinositide 3-Kinase (PDB: 5DXT) through H-bonding with ASP933 and Lys802 amino-acid residues (Fig. 3). Compound **6d** may rescue the inhibition of the class I PI3K signaling pathway on autophagy by interfering with the IL-13-dependent activation of protein kinase B (PKB)

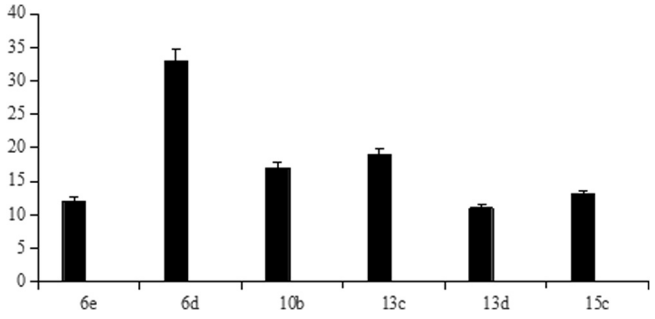


Fig. 2. Autophagy inducing evaluation by MDC fluorescence staining analysis.

Table 1
Myocardial protective activity of homoisoflavonoid analogues (EC_{50}, μM).

Compds	**6a**	**6b**	**6c**	**6d**	**6e**	**6f**	**10a**
EC_{50}	>100	24.4	>100	12.1	15.7	12.7	32.1
Compds	**10b**	**10c**	**10d**	**13a**	**13b**	**13c**	**13d**
EC_{50}	12.7	>100	>100	>100	>100	23.8	74.3
Compds	**14a**	**14b**	**14c**	**15a**	**15b**	**15c**	PC[a]
EC_{50}	>100	>100	>100	>100	>100	19.9	12.6

[a] Positive control: diazoxide.

Table 2
Evaluation of neuroprotective efficacy and cytotoxicity of six selected compounds.

Compound	Neuroprotective efficacy: Cell Viability			Cytotoxicity: inhibitory rate @ 50 μM	
	MPP^+	MPP^++compd @ 5 μM	MPP^++compd @ 50 μM	SH-SY5Y	MCF-7
6d	55.85	60.27 ± 2.06	60.9 ± 2.14	10.71%	41.62%
6e	55.23	62.07 ± 1.24	48.39 ± 1.08	15.31%	49.47%
10b	41.14	38.15 ± 1.14	37.45 ± 2.23	7.41%	28.80%
13c	55.23	62.42 ± 1.91	48.91 ± 2.12	21.58%	21.52%
13d	40.71	37.83 ± 2.26	42.19 ± 1.81	18.95%	46.59%
15c	40.71	42.54 ± 1.91	35.48 ± 0.88	24.12%	77.12%

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Table 3
Selection of the protein targets found by the reverse docking Sea-docking for six selected compounds.

Compds	Reverse docking procedure targets
6d	GPR35_HUMAN, CP1B1_HUMAN, ABCG2_HUMAN, ERR1_HUMAN, MP2K1_HUMAN, MRP1_HUMAN, CALM_HUMAN, IL5_MOUSE, ERR2_HUMAN, RNH1_HUMAN, NQO1_HUMAN, MDR1_HUMAN, CBR1_HUMAN, MDR1A_MOUSE, KCC2B_HUMAN, KDM4E_HUMAN, LOX15_HUMAN, ACE_MOUSE, NOX4_HUMAN, CAH3_HUMAN, S22AC_HUMAN, LGUL_HUMAN, LOX12_HUMAN, CP2CJ_HUMAN, SHBG_HUMAN, PK3CA_HUMAN, PDE3B_HUMAN, PA21B_HUMAN, ESR2_HUMAN, ALDH2_HUMAN, PERM_HUMAN, PK3CG_HUMAN
6e	IL5_MOUSE, CALM_HUMAN, CBR1_HUMAN, NQO1_HUMAN, PK3CA_HUMAN
10b	ALDH2_HUMAN, CP1B1_HUMAN, PPAC_HUMAN, MIF_HUMAN, XDH_HUMAN, MDR1A_MOUSE, AK1BA_HUMAN, IL5_MOUSE, CDK6_HUMAN, CALM_HUMAN, GPR35_HUMAN, MRP1_HUMAN, WEE1_HUMAN, AOFB_HUMAN, ABCG2_HUMAN, CBR1_HUMAN, ERR1_HUMAN, NQO1_HUMAN, S22AC_HUMAN, LOX12_HUMAN, ERR2_HUMAN, NOX4_HUMAN, CASP2_HUMAN, CHLE_HUMAN, UPP1_MOUSE, ALDR_HUMAN, DHB3_HUMAN, AOFA_HUMAN
13c	CBR1_HUMAN, PK3CA_HUMAN
13d	IL5_MOUSE, PK3CA_HUMAN, CBR1_HUMAN, ABCG2_HUMAN, ALDH2_HUMAN
15c	CP1B1_HUMAN, NCEH1_HUMAN, CBR1_HUMAN, ERR1_HUMAN, IL5_MOUSE, NQO1_HUMAN, ERR2_HUMAN, ABCG2_HUMAN, ALDH2_HUMAN, AOFA_HUMAN, PK3CA_HUMAN, CAH13_MOUSE

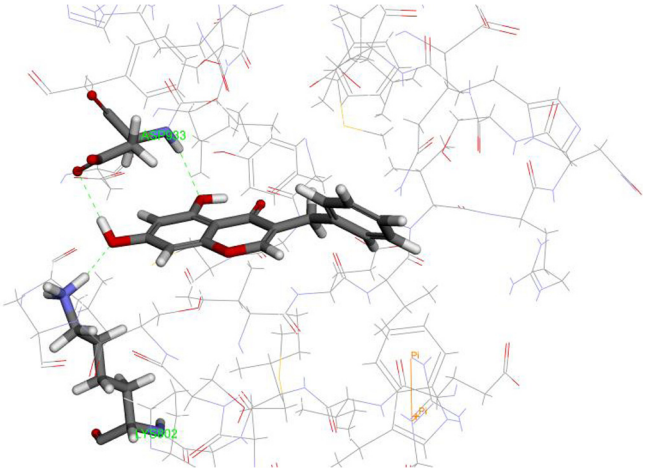

Fig. 3. Compound **6d** docked in the active site of Phosphoinositide 3-Kinase (PK3CA_HUMAN).

and stimulation of the expression of Beclin 1 and inducing cell-protective autophagy. Finally, We used MDC (Monodansylcadaverine) to examine whether compound **6d** could induce autophagy and observed increasing green fluorescent dots under fluorescence microscope (Fig. 4). Quantitative analysis of autophagy was later

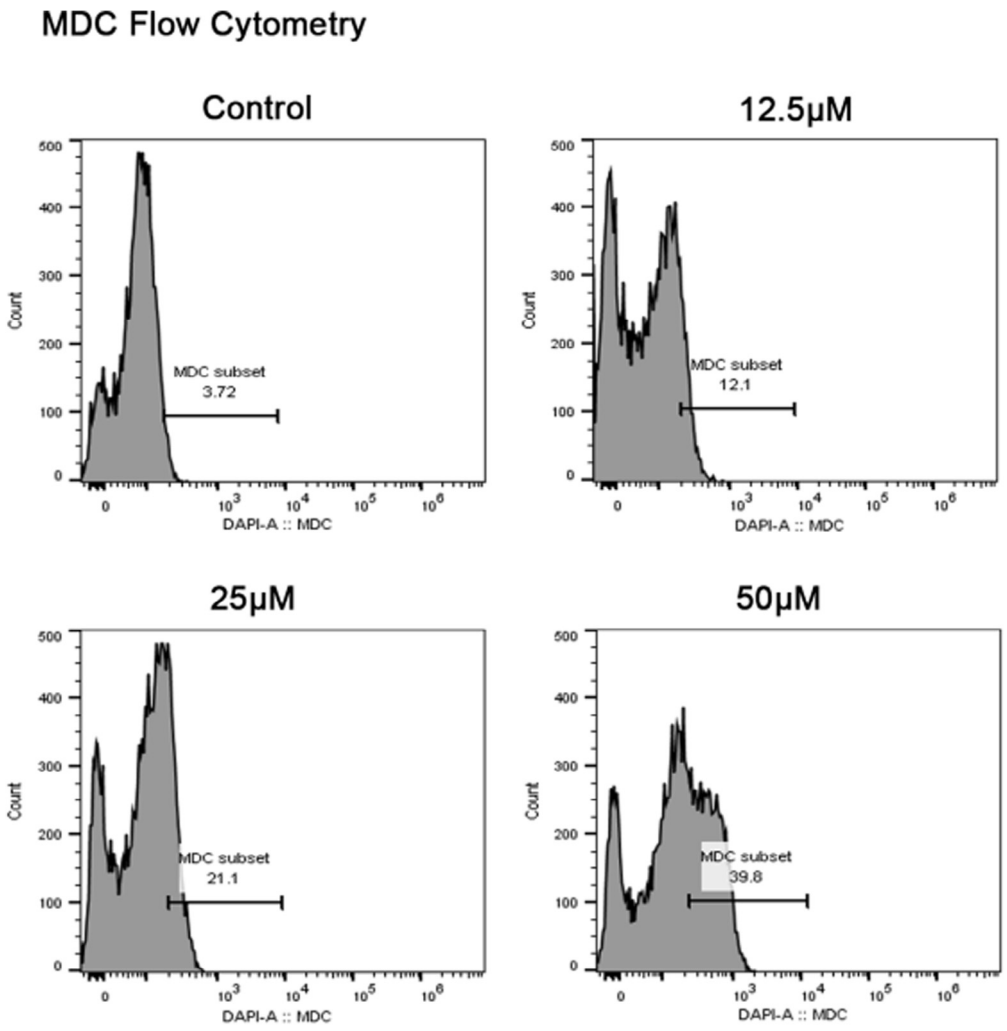

Fig. 4. SH-SY5Y cells were treated with compound **6d** for indicated times and stained with MDC, then the MDC positive ratios were analyzed by flow cytometry.

employed, and we found that MDC positive ratios were markedly enhanced after treatment with compound **6d**.

In conclusion, we described the synthesis of twenty new homonisoflavonoid analogues, including different substitution types on rings A and B, as well as heteroaromatic scaffold. The advantages of these synthetic process included high bond-forming efficiency, high yields, simple work-up procedure, and mild reaction conditions. The myocardial protection activities of these homonisoflavonoid derivatives were evaluated and a primary structure activity relationship was discussed. Six myocardial protective compounds again showed promising neural cell protection activities on a MPP+ induced SH-SY5Y cell injury model. The action mechanism of these cell protective homoisoflavonoid analogues may involving autophagy regulating via class I PI3K signaling pathway. This study provides the potential usage of homoisoflavonoids as cell protective agents for further drug candidate development.

Acknowledgment

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A. Supplementary material

Supplementary data (experimental part, ^{1}H and ^{13}C NMR spectra for compounds **6a**–**f**, **10a**–**d**, **13a**–**d**, **14a**–**c**, and **15a**–**c**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.01.086.

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