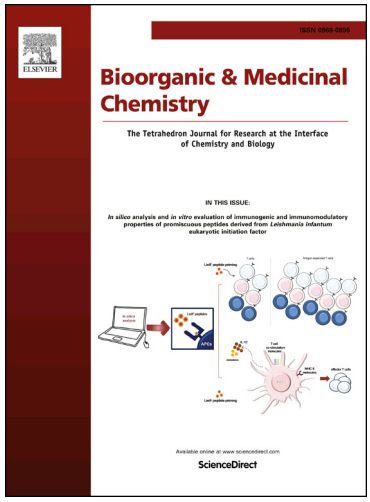

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Design, synthesis and evaluation of 4′-OH-flurbiprofen-chalcone hybrids as potential multifunctional agents for Alzheimer's disease treatment

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Abstract

A series of 4′-OH-flurbiprofen-chalcone hybrids were designed, synthesized and evaluated as potential multifunctional agents for the treatment of Alzheimer's disease. The biological screening results indicated that most of these hybrids exhibited good multifunctional activities. Among them, compounds **7k** and **7m** demonstrated the best inhibitory effects on self-induced A$\beta_{1\text{-}42}$ aggregation (60.0% and 78.2%, respectively) and Cu^{2+}-induced A$\beta_{1\text{-}42}$ aggregation (52.4% and 95.0%, respectively). Moreover, these two representative compounds also exhibited good antioxidant activities, MAO inhibitions, biometal chelating abilities and anti-neuroinflammatory activities *in vitro*. Furthermore, compound **7m** displayed appropriate blood-brain barrier permeability. These multifunctional properties highlight compound **7k** and **7m** as promising candidates for further development of multi-functional drugs against AD.

Keywords:

Alzheimer's disease; Flurbiprofen-chalcone hybrids; Multifunctional agents; Monoamine oxidase inhibitors; Aβ aggregation inhibitors; Anti-inflammatory agents.

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia, which is a fatal, chronic, and neurodegenerative disease in the brain.[1] As the leading cause of death among the elderly, AD is thought to affect about 46.8 million people worldwide in 2015, and the annual cost estimated to exceed 180 billion dollars in the America alone.[2] However, due to the complex pathologies of AD, the therapeutic options for this disease, including cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonist, are mainly focused on the symptomatic aspects, and could not prevent progressive neurodegeneration effectively.[3]

Although the pathogenesis of AD is still unknown, many factors, such as β-amyloid (Aβ) deposits, tau hyper phosphorylation, oxidative stress, and low levels of acetylcholine have been described to play definitive roles in its etiology.[4] Among these multiple factors, Aβ deposits is now widely regarded as central to the development of AD. Aβ is generated by the proteolytic processing of the APP, 90% of Aβ produced is Aβ_{1-40}, whereas a small proportion is Aβ_{1-42}, and Aβ_{1-42} is much more prone to aggregate as fibrils.[5] Amyloid hypothesis indicated that the production of Aβ_{1-42} is increased by the mutations of APP in AD. A sustained imbalance between production and clearance of Aβ_{1-42} leads to accumulation of Aβ_{1-42} monomers, oligomers, and finally large insoluble amyloid fibrils.[6] Amyloid deposits, then, may lead to cerebrovascular compromise, neuroinflammation, abnormal calcium homeostasis and neurodegeneration.[7] Therefore, reducing the aggregation of Aβ_{1-42} is a potential therapeutic strategy for the treatment of AD.

Additionally, oxidative stress is one of the earliest events in AD pathogenesis.[8] The “oxidative stress hypothesis” states that the endogenous antioxidant protection system progressively decays and may be further diminished in AD. The AD brains exhibit constant evidence of reactive nitrogen species (RNS)-mediated injury and reactive oxygen species (ROS)-mediated injury, which can lead to deleterious effects on the cellular components and damages on biological molecules such as membranes, DNA and lipids.[9,10] Recent studies also have indicated that oxidative stress could enhance the development of amyloid plaques and neurofibrillary tangles in AD.[11] Furthermore, more evidence have indicated that the dyshomeostasis of metal ions, such as Cu^{2+}, Fe^{2+}, Zn^{2+} and Al^{3+}, clearly exists in the brains of AD patients, especially in Aβ plaques. It is well known that these biometals can promote the aggregation of Aβ peptides and lead to the formation of ROS and oxidative stress as well.[12] Moreover, another important pathogenic factor in AD is the local

inflammatory response, particularly involving microglia.[13] Study revealed that abnormal Aβ deposits could stimulate microglia, then the microglia multiplied and adopted an activated state. The activated microglia could produce proinflammatory cytokines and neurotoxic mediators including nitric oxide that causes the neuronal cell death.[14] Upregulation of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and reactive oxygen species has been described to associate closely with Aβ release and deposition.[15,16] So it is apparent that drugs with protection of neuronal cells from oxidative stress, modulation of biometals and anti-inflammatory activities are necessary in AD patients.

Monoamine oxidase A and B (MAO-A and MAO-B) are integral proteins of outer mitochondrial membranes, and critically involved in the oxidative deamination of neurotransmitters and xenobiotic amines.[17] It is well known that most of the AD patients suffer from psychological symptom of depression. Selective MAO-A inhibitors are used in the treatment of depression and anxiety, and selective MAO-B inhibitors have been demonstrated to significantly slow down the progression of neurodegenerative disorders, such as Parkinson's disease (PD) and AD.[18] Furthermore, recent studies also have indicated that MAO could promote oxidative stress by generating cytotoxic free radicals and hydrogen peroxide (H_2O_2) when highly expressed in neuronal tissues, this can force increased neuronal cell death.[19] Thus, MAO inhibitors are considered potential candidates for anti-Alzheimer drugs.

As one of the major complex diseases, AD is currently the most frustrating areas of drug discovery.[20] Because of the multifactorial pathological nature and complex network of AD, the traditional modulation of a single target seems not so effective and has been appraised as a major cause of the current clinical failures.[21] In recent years, a new therapeutic strategy based on the multi-target-directed ligands (MTDLs) has drawn considerable attention for its potential advancements in the treatment of AD.[22,23] To obtain novel MTDLs, a design strategy is usually applied in which distinct pharmacophores of different agents are conjugated into the same chemical structure to afford hybrid molecules.[24]

A variety of recent studies have showed that prolonged use of some nonsteroidal anti-inflammatory drugs (NSAIDs) could diminish the risk of AD, delay dementia onset, slow its progression and reduce the severity of cognitive symptoms.[25,26] Among these NSAIDs, flurbiprofen raised particular interest because of its multiple actions on key AD hallmarks.[27] Evidence then

emerged that flurbiprofen had the ability to selectively lower Aβ_{1-42} peptide production, reduce the pathological levels of tau hyperphosphorylation and glutamine, affect the cellular component of neuroinflammation and reduce microglia activation.[13,27,28] So flurbiprofen would seem an ideal candidate as a disease modifying agent in AD. However, the low oral absorption, poor solubility and insufficient blood-brain barrier permeability of flurbiprofen restrict its clinical uses as an anti-AD drug.[29] Chalcones (1,3-diaryl-2-propen-1-ones), which belong to the flavonoid family, have a chemical structure of two aromatic rings connected by an α, β-unsaturated carbonyl group. Because of the flexible structure, chalcones have sufficient brain permeability and exhibit diverse biological activities, such as anti-inflammatory, anti-oxidant and neuroprotective properties.[30-32] Moreover, recent study has indicated that chalcones have properties as Aβ-imaging tracers with high brain uptake and they have high affinity for Aβ aggregates also.[33] Furthermore, in our previous work, the multifunctional agents with phenolic hydroxyl groups showed higher antioxidant activities.[34] Therefore, in this paper, 4′-OH-flurbiprofen was selected to combine with chalcones to obtain a series of 4′-OH-flurbiprofen-chalcone hybrids that are expected to act as multifunctional agents with inhibitory effects on Aβ aggregation, inhibition of MAO, anti-neuroinflammatory, anti-oxidative activities, biometal chelating properties and the abilities to cross the blood–brain barrier (BBB). The design strategy for 4′-OH-flurbiprofen-chalcone hybrids is depicted in **Figure 1**.

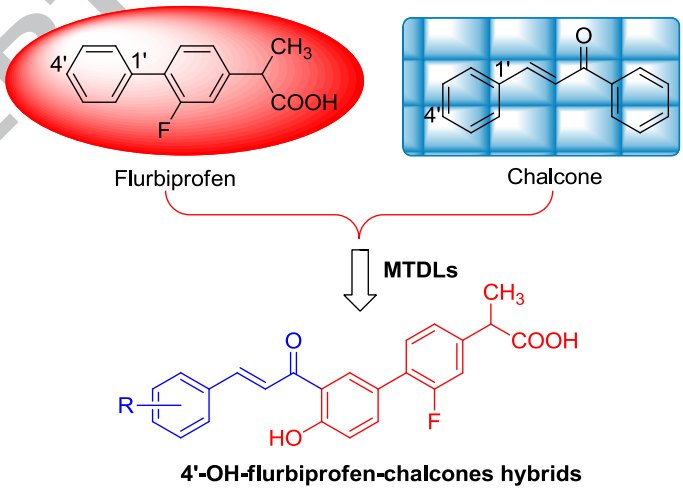


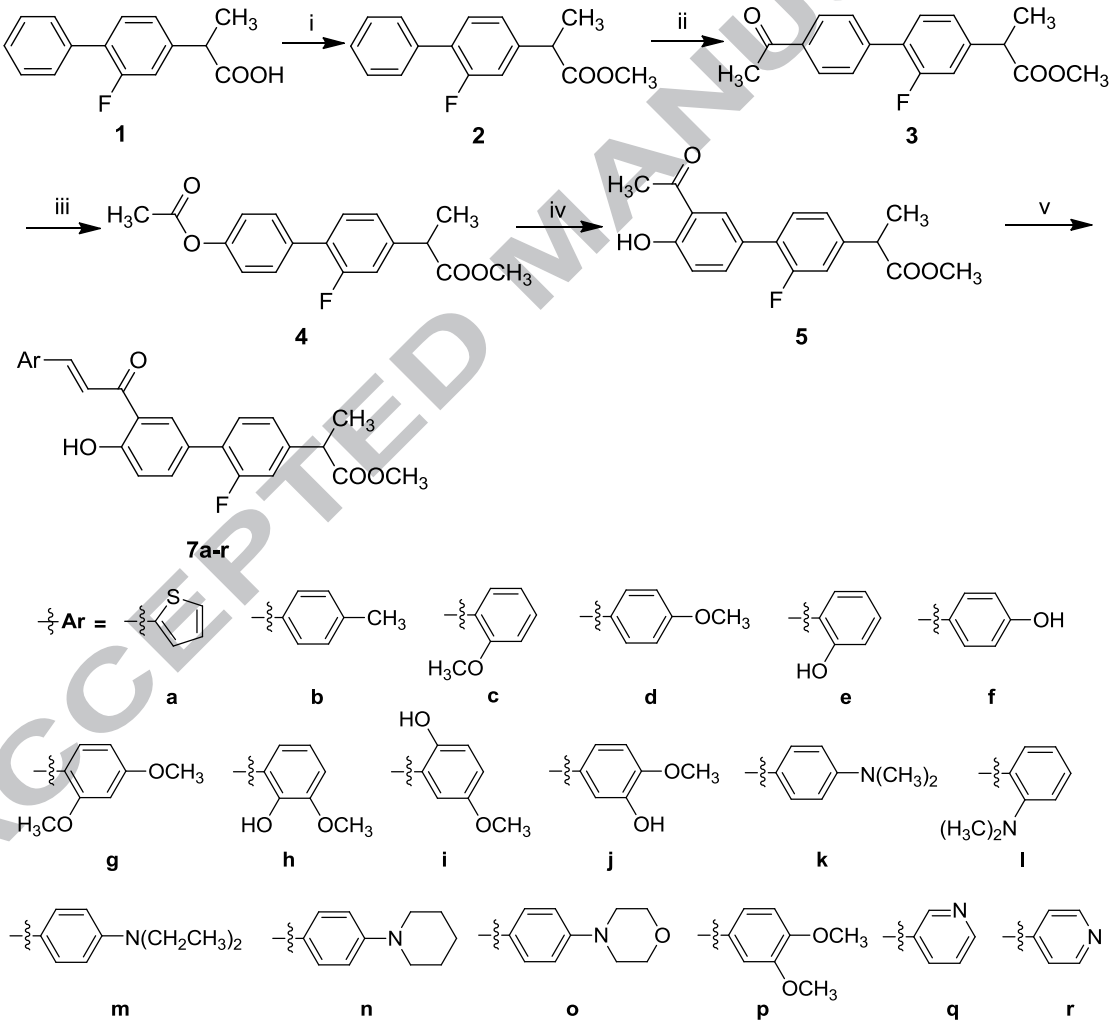
Figure 1. Design strategy for 4′-OH-flurbiprofen-chalcone hybrids.

2. Results and discussion

2.1. Chemistry

The syntheses of the 4′-OH-flurbiprofen-chalcone hybrids (**7a-r**) are shown in **Scheme 1**, for

which Flurbiprofen (**1**) was used as the starting material. The key intermediate **5** was prepared with four steps. At first the carboxyl group of compound **1** was protected by methylation to form methyl ester **2**, and then followed by Friedel-Crafts acylation and Baeyer-Villiger oxidation rearrangement to yield methyl ester **4**[35], the intermediate **5** was finally obtained from methyl ester **4** after Fries rearrangement[36]. The target compounds **7a-r** were synthesized with the general procedure by condensation the intermediate **5** with the corresponding aromatic aldehyde (**6a-r**) in the presence of KOH[34]. All the target compounds have not previously been reported in the literature, and their structures were characterized by ^{1}H NMR, ^{13}C NMR and ESI-MS. The purity of all final compounds was determined by high-performance liquid chromatography (HPLC) analysis to be over 96%.



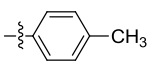
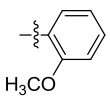
Scheme 1. Synthesis of 4′-OH-flurbiprofen-chalcone hybrids **7a-r**. *Reagents and conditions*: (i) CH_3OH, H_2SO_4, reflux for 4 h; (ii) CH_3COCl, $AlCl_3$, dry CH_2Cl_2, reflux for 12 h; (iii) *m*-CPBA, CH_2Cl_2, at r.t. for 60 h; (iv) Ac_2O, $AlCl_3$, at 90 ^{o}C for 3 h; (v) aromatic aldehyde (**6a-r**), 50%KOH, CH_3OH, at r.t. for 48-72 h; then neutralization with 10%HCl.

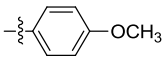
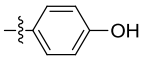
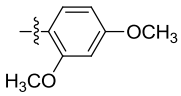
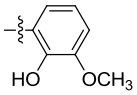
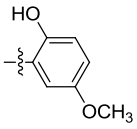
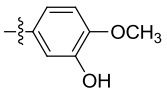
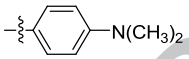
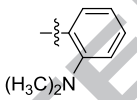
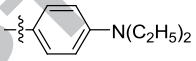
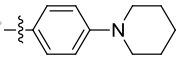
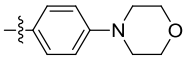
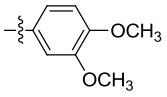
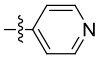
2.2. Pharmacology

2.2.1. Antioxidant activity assay

The *in vitro* antioxidant activities of the 4′-OH-flurbiprofen-chalcone hybrids were determined by following the ORAC-FL method (oxygen radical absorbance capacity by fluorescein).[37] Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), a water-soluble vitamin E analogue, was used as a standard, and the antioxidant activity was expressed as Trolox equivalent. Flurbiprofen was also tested, with an ORAC-FL value of 0.14 Trolox equivalents. As shown in **Table 1**, all of the target compounds displayed moderate to excellent antioxidant activities with ORAC-FL values of 0.42-3.50 Trolox equivalents. In particular, compounds **7f**, **7e** and **7m** showed the most potent antioxidant activities with ORAC-FL values of 3.50, 3.40 and 3.30 Trolox equivalents respectively. And the representative compound **7k** also displayed significant antioxidant activity with a value 3.10-fold of Trolox. When compared the ORAC-FL values of flurbiprofen and its hybrids, all the hybrids exhibited higher activities. It revealed that the introduction of chalcone unit and the free 4'-OH in the flurbiprofen nucleus was crucial for the radical scavenging ability. And it was noticeable that the hybrids contained two phenolic hydroxyl groups possessed higher antioxidant activities than those with only one phenolic hydroxyl groups. Furthermore, the hybrid compounds contained a dimethylamino group or diethylamino group at 4'-position of chalcone moiety (**7k** and **7m**) showed remarkable antioxidant activities. Thus, the phenolic hydroxyl groups and the dimethylamino group or diethylamino group at 4'-position of the chalcone moiety were important to the improvement of antioxidant activity.

Table 1. The yield, purity and oxygen radical absorbance capacity (ORAC, Trolox equivalents) of 4′-OH-flurbiprofen-chalcone hybrids and reference compounds.

Compound	Ar	Yield (%)[a]	Purity (%)	ORAC[b]
7a		44.7	98.1	1.20 ± 0.02
7b		28.2	97.6	0.42 ± 0.02
7c		51.2	98.4	1.06 ± 0.01

7d		49.7	98.2	0.61 ± 0.01
7e		52.9	96.5	3.40 ± 0.02
7f		32.8	98.6	3.50 ± 0.01
7g		33.8	98.4	1.05 ± 0.02
7h		53.7	97.2	1.60 ± 0.01
7i		56.6	96.8	2.50 ± 0.01
7j		47.9	98.1	2.00 ± 0.01
7k		59.9	98.6	3.10 ± 0.03
7l		64.3	96.8	0.77 ± 0.02
7m		64.5	98.9	3.30 ± 0.03
7n		68.2	98.7	1.10 ± 0.02
7o		38.6	98.6	2.23 ± 0.02
7p		45.0	98.8	1.56 ± 0.01
7q		42.1	96.4	1.10 ± 0.03
7r		42.1	96.8	0.90 ± 0.02
Flurbiprofen	-	-	-	0.14 ± 0.01

[a] The yields refers to the general procedure step.

[b] The mean ± SD of the three independent experiments. Data are expressed as μM of Trolox equivalent/μM of tested compound.

2.2.2. Inhibition of self- and Cu^{2+}-induced Aβ_{1-42} aggregation

The inhibitory activities of the 4′-OH-flurbiprofen-chalcone hybrids on self- and Cu^{2+}-induced Aβ_{1-42} aggregation were evaluated by using thioflavin T fluorescence method.[38-40] Curcumin and donepezil were used as the reference compounds. Inhibitory activities were summarized in **Table 2** as inhibition ratios at a test concentration of 25 μM. It indicated that these hybrids exhibited slight to excellent inhibitory activities of self- and Cu^{2+}-induced Aβ_{1-42} aggregation (16.6-78.2% and 8.7-95.0%, respectively), compared with that of curcumin (40.6% and 69.5 %, respectively). For the inhibition of self-induced Aβ_{1-42} aggregation, the representative compounds **7k** and **7m** exhibited the highest inhibitory activities with respective inhibition ratio of 60.0% and 78.2%, and they also displayed the most potent inhibition effects on Cu^{2+}-induced Aβ_{1-42} aggregation with the percentage of 52.4% and 95.0% respectively. Compared with the lead compound flurbiprofen (12.3 and 6.2%, respectively), all of the hybrids exhibited better inhibitory activities. It revealed that the introduction of chalcone moiety increased Aβ_{1-42} aggregation inhibitory efficiency. Noticeably, the potencies of hybrids which contained substituted amino groups at 4'-position of chalcone nucleus (**7k**, **7m** and **7n**) were much higher than other hybrids, however the hybrid contained a dimethylamino group at 2'-position of chalcone unit (**7l**) showed weak inhibitory activities. This result might be reasonably attribute to the substituted amino groups at 4'-position of chalcones nucleus, which can enhance the inhibition effect by forming hydrogen bond and reduce the speed and degree of Cu^{2+}-induced Aβ_{1-42} aggregation by chelating effect.

2.2.3. Recombinant human MAO-A and -B inhibition studies

To complete the study of the multifunctional biological profile of the hybrid compounds, the MAO-A and -B inhibitory properties were evaluated at a test concentration of 10 μM, with clorgyline, rasagiline and iproniazid as reference compounds.[41,42] As shown in **Table 2**, the results were not so satisfactory, and most of the hybrid compounds displayed moderate potency. The representative compounds **7k** and **7m** exhibited the best MAO-A inhibitory activities (73.1% and 75.5%, respectively), and they also displayed moderate MAO-B inhibitory activities with the respective percentage of 47.5% and 48.5%. Compound **7q** showed the best MAO-B inhibitory activities (70.7%). It revealed that the dimethylamino and diethylamino group at 4'-position of chalcones nucleus could increase the MAO-A inhibitory activity and the 3-pyridyl at 4'-position of chalcones nucleus could increase the MAO-B inhibitory activity.

Table 2. Inhibition of self- and Cu^{2+}-induced Aβ_{1-42} aggregation, recombinant human MAO-A and -B of 4′-OH-flurbiprofen-chalcone hybrids and reference compounds.

Compound	%Inhibition of Aβ_{1-42} aggregation[a]		%Inhibition of MAO	
	Self-induced[b, f]	Cu^{2+}-induced[c, f]	MAO-A[d, f]	MAO-B[d, f]
7a	16.6 ± 3.2	12.1 ± 2.7	17.0 ± 2.4	51.4 ± 2.1
7b	23.6 ± 2.5	18.7 ± 3.1	38.0 ± 1.4	44.6 ± 0.9
7c	25.1 ± 2.2	18.9 ± 2.8	29.3 ± 2.5	28.4 ± 1.4
7d	32.9 ± 1.2	29.1 ± 1.6	57.0 ± 1.9	24.6 ± 1.5
7e	23.1 ± 1.4	16.3 ± 3.0	4.7 ± 2.6	28.1 ± 1.3
7f	18.7 ± 1.9	26.1 ± 2.1	24.0 ± 0.7	43.8 ± 3.0
7g	18.8 ± 1.5	45.3 ± 1.7	63.5 ± 2.9	17.3 ± 1.2
7h	29.0 ± 1.5	13.4 ± 2.7	11.0 ± 2.6	12.3 ± 1.9
7i	38.1 ± 2.0	22.2 ± 1.1	13.3 ± 1.3	7.5 ± 1.8
7j	31.0 ± 1.0	14.6 ± 1.8	41.5 ± 1.8	16.3 ± 1.6
7k	60.0 ± 1.3	52.4 ± 1.9	73.1 ± 2.4	47.5 ± 0.8
7l	36.8 ± 0.8	20.1 ± 1.7	12.4 ± 2.7	19.7 ± 0.6
7m	78.2 ± 1.4	95.0 ± 1.3	75.5 ± 1.9	48.5 ± 0.6
7n	31.5 ± 0.9	53.8 ± 1.5	53.4 ± 1.6	44.0 ± 2.3
7o	51.3 ± 1.6	47.2 ± 1.0	42.9 ± 1.1	53.6 ± 2.2
7p	14.2 ± 1.3	24.5 ± 0.8	48.3 ± 0.8	6.8 ± 1.5
7q	8.3 ± 0.7	47.0 ± 1.4	30.7 ± 1.6	70.7 ± 1.6
7r	18.2 ± 1.1	52.8 ± 1.7	24.0 ± 1.2	43.8 ± 2.5
Flurbiprofen	12.3 ± 1.6	6.2 ± 2.3	NT[g]	NT[g]
Curcumin	40.6 ± 0.9	69.5 ± 2.6	NT[g]	NT[g]
Clorgyline	NT[g]	NT[g]	0.0027 ± 0.0001 μM[e]	20.8 ± 0.27μM[e]
Rasagiline	NT[g]	NT[g]	0.587 ± 0.038 μM[e]	0.0281 ± 0.0068 μM[e]
Iproniazid	NT[g]	NT[g]	5.48 ± 0.03 μM[e]	1.35 ± 0.02 μM[e]
Donepezil	<5.0	<5.0	NT[g]	NT[g]

[a] For inhibition of Aβ aggregation, the thioflavin-T fluorescence method was used.

[b] Inhibition of self-induced Aβ_{1-42} aggregation (25 μM) by tested inhibitors at 25 μM.

[c] Inhibition of Cu^{2+}-induced Aβ_{1-42} aggregation. The concentration of tested compounds and Cu^{2+} were 25 μM.

[d] Percentages are the percent inhibition of MAO by tested compounds at 10 μM.

[e] IC_{50} values of the compounds

[f] The mean ± SD of the three independent experiments.

[g] NT = not tested.

2.2.4. *In vitro* anti-neuroinflammatory activity assay

To evaluate the anti-neuroinflammatory activity of these hybrid compounds, the *in vitro* neurocytotoxicity, inhibition of LPS-induced NO and TNF-α production were investigated, with **7k** and **7m** as representative compounds and flurbiprofen as reference compound. The *in vitro*

cytotoxicity assay was carried out by the MTT method.[43] As shown in **Figure 2**, **7k**, **7m** and flurbiprofen did not alter BV-2 cell viability up to the concentration of 10 μM. With increased concentration to 40 μM, **7k** and **7m** induced a decrease of cell viability (69.1% and 64.9%, respectively), while flurbiprofen still did not alter cell viability. Furthermore, as shown in **Figure 3**, there was no significant difference in cell viability in BV-2 cells treated with LPS (0.1 μg/mL) and different concentrations of flurbiprofen (2.5 and 10.0 μM), **7k** and **7m** (0.5, 2.5 and 10.0 μM), indicating no cytotoxic effect under the test concentrations. The MTT assay results suggest that compounds **7k, 7m** and flurbiprofen are not toxic toward the BV-2 cell line up to the concentration of 10 μM, and LPS (0.1 μg/mL) could not alter cell viability also.

Inhibition of LPS-induced NO production was measured by using Griess reaction method.[44-46] BV2-cells were stimulated with LPS in the presence of test samples for 24 h and the amounts of NO released from cells was measured by the Griess method. As shown in **Figure 4**, compared with the parent compound flurbiprofen which showed good inhibitory activity on LPS-induced NO production (11.8% and 23.0% when the cells were treated with 2.5 and 10.0 μM of flurbiprofen), both **7k** and **7m** exhibited better activities. And their inhibitory activities were not due to cytotoxic effects. **7m** displayed higher NO inhibitory activity with the respective percentages of 30.1%, 46.3% and 68.4% when the cells were treated with 0.5, 2.5 and 10.0 μM, and **7k** exhibited slightly lower activities with the inhibition ratios of 25.7%, 36.0% and 64.7%. Additionally, inhibition of LPS-induced TNF-α production was measured by the Enzyme-linked immunosorbent assay (ELISA).[47] As shown in **Figure 5**, compared with flurbiprofen (9.6% and 30.3% when the cells were treated with 2.5 and 10.0 μM of flurbiprofen, respectively) compounds **7k** and **7m** also showed significant inhibitory activities on LPS-induced TNF-α production. Compound **7m** displayed higher TNF-α inhibitory activity with the respective percentages of 14.3%, 46.2% and 65.4% when the cells were treated with 0.5, 2.5 and 10.0 μM and **7k** exhibited slightly lower activities with the inhibition ratios of 12.1%, 39.6% and 61.5%. Compared with the lead compound flurbiprofen, both **7k** and **7m** exhibited better inhibitory activities on LPS-induced NO and TNF-α production. This result might be attribute to the poor bioavailability and insufficient blood-brain barrier permeability of flurbiprofen.[29] The results also revealed that hybridization of chalcone moiety and flurbiprofen can increased the inhibition of neuroinflammatory activity significantly *in vitro*.

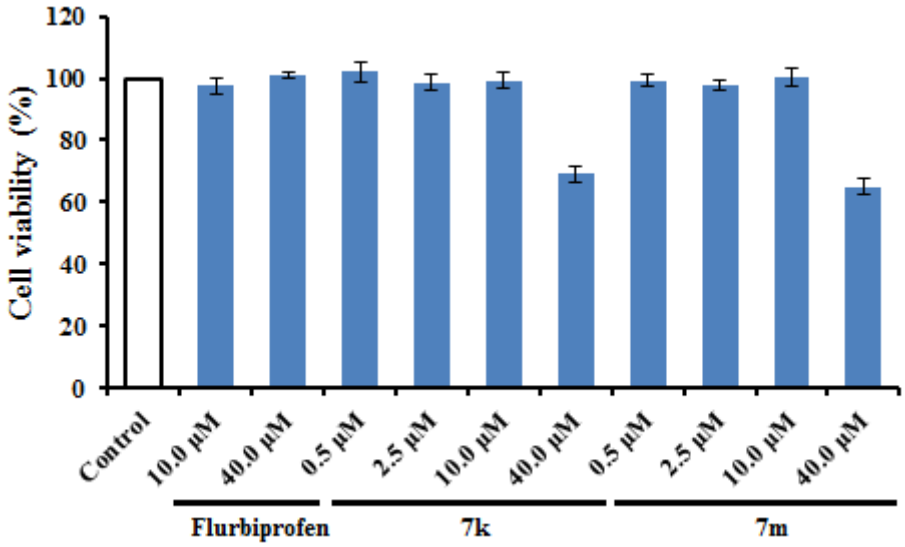


Figure 2. Effects of **7k**, **7m** and flurbiprofen on cell viability in BV-2 microglial cells. Cell viability was determined by MTT assay. The data are expressed as the mean±SD from three independent experiments.

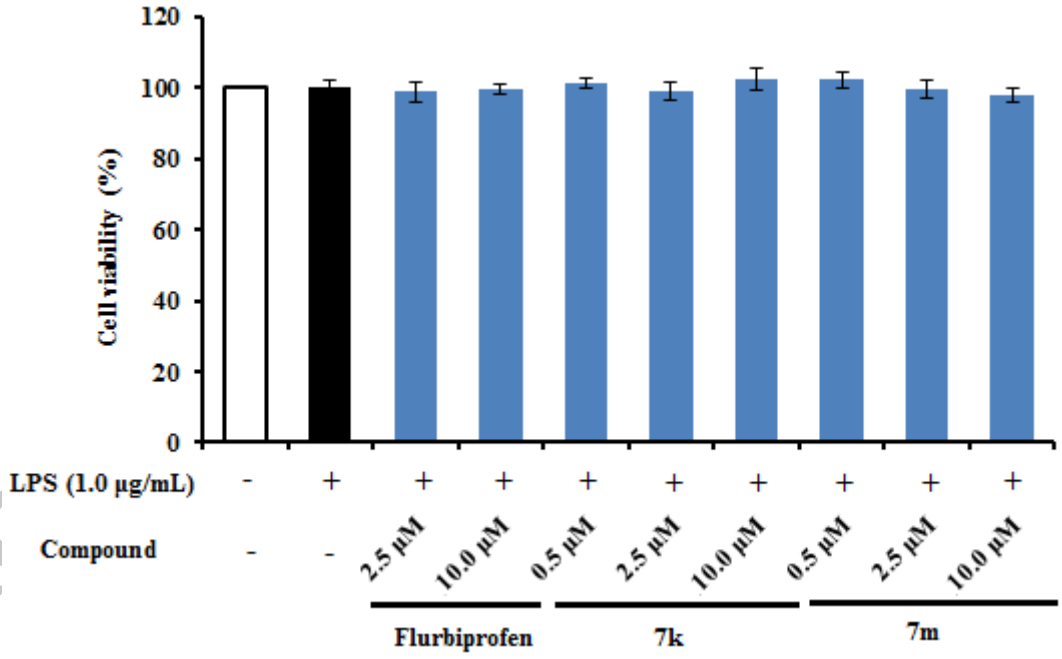


Figure 3. Effect of **LPS**, flurbiprofen and representative compounds on cell viability in BV-2 microglial cells. Cell viability was determined by MTT assay. The data are expressed as the mean±SD from three independent experiments.

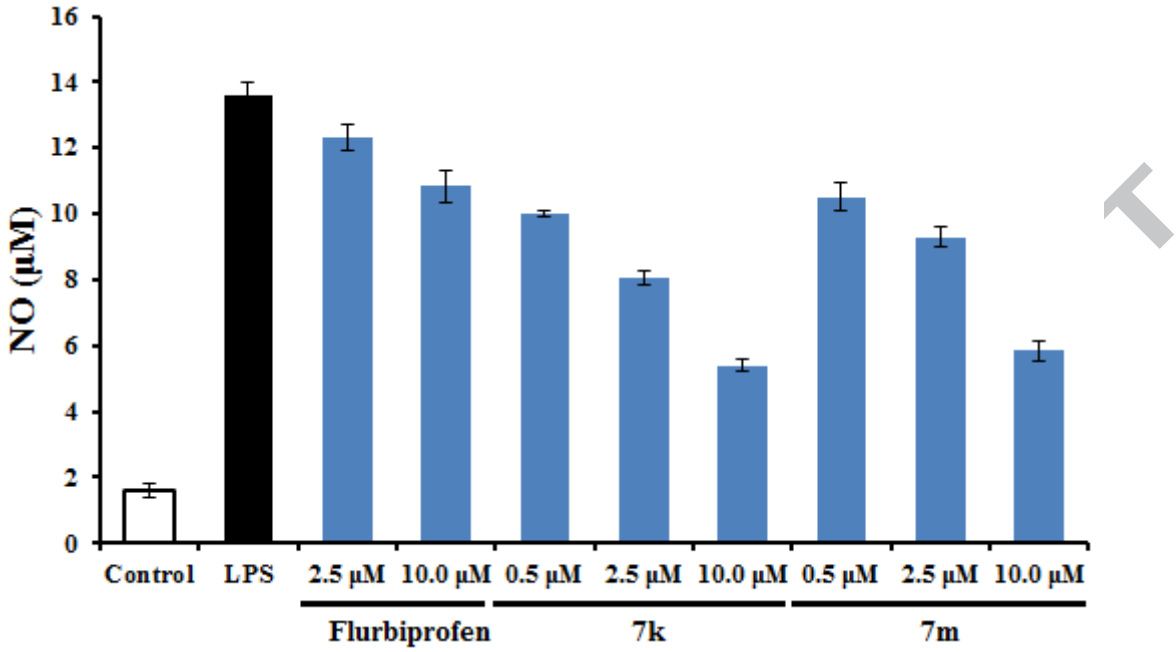


Figure 4. Inhibitory effects of flurbiprofen and **7k**, **7m** on NO production in LPS-activated BV-2 microglial cells. The data are expressed as the mean±SD from three independent experiments.

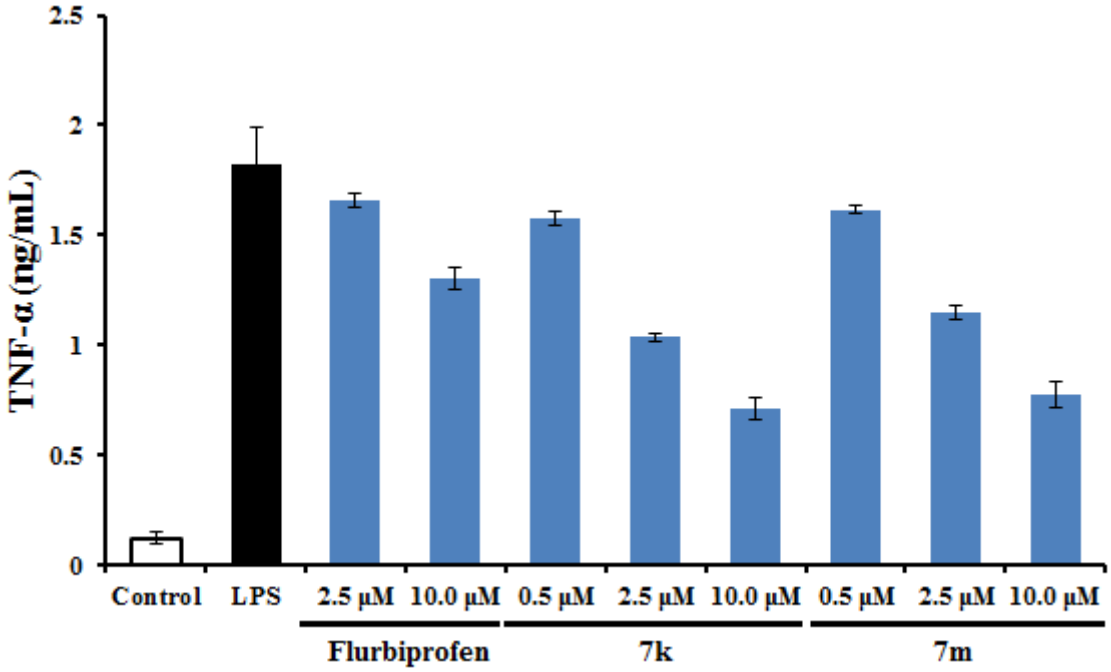


Figure 5. Inhibitory effects of flurbiprofen and **7k**, **7m** on TNF-α expression in LPS-activated BV-2 microglial cells. The data are expressed as the mean±SD from three independent experiments.

2.2.5. Metal-chelating properties studies

The metal-chelating abilities of the 4′-OH-flurbiprofen-chalcone hybrids were evaluated by

UV-visual spectrometry, with **7k** and **7m** as representative compounds, and the results are shown in **Figure 6**. As study had revealed that chalcone derivatives with the dimethylamino group at 4'-position demonstrated good metal binding abilities[48], the electronic spectra of **7k** demonstrated a red shift (the peak at 440 nm shifted to 492 nm and 500 nm, respectively) upon the addition of $CuCl_2$ and $AlCl_3$. However no significant shift was observed upon the addition of $FeSO_4$ and $ZnCl_2$. It revealed that compound **7k** has the selectivity of metal chelation and could interact effectively with the Cu^{2+} and Al^{3+}. Similarly, noticeable optical shifts of compound **7m** was detected after the addition of $CuCl_2$ and $AlCl_3$, the maximum absorption at 453 nm shifted to 500 nm and 516 nm respectively, indicating the formation of a **7m**-Cu^{2+} complex and **7m**-Al^{3+} complex.

Several studies have shown that copper is considered to be the main metal in the development of chelators for potential AD drugs because of its dramatic effects on Aβ homeostasis by increasing the formation of senile plaques, related neuroinflammation and reactive oxygen species producing.[49,50] Therefore, the selective complexation of Cu^{2+} by the compounds **7k** and **7m** could simultaneously stop the Aβ deposition, relieve neuroinflammation and oxidative stress.

To determine the stoichiometry of **7k**-Cu^{2+} complex and **7m**-Cu^{2+} complex, molar ratio method was performed by preparing the methanol solutions of **7k** or **7m** with increasing amounts of $CuCl_2$. The UV spectra were used to obtain the absorbance of the **7k** and **7m** complexes and different concentrations of $CuCl_2$ at 492 nm and 500 nm, respectively. As shown in **Figure 7**, the absorbance initially increased linearly and then became plateaued with the increase of Cu^{2+} concentration. The points for the straight lines to intersect were determined to be at a mole fraction of 0.99 and 1.04 respectively, revealing a 1:1 stoichiometry for **7k**-Cu^{2+} complex and **7m**-Cu^{2+} complex.

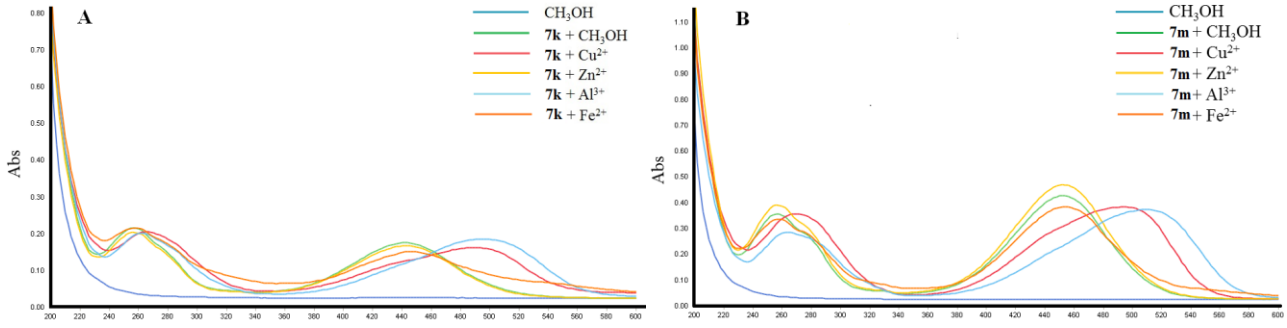


Figure 6. UV spectra of compounds **7k** (**A**) and **7m** (**B**) (37.5 μM in methanol) alone or in the presence of $CuCl_2$, $ZnCl_2$, $FeSO_4$ or $AlCl_3$ (37.5 μM, in methanol).

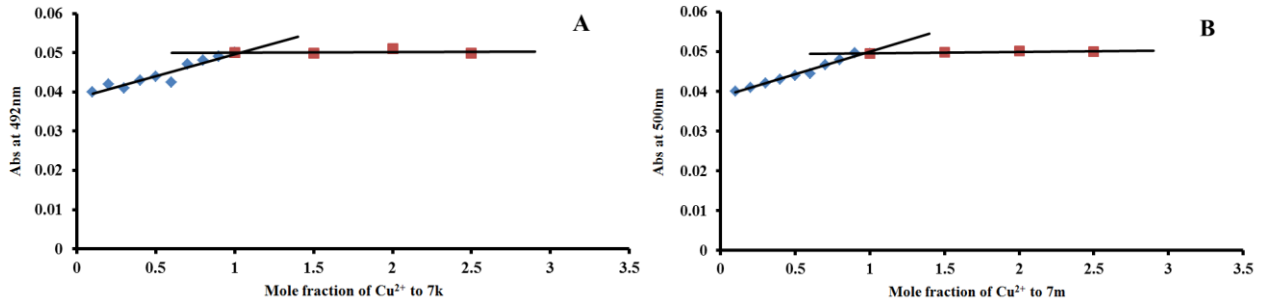


Figure 7. Determination of the stoichiometry of complex-Cu^{2+} by using the molar ratio method of titrating the methanol solution of compounds **7k** (**A**) and **7m** (**B**) with ascending amounts of $CuCl_2$. The final concentration of tested compound was 37.5 μM, and the final concentration of Cu^{2+} ranged from 3.75 to 150 μM.

2.2.6. *In vitro* blood-brain barrier permeation assay

The ability to penetrate brain and reach the therapeutic targets is a necessary prerequisite for successful central nervous system (CNS) drugs. To evaluate the brain penetration of 4′-OH-flurbiprofen-chalcone hybrids, the parallel artificial membrane permeation assay for the blood-brain barrier (PAMPA-BBB) which is wildly used in the early phase of drug discovery was performed.[39,51] To validate the assay, the experimental permeabilities of 11 commercial drugs were compared with their reported values (**Table 3**). And a plot of experimental data versus the bibliographic values showed a good linear correlation: P_e (exp.) = 0.8792 × P_e (bibl.) − 0.0616 (R^2 = 0.9555) (**Figure 8**). From this equation, and taking into consideration the limit established by Di *et al*[49], we determined that compounds with permeabilities above 3.46 × 10^{-6} cm/s could cross the blood-brain barrier (**Table 4**). Then, the representative compounds **7k** and **7m** were tested in the PAMPA-BBB assay. The results presented in **Table 5** showed that **7m** could penetrate into the CNS and reach their therapeutic targets located in the CNS, but unfortunately **7k** exhibited an uncertain BBB permeation in this assay.

Table 3. Permeability P_e (× 10^{-6} cm/s) in the PAMPA-BBB assay for 11 commercial drugs used in the experiment validation.

Commercial drugs	Bibl[a]	PBS/EtOH (70:30)[b]
Verapamil	16	16.20 ± 0.20
Oxazepam	10	9.20 ± 0.16
Diazepam	16	10.90 ± 0.32

Clonidine	5.3	5.50 ± 0.18
Imipramine	13	11.20 ± 0.29
Testosterone	17	15.50 ± 0.30
Caffeine	1.3	1.22 ± 0.04
Enoxacine	0.9	0.55 ± 0.03
Piroxicam	2.5	0.84 ± 0.02
Norfloxacin	0.1	0.33 ± 0.01
Theophylline	0.12	0.17 ± 0.005

[a] Taken from Ref.[50]

[b] Data are the mean ± SD of three independent experiments.

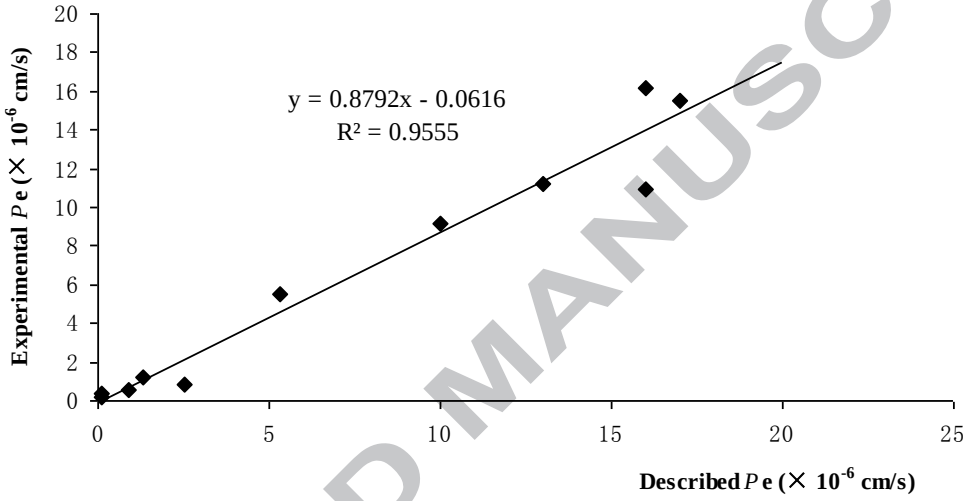


Figure 8. Lineal correlation between experimental and reported permeability of commercial drugs using the PAMPA-BBB assay. P_e (exp) = 0.8792 × P_e (bibl.) − 0.0616 (R^2 = 0.9555).

Table 4. Ranges of permeability of PAMPA-BBB assays (P_e × 10^{-6} cm/s)

High BBB permeation predicted (CNS +)	$P_e > 3.46$
Uncertain BBB permeation (CNS +/-)	$3.46 > P_e > 1.70$
Low BBB permeation predicted (CNS -)	$P_e < 1.70$

Table 5. Permeability results P_e (× 10^{-6} cm/s) from the PAMPA-BBB assay for selected 4'-OH-fluribiprofen-chalcone hybrids with their predicted penetration into the CNS.

Compd.[a]	P_e (× 10^{-6} cm/s)[b]	Prediction
7k	2.25 ± 0.14	CNS +/-
7m	6.66 ± 0.36	CNS +

[a] All compounds were dissolved in DMSO at 5 mg/mL and diluted with PBS/EtOH (70:30). The final concentration of each compound was 100 μg/mL.

[b] Data are the mean ± SD of three independent experiments.

3. Conclusion

In summary, a series of 4′-OH-flurbiprofen-chalcone hybrids were designed, synthesized and evaluated as multifunctional agents for the treatment of AD. The biological screening results indicated that most of the synthesized compounds showed good antioxidant activities, significant inhibitory activities of self- and Cu^{2+}-induced Aβ_{1-42} aggregation *in vitro* and moderate MAO inhibitions. Among them, compounds **7k** and **7m** demonstrated the best inhibitory effects on self-induced Aβ_{1-42} aggregation (60.0% and 78.2%, respectively) and Cu^{2+}-induced Aβ_{1-42} aggregation (52.4% and 95.0%, respectively), displayed excellent antioxidant activities (3.1 and 3.3 Trolox equivalents, respectively) and moderate MAO inhibitions. Moreover, these two representative compounds also possessed the prospective properties of acting as metal chelators and anti-neuroinflammatory agents. Furthermore, compound **7m** displayed appropriate BBB permeability *in vitro*. These properties highlighted that the compounds **7m** and **7k** could serve as new multifunctional candidates for further development in the treatment of AD.

4. Experimental section

4.1. Chemistry

All reagents, unless otherwise noted, were of analytical reagent grade and were obtained from commercial suppliers. All solvents were purified and dried by standard procedures wherever needed. Reaction progress was monitored by thin-layer chromatography (TLC) on silica gel GF254 plates from Qingdao Haiyang Chemical Co. Ltd. (China), and then visualized by UV light (254 nm) or in an iodine chamber. Chromatographic separations were performed on silica gel (230-400 mesh) from Qingdao Haiyang Chemical Co. Ltd. (China). HPLC analysis was carried out on a Shimadzu LC-10Avp plus system with the use of a Kromasil C_{18} column (4.6 mm × 250 mm, 5 μm). Melting points were measured in open glass capillaries on YRT-3 melting-point apparatus (China) and uncorrected. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 25 ^{o}C in $CDCl_3$ or DMSO-d_6 solutions with Varian INOVA spectrometer. Chemical shifts (δ) are reported in ppm from internal Tetramethylsilane (TMS), coupling constants (*J*) are reported in hertz (Hz). Mass spectra were recorded on Agilent-6210 TOF LC-MS Spectrometer.

4.1.1. Methyl 2-(2-fluoro-[1,1'-biphenyl]-4-yl)propanoate (2)

Compound **2** was prepared as previously described.[35] To a stirred solution of flurbiprofen (5.0 g,

20.5 mmol) in methanol (45 mL) was added catalytic amount of conc. H_2SO_4. The solution was refluxed for 4 h and then the solvent was evaporated to dryness under reduced pressure. The residue was diluted with a saturated aqueous solution of Na_2CO_3 (40 mL), and the mixture was extracted with CH_2Cl_2 (40 mL × 2). The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give the crude product **2**, which was used without further purification.

4.1.2. Methyl 2-(4'-acetyl-2-fluoro-[1,1'-biphenyl]-4-yl) propanoate (3)

Compound **3** was prepared according to previously reported.[35] Briefly, $AlCl_3$ (5.44 g, 40.82 mmol) was added to a solution of acetyl chloride (2.89 mL, 40.82 mmol) in dry CH_2Cl_2 (40 mL) at 0-5 ^oC. The mixture was stirred for 30 min, then added the solution of compound **2** (3.00 g, 11.64 mmol) in dry CH_2Cl_2 (10 mL) and refluxed for further 12 h under an argon atmosphere. After complete reaction, the mixture was poured into ice and 30% aqueous HCl (25 mL) was added. Then the mixture was extracted with CH_2Cl_2 (40 mL × 2). The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give the crude product **3**.

4.1.3. Methyl 2-(4'-acetoxy-2-fluoro-[1,1'-biphenyl]-4-yl)propanoate (4)

Compound **4** was prepared as previously described.[35] Briefly, to a stirred solution of compound **3** (3.26 g, 10.83 mmol) in dry CH_2Cl_2 (40 mL) was added 3-chloroperoxybenzoic acid (6.54 g, 38.0 mmol). The solution was stirred for 60 h at room temperature. After complete reaction, saturated aqueous solution of $NaHSO_3$ (50 mL) was added slowly. Then the organic phases were separated and washed with saturated aqueous solution of Na_2CO_3 (40 mL) and brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography using petroleum ether/ethyl acetate (40:1) as eluent, the title product was obtained as white solid, yield 70.3%, mp 79.8-81.1 ^oC. ^1H NMR (400 MHz, $CDCl_3$) δ 7.56-7.54 (m, 2H), 7.39-7.35 (m, 1H), 7.17-7.10 (m, 4H), 3.78-3.73 (m, 1H), 3.70 (s, 3H), 2.32 (s, 3H), 1.53 (d, J = 7.6 Hz, 3H).

4.1.4. Methyl 2-(3'-acetyl-2-fluoro-4'-hydroxy-[1,1'-biphenyl]-4-yl)propanoate (5)

To a stirred solution of compound **4** (3.00 g, 9.48 mmol) in acetic anhydride (1.34 mL, 14.23 mmol) was added $AlCl_3$ (10.11 g, 75.87 mmol), the mixture was heated to 90 ^oC and stirred for 3 h under argon atmosphere. Then the mixture was poured into ice and 37 % aqueous HCl (25 mL) was

added. The mixture was extracted with ethyl acetate (40 mL × 2). The combined organic phases were washed with water and brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. After further purification through silica gel column chromatography using petroleum ether/ethyl acetate (30:1) as eluent, light yellow oil was obtained, yield 50.1%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.30 (brs, 1H), 7.90 (s, 1H), 7.64 (d, J = 8.4 Hz, 1H), 7.37-7.33 (m, 1H), 7.16-7.11 (m, 2H), 7.25 (d, J = 8.4 Hz, 1H), 3.79-3.73 (m, 1H), 3.70 (s, 3H), 2.66 (s, 3H), 1.53 (d, J = 7.6 Hz, 3H).

4.1.5. General procedure for the synthesis of compounds 7a-r

Compound **5** (50 mg, 0.16 mmol), KOH in methanol (50%, 1 mL) were added to methanol (0.5 mL). The mixture was stirred at room temperature for 1 h, and then the corresponding aromatic aldehyde (**6a-r**) (0.15 mmol) was added and stirred for 48-72 h at room temperature. After complete reaction, the solvent was evaporated to dryness under reduced pressure. The residue was diluted with an aqueous solution of KOH (30%, 2 mL), stirred for 1 h, neutralization to pH 6-7 with 10% HCl, then distilled water (15 mL) was added, and the mixture was extracted with ethyl acetate (15 mL × 2), the combined organic phases were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give the corresponding crude compound.

4.1.5.1. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(thiophen-2-yl)acryloyl)-[1,1'-biphenyl]-4-yl)propanoic acid (7a)

Compound **7a** was synthesized from **5** and **6a** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (4:1:0.5%) as eluent, the pure product **7a** was obtained as a yellow solid, yield 44.7%, mp 153.6-155.1 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.91 (brs, 1H), 8.07 (d, J = 16.0 Hz, 1H), 8.01 (s, 1H), 7.65 (d, J = 8.8 Hz, 1H), 7.45-7.40 (m, 3H), 7.42 (d, J = 16.0 Hz, 1H), 7.22-7.18 (m, 2H), 7.11-7.09 (m, 2H), 3.82-3.81 (m, 1H), 1.58 (d, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.1, 179.9, 163.1, 159.6, 141.1, 140.0, 138.2, 136.7, 132.9, 130.5, 129.8, 129.8, 129.7, 127.0, 126.1, 123.9, 119.8, 118.8, 118.5, 115.5, 44.8, 17.9; ESI-MS m/z: 397.3 $[M+H]^+$.

4.1.5.2. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(p-tolyl)acryloyl)-[1,1'-biphenyl]-4-yl) propanoic acid (7b)

Compound **7b** was synthesized from **5** and **6b** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (6:1:0.5%) as eluent, the pure product **7b** was obtained as a slight yellow solid, yield 28.2%, mp

107.8-109.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.91 (brs, 1H), 7.99 (s, 1H), 7.88 (d, J = 15.6 Hz, 1H), 7.57-7.50 (m, 3H), 7.40 (d, J = 15.6 Hz, 1H), 7.33-7.31 (m, 1H), 7.18-7.16 (m, 4H), 7.05-7.03 (m, 1H), 3.76-3.72 (m, 1H), 2.21 (s, 3H), 1.41 (d, J = 6.4 Hz, 3H); ESI-MS m/z: 405.2 $[M+H]^+$.

4.1.5.3. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(2-methoxyphenyl)acryloyl)-[1,1'-biphenyl]-4-yl)propanoic acid (7c)

Compound **7c** was synthesized from **5** and **6c** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (5:1:0.5%) as eluent, the pure product **7c** was obtained as a yellowish-brown solid, yield 51.2%, mp 149.5-151.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.98 (br, 1H), 8.24 (d, J = 15.6 Hz, 1H), 8.08 (s, 1H), 7.76 (d, J = 15.6 Hz, 1H), 7.65-7.51 (m, 2H), 7.43-7.35 (m, 2H), 7.20-7.16 (m, 2H), 7.09 (d, J = 8.8 Hz, 1H), 6.99-6.92 (m, 2H), 3.92 (s, 3H), 3.82-3.79 (m, 1H), 1.57 (d, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.3, 179.7, 163.1, 159.7, 159.0, 141.5, 140.9, 136.5, 132.3, 130.4, 130.2, 129.6, 127.1, 125.9, 123.8, 123.5, 120.8, 120.5, 120.1, 118.7, 115.5, 111.3, 55.58, 44.8, 18.0; ESI-MS m/z: 421.1 $[M+H]^+$.

4.1.5.4. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(4-methoxyphenyl)acryloyl)-[1,1'-biphenyl]-4-yl)propanoic acid (7d)

Compound **7d** was synthesized from **5** and **6d** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (5:1:0.5%) as eluent, the pure product **7d** was obtained as a yellow solid, yield 49.7%, mp 150.8-152.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.00 (br, 1H), 8.03 (s, 1H), 7.89 (d, J = 15.6 Hz, 1H), 7.62-7.58 (m, 3H), 7.49 (d, J = 15.6 Hz, 1H), 7.40-7.37 (m, 1H), 7.20-7.16 (m, 2H), 7.07-7.06 (m, 1H), 6.91-6.90 (m, 2H), 3.82 (s, 3H), 3.80-3.77 (m, 1H), 1.56 (d, J = 7.2Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.5, 179.8, 163.1, 162.0, 159.6, 145.8, 141.1, 136.5, 130.6 (2C), 130.5, 129.9, 127.2, 127.0, 125.9, 123.8, 119.9, 118.7, 117.2, 115.4, 114.5 (2C), 55.4, 44.9, 18.0; ESI-MS m/z: 421.2 $[M+H]^+$.

4.1.5.5. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(2-hydroxyphenyl)acryloyl)-[1,1'-biphenyl]-4-yl)propanoic acid (7e)

Compound **7e** was synthesized from **5** and **6e** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (2:1:0.5%) as eluent, the pure product **7e** was obtained as a reddish brown solid, yield 52.9%, mp

102.8-104.6 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.65 (brs, 1H), 10.47 (br, 1H), 8.27 (s, 1H), 8.19 (d, J = 15.6 Hz, 1H), 8.03 (d, J = 15.6 Hz, 1H), 7.93 (d, J = 7.6 Hz, 1H), 7.71 (d, J = 8.4 Hz, 1H), 7.56-7.52 (m, 1H), 7.31-7.23 (m, 3H), 7.10 (d, J = 8.4 Hz, 1H), 6.5 (d, J = 7.6 Hz, 1H), 6.88-6.85 (m, 1H), 3.84 (q, J = 7.2Hz, 1H), 1.41 (d, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.9, 175.5, 161.5, 159.1, 157.8, 143.4, 140.8, 136.5, 130.9, 130.8, 129.3, 126.2, 125.9, 124.2, 121.4 (2C), 120.7, 119.6, 118.2, 116.5, 115.3, 44.6, 18.6; ESI-MS m/z: 407.3 $[M+H]^+$.

4.1.5.6. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(4-hydroxyphenyl)acryloyl)-[1,1'-biphenyl]-4-yl)propanoic acid (7f)

Compound **7f** was synthesized from **5** and **6f** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (3:1:0.5%) as eluent, the pure product **7f** was obtained as a reddish brown solid, yield 32.8%, mp 84.5-86.8 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.88 (brs, 1H), 12.63 (brs, 1H), 10.33 (brs, 1H), 8.34 (s, 1H), 7.91 (d, J = 15.6 Hz, 1H), 7.83 (d, J = 15.6 Hz, 1H), 7.80 (d, J = 8.4 Hz, 2H), 7.65 (m, 1H), 7.56-7.53 (m, 1H), 7.25-7.23 (m, 2H), 7.08 (d, J = 8.4 Hz ,1H), 6.85-6.83 (m, 2H), 3.77(q, J = 7.2 Hz, 1H), 1.41 (d, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 193.7, 175.3, 161.8, 161.0, 159.2, 146.3, 143.3, 136.6, 132.0, 131.9, 131.1, 130.8, 126.2, 125.9, 125.8, 124.1, 121.0, 118.1, 118.0, 116.2, 116.0, 115.2, 44.5, 18.6. ESI-MS m/z: 407.2 $[M+H]^+$.

4.1.5.7. (*E*)-2-(3'-(3-(2,4-dimethoxyphenyl)acryloyl)-2-fluoro-4'-hydroxy-[1,1'-biphenyl]-4-yl)propanoic acid (7g)

Compound **7g** was synthesized from **5** and **6g** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (4:1:0.5%) as eluent, the pure product **7g** was obtained as a reddish brown solid, yield 33.8%, mp 165.2-166.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.14 (brs, 1H), 8.18 (d, J = 15.2 Hz ,1H), 8.08 (s, 1H), 7.68 (d, J = 15.2 Hz, 1H), 7.64-7.56 (m, 2H), 7.43-7.39 (m, 1H), 7.20-7.16 (m, 2H), 7.07 (d, J = 8.8 Hz, 1H), 6.52 (d, J = 8.8Hz, 1H), 6.46-6.46 (m, 1H), 3.90 (s, 3H), 3.84 (s, 3H), 3.87-3.79 (m, 1H), 1.57 (d, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.1, 179.8, 163.5, 163.0, 160.7, 159.7, 141.6, 140.9, 136.2, 131.4, 130.4, 130.0, 127.1, 125.8, 123.8, 120.2, 118.6, 117.7, 116.7, 115.4, 105.6, 98.34, 55.6, 55.5, 44.8, 18.0. ESI-MS m/z: 451.2 $[M+H]^+$.

4.1.5.8. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(2-hydroxy-3-methoxyphenyl)acryloyl)-[1,1'-biphenyl]-4-yl)propanoic acid (7h)

Compound **7h** was synthesized from **5** and **6h** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (3:1:0.5%) as eluent, the pure product **7h** was obtained as a yellowish-brown solid, yield 53.7%, mp 159.8-161.8 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.62 (brs, 2H), 9.56 (brs, 1H), 8.27 (s, 1H), 8.23 (d, J = 15.6 Hz, 1H), 7.99 (d, J = 15.6 Hz, 1H), 7.71 (d, J = 8.4 Hz, 1H), 7.55-7.54 (m, 2H), 7.26-7.23 (m, 2H), 7.11-7.05 (m, 2H), 6.82-6.86 (m, 1H), 3.84 (s, 3H), 3.79-3.74 (m, 1H), 1.41 (d, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 193.9, 175.2, 161.4, 159.2, 148.3, 147.2, 143.2, 140.5, 136.5, 131.1, 130.8, 126.2, 126.1, 124.2, 121.7, 121.5, 121.1, 120.3, 119.4, 118.3, 115.3, 114.3, 56.3, 44.4, 18.6. ESI-MS m/z: 437.1 $[M+H]^+$.

4.1.5.9. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(2-hydroxy-5-methoxyphenyl)acryloyl)-[1,1'-biphenyl] -4-yl)propanoic acid (7i)

Compound **7i** was synthesized from **5** and **6i** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (4:1:0.5%) as eluent, the pure product **7i** was obtained as a reddish brown solid, yield 56.6%, mp 169.8-172.1 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.50 (brs, 2H), 10.00 (brs, 1H), 8.27 (s, 1H), 8.17 (d, J = 15.6 Hz, 1H), 8.02 (d, J = 15.6 Hz, 1H), 7.70 (d, J = 8.4 Hz, 1H), 7.56-7.52 (m, 1H), 7.46-7.45 (m, 1H), 7.26-7.23 (m, 2H), 7.10 (d, J = 8.4 Hz, 1H), 6.95-6.88 (m, 2H), 3.74 (s, 3H), 3.73-3.69 (m, 1H), 1.41 (d, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 194.0, 175.6, 161.4, 159.1, 152.5, 152.1, 143.5, 140.7, 136.5, 130.9, 130.7, 126.2, 125.9, 124.2, 121.6, 121.4, 120.9, 119.7, 118.2, 117.4, 115.2, 112.6, 56.0, 44.7, 18.6. ESI-MS m/z: 437.2 $[M+H]^+$.

4.1.5.10. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(3-hydroxy-4-methoxyphenyl)acryloyl)-[1,1'-biphen-yl]4-yl)propanoic acid (7j)

Compound **7j** was synthesized from **5** and **6j** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (4:1:0.5%) as eluent, the pure product **7j** was obtained as a yellowish-brown solid, yield 47.9%, mp 177.3-179.6 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.74 (brs, 2H), 9.21 (brs, 1H), 8.32 (s, 1H), 7.87 (d, J = 15.2 Hz, 1H), 7.76 (d, J = 15.2 Hz, 1H), 7.70 (d, J = 8.4 Hz, 1H), 7.57-7.53 (m, 1H), 7.43 (s, 1H), 7.34 (d, J = 8.0 Hz, 1H), 7.26-7.23 (m, 2H), 7.09 (d, J = 8.4 Hz, 1H), 7.00 (d, J = 8.0 Hz, 1H), 3.84 (s, 3H), 3.78-3.76 (m, 1H), 1.41 (d, J = 6.8Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 193.6, 175.4, 161.6, 159.2, 151.0, 147.0, 146.9, 143.1, 136.7, 130.9, 127.7, 126.2, 126.1, 124.2, 123.4,

123.1, 121.5, 118.1, 115.4, 115.2, 112.1, 112.0, 55.9, 44.5, 18.6. ESI-MS m/z: 437.2 $[M+H]^+$.

4.1.5.11. (*E*)-2-(3'-(3-(4-(dimethylamino)phenyl)acryloyl)-2-fluoro-4'-hydroxy-[1,1'-biphenyl]-4-yl)propanoic acid (7k)

Compound **7k** was synthesized from **5** and **6k** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (3:1:0.5%) as eluent, the pure product **7k** was obtained as a reddish brown solid, yield 59.9%, mp 81.5-82.9 ^oC. ^1H NMR (400 MHz, $CDCl_3$) δ 13.32 (brs, 1H), 8.04 (s, 1H), 7.89 (d, J = 15.6 Hz, 1H), 7.62-7.52 (m, 3H), 7.40 (m, 1H), 7.40-7.36 (m, J = 15.6 Hz, 1H), 7.20-7.18 (m, 2H), 7.07 (d, J = 8.8 Hz, 1H), 6.64 (d, J = 8.8 Hz, 2H), 3.83-3.79 (m, 1H), 3.00 (s, 6H), 1.58 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.3, 179.7, 163.1, 159.7, 152.3, 146.9, 140.9, 134.0, 131.0 (2C), 130.6, 129.8, 127.3, 125.7, 123.8, 122.1, 120.2, 118.6, 115.4, 113.7, 111.7 (2C), 44.8, 40.0 (2C), 18.0. ESI-MS m/z: 434.2 $[M+H]^+$.

4.1.5.12. (*E*)-2-(3'-(3-(2-(dimethylamino)phenyl)acryloyl)-2-fluoro-4'-hydroxy-[1,1'-biphenyl]-4-yl)propanoic acid (7l)

Compound **7l** was synthesized from **5** and **6l** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (3:1:0.5%) as eluent, the pure product **7l** was obtained as a reddish brown solid, yield 64.3%, mp 77.2-78.1 ^oC. ^1H NMR (400 MHz, $CDCl_3$) δ 13.04(brs, 1H), 8.32 (d, J = 15.6 Hz, 1H), 8.09 (s, 1H), 7.65 (d, J = 15.6 Hz, 1H), 7.69-7.63 (m, 2H), 7.43-7.34 (m, 2H), 7.21-7.16 (m, 2H), 7.11-7.06 (m, 2H), 7.04-7.00 (m, 1H), 3.81-3.79 (m, 1H), 2.80 (s, 6H), 1.57 (d, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.1, 179.8, 163.1, 159.3, 154.5, 144.2, 141.1, 136.6, 131.5, 130.4, 130.0, 128.6, 127.9, 127.0, 126.0, 123.8, 122.0, 120.1, 118.9, 118.7, 118.5, 115.4, 45.1 (2C), 44.9, 18.0. ESI-MS m/z: 434.3 $[M+H]^+$.

4.1.5.13. (*E*)-2-(3'-(3-(4-(diethylamino)phenyl)acryloyl)-2-fluoro-4'-hydroxy-[1,1'-biphenyl]-4-yl) propanoic acid (7m)

Compound **7m** was synthesized from **5** and **6m** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (3:1:0.5%) as eluent, the pure product **7m** was obtained as a reddish brown solid, yield 64.5%, mp 84.7-85.9 ^oC. ^1H NMR (400 MHz, $CDCl_3$) δ 13.33 (brs, 1H), 8.05 (s, 1H), 7.92 (d, J = 15.6 Hz, 1H), 7.61(d, J = 8.8 Hz, 1H), 7.54(d, J = 8.8 Hz, 2H), 7.43-7.39 (m, 1H), 7.41 (d, J = 15.6 Hz, 1H),

7.21-7.17 (m, 2H), 7.07 (d, *J* = 8.4 Hz, 1H), 6.65-6.63 (m, 2H), 3.82 (q, *J* = 7.2 Hz, 1H), 3.39 (q, *J* = 6.8 Hz, 4H), 1.58 (d, *J* = 7.2 Hz, 3H), 1.19 (q, *J* = 6.8Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.2, 179.9, 163.0, 159.1, 150.1, 147.1, 140.9, 135.9, 131.3, 130.5, 129.7, 127.2, 125.7, 123.8, 121.4, 120.3, 118.6, 115.3, 113.1, 111.2 (2C), 44.9, 44.5 (2C), 18.0, 12.5(2C). ESI-MS m/z: 462.2 $[M+H]^+$.

4.1.5.14. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(4-(piperidin-1-yl)phenyl)acryloyl)-[1,1'-biphenyl]-4-yl)propanoic acid (7n)

Compound **7n** was synthesized from **5** and **6n** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (2:1:0.5%) as eluent, the pure product **7n** was obtained as a reddish brown solid, yield 68.2%, mp 98.1-99.6 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.21 (brs, 1H), 8.05 (s, 1H), 7.89 (d, *J* = 16.0 Hz, 1H), 7.61 (d, *J* = 7.8 Hz, 1H), 7.54 (d, *J* = 8.4 Hz, 2H), 7.45-7.40 (m, 1H), 7.41 (d, *J* = 16.0 Hz, 1H), 7.21-7.17 (m, 2H), 7.07 (d, *J* = 8.4 Hz, 1H), 6.86 (d, *J* = 8.4 Hz, 2H), 3.82-3.81 (m, 1H), 3.31 (s, 4H), 1.68-1.64 (m, 6H), 1.57 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.4, 179.8, 163.0, 159.1, 153.3, 146.6, 141.0, 136.1, 130.81(2C), 130.5, 129.8, 127.1, 125.8, 123.8, 123.7, 120.2, 118.6, 115.4, 114.7, 114.4 (2C), 48.7 (2C), 44.9, 25.3 (2C), 24.2, 18.0. ESI-MS m/z: 474.2 $[M+H]^+$.

4.1.5.15. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(4-morpholinophenyl)acryloyl)-[1,1'-biphenyl]-4-yl) propanoic acid (7o)

Compound **7o** was synthesized from **5** and **6o** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (3:1:0.5%) as eluent, the pure product **7o** was obtained as a reddish brown solid, yield 38.6%, mp 181.7-182.5 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.12 (brs, 1H), 8.06 (s, 1H), 7.91 (d, *J* = 15.2 Hz, 1H,), 7.64 (d, *J* = 8.4 Hz, 2H), 7.59 (d, *J* = 8.8 Hz, 2H), 7.48 (d, *J* = 15.2 Hz, 1H), 7.44-7.40 (m, 1H), 7.23-7.18 (m, 2H), 7.10 (d, *J* = 8.8 Hz, 1H), 6.88 (d, *J* = 8.8 Hz, 2H), 3.87-3.80 (m, 5H), 3.28-3.26 (m, 4H), 1.59 (d, *J* = 7.2Hz ,3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.5, 179.2, 163.1, 159.2, 153.3, 146.1, 141.0, 136.3, 130.6 (2C), 130.5, 129.9, 127.2, 125.9, 125.2, 123.8, 120.1, 118.7, 115.9, 115.5, 114.4 (2C), 65.5 (2C), 47.7 (2C), 44.7, 18.0. ESI-MS m/z: 476.3 $[M+H]^+$.

4.1.5.16. (*E*)-2-(3'-(3-(3,4-dimethoxyphenyl)acryloyl)-2-fluoro-4'-hydroxy-[1,1'-biphenyl]-4-yl) propanoic acid (7p)

Compound **7p** was synthesized from **5** and **6p** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid

(4:1:0.5%) as eluent, the pure product **7p** was obtained as a reddish brown solid, yield 45.0%, mp 174.8-176.1 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.98 (brs, 1H), 8.06 (s, 1H), 7.91 (d, J = 15.2 Hz, 1H), 7.64 (d, J = 8.4 Hz, 1H), 7.48(d, J = 15.2 Hz, 1H), 7.43-7.39 (m, 1H), 7.28-7.26 (m, 1H), 7.21-7.15 (m, 3H), 7.10 (d, J = 8.4Hz, 1H), 6.90 (d, J = 8.4 Hz ,1H), 3.95 (s, 3H), 3.93 (s, 3H), 3.81 (q, J = 6.8 Hz, 1H), 1.58 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.5, 179.8, 163.1, 159.2, 151.7, 149.2, 141.0, 140.9, 136.6, 130.5, 129.9, 127.4, 127.1, 125.9, 123.8, 123.7, 119.9, 118.8, 117.5, 115.4, 111.1, 110.4, 56.0, 55.9, 44.8, 17.9. ESI-MS m/z: 451.2 $[M+H]^+$.

4.1.5.17. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(pyridin-3-yl)acryloyl)-[1,1'-biphenyl]-4-yl)propanoic acid (7q)

Compound **7q** was synthesized from **5** and **6q** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (3:1:0.5%) as eluent, the pure product **7q** was obtained as a yellow solid, yield 42.1%, mp 196.7-198.1 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.48 (brs, 2H), 9.04 (s, 1H), 8.62 (s, 1H), 8.38-8.32 (m, 2H), 8.21 (d, J = 14.4 Hz, 1H), 7.87 (d, J = 14.4 Hz, 1H), 7.17 (brs, 1H), 7.53-7.49 (m, 2H), 7.24 (brs, 2H), 7.12 (brs, 2H), 3.86-3.75 (m, 1H), 1.40 (m, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 193.4, 175.7, 161.1, 159.1, 151.5, 150.9, 143.7, 141.6, 136.8, 135.6, 131.1, 130.9, 130.6, 126.4, 125.8, 124.3, 124.2 (2C), 121.4, 118.2, 115.2, 44.8, 18.7. ESI-MS m/z: 392.2 $[M+H]^+$.

4.1.5.18. (*E*)-2-(2-fluoro-4'-hydroxy-3'-(3-(pyridin-4-yl)acryloyl)-[1,1'-biphenyl]-4-yl)propanoic acid (7r)

Compound **7r** was synthesized from **5** and **6r** according to the general procedure. After purification by chromatography on silica gel using petroleum ether/ethyl acetate/acetic acid (3:1:0.5%) as eluent, the pure product **7r** was obtained as a yellowish-brown solid, yield 42.1%, mp 151.2-152.6 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.48 (brs, 2H), 9.04 (s, 1H), 8.62 (s, 1H), 8.38-8.32 (m, 2H), 8.21 (d, J = 14.4 Hz, 1H), 7.87 (d, J = 14.4 Hz, 1H), 7.17 (brs, 1H), 7.53-7.49 (m, 2H), 7.24 (brs, 2H), 7.12 (brs, 2H), 3.86-3.75 (m, 1H), 1.40 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 193.4, 175.7, 161.1, 159.1, 151.5, 150.9, 143.7, 141.6, 136.8, 135.6, 131.1, 130.9, 130.6, 126.4, 125.8, 124.3, 124.2 (2C), 121.4, 118.2, 115.2, 44.8, 18.7. ESI-MS m/z: 392.3 $[M+H]^+$.

4.2. Biological evaluation

4.2.1. Oxygen radical absorbance capacity (ORAC-FL) assay

The antioxidant potency was determined by the oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay with slight modification.[37] Fluorescein (FL) and 6-Hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (trolox) were purchased from TCI (Shanghai) Development, 2,2′-Azobis (amidino propane) dihydrochloride (AAPH) was purchased from Accela ChemBio Co. Ltd. The reaction was carried out in 75 μL phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. Antioxidant (20 μL) and FL (120 μL, 150 nM final concentration) were placed in the wells of a black 96-well plate and the mixture was pre-incubated for 15 min at 37 ^{o}C. Then AAPH solution (60 μL, 12 mM final concentration) was added rapidly using an autosampler. The plate was immediately placed in a Varioskan Flash Multimode Reader (Thermo Scientific) and the fluorescence recorded every minute for 90 min with excitation at 485 nm and emission at 535 nm. Trolox was used as standard (1–8 μM, final concentration). A blank (FL + AAPH) using phosphate buffer instead of antioxidant and trolox calibration were carried out in each assay. The samples were measured at different concentration (1–10 μM). All the reaction mixture was prepared in duplicate, and at least three independent assays were measured for each sample. Antioxidant curves (fluorescence versus time) were normalized to the curve of the blank in the same assay, and the area under the fluorescence decay curve (AUC) was calculated. The net AUC of a sample was obtained by subtracting the AUC of the blank. ORAC-FL values were expressed as Trolox equivalents by using the standard curve calculated for each sample, where the ORAC-FL value of Trolox was taken as 1, indicating the antioxidant potency of the tested compounds.

4.2.2. Inhibition of self- and Cu^{2+}-induced A$\beta_{1–42}$ aggregation

Inhibition of Aβ_{1-42} aggregation was measured by using a Thioflavin T-based fluorometric assay.[38-40] Thioflavin T (Basic Yellow 1, ThT) was purchased from TCI (Shanghai) Development. 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was purchased from Energy Chemical. β-Amyloid$_{1-42}$ (A$\beta_{1–42}$), supplied as trifluoroacetate salt, was purchased from GL Biochem (Shanghai) Ltd. Briefly, A$\beta_{1–42}$ was dissolved in HFIP (1 mg/mL) and incubation for 24 h at room temperature, then the solvent was evaporated. And it was dissolved in dry DMSO to a final stock concentration of 200 μM and kept frozen at -80 ^{o}C. Tested compounds were dissolved in DMSO in 2.5 mM for storage and diluted with phosphate buffer solution (pH 7.4) before use. For the self-induced aggregation assay, A$\beta_{1–42}$ (20 mL, 25 mM, final concentration) together with test compounds (20 mL, 25 mM, final concentration) were incubated in 50 mM phosphate buffer solution (pH 7.40) at 37 ^{o}C for 24 h. The

plate was sealed with a transparent heat-resistant plastic film to minimize evaporation effect. After the incubation, 160 μL of 5 μM ThT in 50 mM glycine-NaOH buffer (pH 8.5) was added. Each assay was run in triplicate. Fluorescence was measured on a Varioskan Flash Multimode Reader (Thermo Scientific) with excitation and emission wavelengths at 446 nm and 490 nm, respectively. The percent inhibition due to the presence of the inhibitor was calculated by the following expression: $(1\text{-}IF_i/IF_c) \times 100$, in which IF_i and IFc are the fluorescence intensities obtained for $A\beta_{1-42}$ in the presence and in the absence of inhibitors after subtracting the background, respectively.

As for the Cu^{2+} induced $A\beta_{1-42}$ aggregation assay, solutions of Cu^{2+} were prepared from standards to concentration of 75 μM using the HEPES buffer (20 mM, pH 6.60, 150 mM NaCl). The $A\beta_{1-42}$ stock solution was diluted with HEPES buffer (20 mM, pH 6.60, 150 mM NaCl). The mixture of $A\beta_{1-42}$ (20 μL, 25 mM, final concentration) and Cu^{2+} (20 μL, 25 μM, final concentration), with or without the tested compound (20 μL, 25 μM, final concentration) was incubated at 37 ^{o}C for 24 h. Then, 190 μL of 5 μM ThT in 50 mM glycine-NaOH buffer (pH 8.50) was added. Each assay was run in triplicate. The detection method was the same as that of self-induced $A\beta_{1-42}$ aggregation assay.

4.2.3. *In vitro* inhibition of monoamine oxidase[41,42]

Recombinant human MAO-A and MAO-B were obtained from commercial sources (Sigma–Aldrich Co.). All test compounds were prepared in DMSO and diluted with potassium phosphate buffer (pH 7.4) before use. All the enzymatic reactions were conducted in potassium phosphate buffer (100 mM, pH = 7.4, made isotonic with KCl 20.2 mM) to a final volume of 500 μL containing kynuramine (45 μL for MAO-A and 30 μL for MAO-B), various concentrations of the test inhibitors (0-100 μL) and lower than 4% DMSO as cosolvent. The reactions were initiated by the addition of MAO-A or -B (7.5 μg/mL) and were subsequently incubated for 30 min at 37 °C. The reactions were terminated by the addition of 400 μL NaOH (2.0 M) and 1000 μL water, centrifuged for 10 min at 16000 g. The concentrations of 4-hydroxyquinoline genarated from MAO in the supernatants were measured by using the Varioskan Flash Multimode Reader (Thermo Scientific) with excitation and emission wavelengths at 310 nm and 400 nm, respectively. The percent inhibition due to the presence of the inhibitor was calculated by the following expression: $(1\text{-}IF_i/IF_c) \times 100$, in which IF_i and IFc are the fluorescence intensities obtained for kynuramine in the presence and in the absence of inhibitors after subtracting the background, respectively.

4.2.4. *In vitro* anti-inflammatory activity studies

To evaluate the anti-inflammatory activity of these hybrid compounds, the *in vitro* cytotoxicity and inhibition of LPS-induced NO and TNF-α production were investigated. BV-2 cell-line was obtained from ATCC, and cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/mL) and streptomycin (10 μg/mL) at 37 ^{o}C in a humidified incubator with 5% CO_2. DMEM was purchased from Gibco and lipopolysaccharide (LPS) was purchased from Sigma. Nitric Oxide Assay Kits were purchased from Nanjing Jiancheng Bioengineering Institute and TNF alpha Rat ELISA Kits were purchased from Abcam. All test samples were prepared in DMSO at various concentrations (0.5, 2.5 and 10.0 μM) before addition to the experimental wells.

The *in vitro* cytotoxicity assay was carried out by the MTT method.[43] BV-2 cells prepared in DMEM (100 μL) were cultured in 96-well cell culture microplate and incubated at 37 ^{o}C for 24 h in a humidified 5% CO_2 atmosphere. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. The test samples at a volume of 10 μL were added to confluent cells in triplicate wells. After the addition of the test samples, the treated wells were incubated for 30 minutes. And then LPS (10 μL, 1.0 μg/mL) was added, the treated wells were incubated for another 24 h. After the incubation period, 100 μL of MTT solution was added to each well. The wells were incubated for 4 h. The crystals formed were dissolved in 200 μL of DMSO and the absorbance was detected at 490 nm using a spectrophotometer. Results are expressed as percent viability compared to untreated cells.

Inhibition of LPS-induced NO production was measured by using a Griess reaction method.[44-46] BV-2 cells prepared in DMEM (100 μL) were cultured in 96-well cell culture microplate and incubated at 37 ^{o}C for 24 h in a humidified 5% CO_2 atmosphere. $NaNO_2$ was prepared in the mixture of PBS and DMEM at various concentrations (0, 1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 μM) before addition to the experimental wells. The test samples at a volume of 10 μL were added to confluent cells in triplicate wells. After the addition of the test samples, the treated wells were incubated for 30 minutes. And then LPS (10 μL, 1.0 μg/mL) was added, BV2-cells were stimulated with LPS in the presence of test samples for 24 h. NO released from BV-2 cells was measured by the determination of $NaNO_2$ concentration in culture supernatant. Samples (50 μL) of culture media were incubated with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene

diamine in 2.5% phosphoric acid solution) at room temperature for 10 min. Absorbance at 540 nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as standard.

Inhibition of LPS-induced TNF-α production was measured by using a Enzyme-linked immunosorbent assay (ELISA).[47] BV-2 cells prepared in DMEM (100 μL) were cultured in 96-well cell culture microplate and incubated at 37 ^{o}C for 24 h in a humidified 5% CO_2 atmosphere. The test samples at a volume of 10 μL were added to confluent cells in triplicate wells. After the addition of the test samples, the treated wells were incubated for 30 minutes. And then LPS (10 μL, 1.0 μg/mL) was added, BV2-cells were stimulated with LPS in the presence of test samples for 24 h. And then the TNF-α released from BV-2 cells was measured by the ELISA assay according to the manufacturer's instructions.

4.2.5. Metal-chelating studies

The chelating studies were performed with a Varioskan Flash Multimode Reader (Thermo Scientific). The UV absorption spectra of compounds (**7k** or **7m**) alone or in the presence of $CuCl_2$, $ZnCl_2$, $AlCl_3$, and $FeSO_4$ were recorded with wavelength ranging from 200 to 600 nm after incubating for 30 min at room temperature. The final volume of reaction mixture was 200 μL, and the final concentrations of the tested compound and metals were 37.5 μM. Numerical subtraction of the spectra of the metal alone and the compound alone from the spectra of the mixture gave the difference UV–vis spectra due to complex formation.

The stoichiometry of the compound-Cu^{2+} complex was determined by molar ration method. The methanol solution of tested compound was titrated with ascending of $CuCl_2$. And the final concentration of tested compound was 37.5 μM, the final concentration of Cu^{2+} ranged from 3.75 to 150 μM. The UV spectra were recorded and treated by numerical subtraction of $CuCl_2$ and tested compound at corresponding concentrations, plotted versus the mole fraction of tested compound.

4.2.6. *In vitro* blood-brain barrier permeation assay

The *in vitro* blood–brain barrier penetration of target compounds **7k** and **7m** was evaluated by using the parallel artificial membrane permeation assay of the blood–brain barrier (PAMPA-BBB) which is wildly used in the early phase of drug discovery.[39,51] Porcine brain lipid (PBL) was purchased from Avanti Polar Lipids. The donor plate (MATRNPS50) and the acceptor plate (PVDF

membrane, pore size 0.45 μm, MAIPN4550) were purchased from Millipore. Filter PDVF membrane units (diameter 25 mm, pore size 0.45 μm) from Pall Corporation were used to filter the samples. And commercial drugs were purchased from Sigma and Alfa Aesar. Test compounds were dissolved in DMSO at 5 mg/mL and diluted 50-fold in PBS/EtOH (70:30) to a final concentration of 100 μg/mL. Then the compound solution (100 μg/mL, 350 μL) was added to the donor wells. The acceptor wells were filled with 200 μL of PBS/EtOH (70:30) and the filter membrane was coated with PBL in dodecane (selected empirically as 4 μL volume of 20 μg/mL PBL in dodecane) and acceptor wells were filled with 200 μL of PBS/EtOH (70:30). The acceptor filter plate was carefully put on the donor plate to form a sandwich (consisting of the aqueous donor with test compound on the bottom, lipid membrane in the middle and the aqueous acceptor on the top), which was left undisturbed for 18 h at 25 °C. After incubation, the donor and acceptor plates were separated carefully and the concentration of drug in the donor and acceptor wells was determined using the Varioskan Flash Multimode Reader (Thermo Scientific). Every sample was analyzed at ten wavelengths in four wells and in at least three independent runs. P_e was calculated using the following expression:

$$P_e = -\ln [1 - C_A(t)/C_{equilibrium}]/[A \times (1/V_D + 1/V_A) \times t]$$

$$C_{equilibrium} = [C_D(t) \times V_D + C_A(t) \times V_A]/(V_D + V_A)$$

Where P_e is permeability in the unit of cm/s. A is effective filter area and t is the permeation time. V_D is the volume of donor well and V_A is the volume of acceptor well. $C_A(t)$ is the compound concentration in acceptor well at time t, and $C_D(t)$ is the compound concentration in donor well at time t. Results are given as the mean ± SD.

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Supplementary data

Supplementary data (the ^{1}H NMR and ^{13}C NMR spectra of target compounds) associated with this article can be found, in the online version.

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