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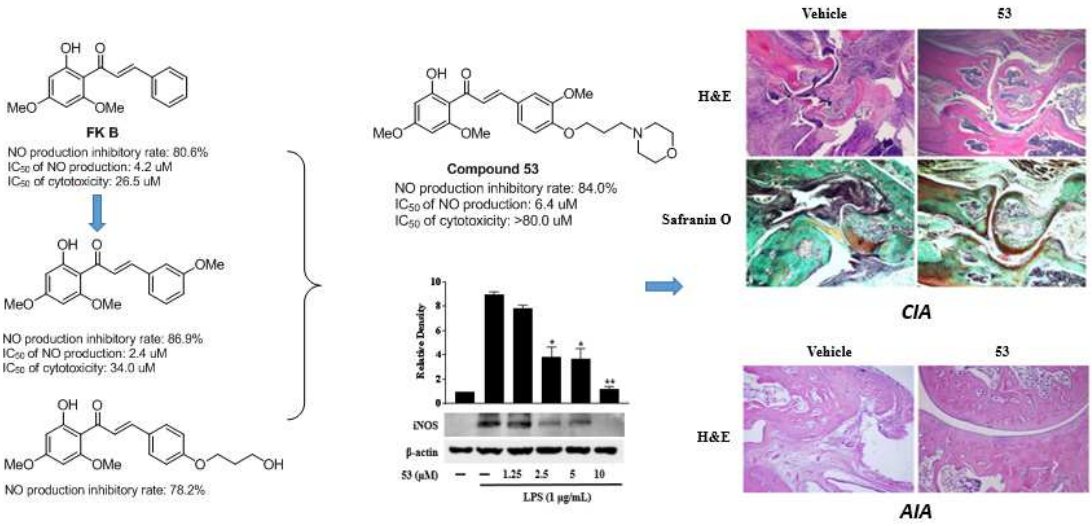
Graphical abstract



Development of a novel nitric oxide (NO) production inhibitor with potential therapeutic effect on chronic inflammation

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Abstract:

Inflammation is a complex biological response to stimuli. Activated macrophages induced excessively release of pro-inflammatory cytokines and mediators such as endogenous radical nitric oxide (NO) play a significant role in the progression of multiple inflammatory diseases. Both natural and synthetic chalcones possess a wide range of bioactivities. In this work, thirty-nine chalcones and three related compounds, including several novel ones, based on bioactive kava chalcones were designed, synthesized and their inhibitory effects on NO production in RAW 264.7 cells were evaluated. The novel compound

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxy-4-(3-morpholinopropoxy)phenyl)prop-2-en-1-one (**53**) exhibited a better inhibitory activity (84.0 %) on NO production at 10 μM (IC_{50} = 6.4 μM) with the lowest cytotoxicity (IC_{50} > 80 μM) among the tested compounds. Besides, western blot analysis indicated that compound **53** was a potent down-regulator of inducible nitric oxide synthase (iNOS) protein. Docking study revealed that compound **53** also can dock into the active site of iNOS. Furthermore, at the dose of 10 mg/kg/day, compound **53** could both significantly suppress the progression of inflammation on collagen-induced arthritis (CIA) and adjuvant-induced arthritis (AIA) models. In addition, the structure-activity relationship (SAR) of the kava chalcones based analogs was also depicted.

Keywords:

inflammation; nitric oxide (NO); kava chalcones; inducible nitric oxide synthase (iNOS); structure-activity relationship (SAR)

1. Introduction

Inflammation is a primary response of organisms to various inflammatory agents, such as bacteria, viruses, radiation and so on. The typical physiological features of inflammation are characterized by redness, swelling, fever, pain, and even loss of organ functions[1]. Previous studies indicated that activated macrophages induce excessively release of pro-inflammatory cytokines and mediators such as endogenous radical nitric oxide (NO), tumor necrosis factors-α (TNF-α), prostaglandin E2 (PGE2) and interleukin-1β (IL-1β) play a significant role in the progression of multiple inflammatory diseases[2-5]. The oxidation of L-arginine to L-citrulline producing NO in the process is catalyzed by the inducible nitric oxide synthase (iNOS)[6, 7]. Pro-inflammatory cytokines and lipopolysaccharide (LPS) can activate the iNOS to overproduce NO then further induce the injury at the inflammatory sites[8, 9]. Hence, suppression of the expression and/or activity of iNOS and reduction the overexpression of NO by drugs are crucial aspects of inflammation control[7, 10, 11].

Chalcones, which are known as compounds derived from 1,3-diphenylprop-2-en-1-one with two aromatic rings linked by conjugated three carbon α,β unsaturated carbonyl bridge (**Figure 1**), are a set of molecules that belong to the flavonoid family[12, 13]. This class of molecules attracts considerable attention and has been proved to possess a wide range of bioactivities such as anti-tumor, antibacterial, anti-tubercular, anti-inflammatory, anti-virus, anti-malarial, antileishmanial, antioxidant and so on[12, 14-20]. In order to enhance their bioactivities and to develop potential therapeutic candidates, synthetic modifications of chalcones have also been intensively carried out[21-25].

Kava, scientifically named as *Piper methysticum*, is an ancient perennial shrub which is grown in the South Pacific. It is traditionally used for religious and ceremonial events, medicinal purposes and social gatherings in this region[26-28]. The chemical constituents and their biological functions of kava extracts have been extensively studied in recent decades. Chalcones and kavalactones are two major phytochemicals present within this plant[29]. Kava chalcones (identified as **FK A**, **B**,

and **C**, **Figure 1**) from the kava extracts exhibit the activities of anti-tumor, anti-inflammation and so on[30, 31]. Despite the increasing interest in the biological profile of kava chalcones, little attention has been paid to the structural modifications and structure-activity relationship of kava chalcones associated with their activities, such as anti-inflammation[21, 31-34].

In the continuous effort to explore novel chemical entities for the treatment of inflammation and related diseases[11, 35, 36], we were interested in designing and synthesizing novel kava chalcones analogs. In this work, our design of target compounds started from three points mainly based on scaffold-hopping method: i) modification of B ring while keeping the special substitutions of A ring; ii) introduction of electron-donating and electron-withdrawing groups or atoms at the middle double bond to interrupt the rigid plane structure of chalcone[37]; iii) modification of A ring to check the importance of the special substitutions. Then the inhibitory effects on NO production of these compounds in LPS-induced RAW 264.7 cells were evaluated. Among these derivatives, a novel compound **53** was identified as the most potent chemical molecule. The IC_{50} value of compound **53** on NO production was 6.4 μM with much lower cytotoxicity than any other compound. Western blot result revealed that compound **53** inhibited the expression of LPS-induced iNOS protein in a dose-dependent manner. Besides, the inhibitory effect was further confirmed by docking study which showed that compound **53** could bind into the active site of iNOS. Furthermore, compound **53** significantly suppressed the progression of inflammation in both two rheumatoid arthritis models of collagen-induced arthritis (CIA) and adjuvant-induced arthritis (AIA). The structure-activity relationship (SAR) of these compounds was also discussed.

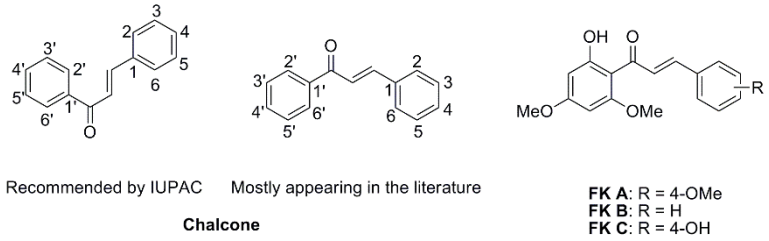


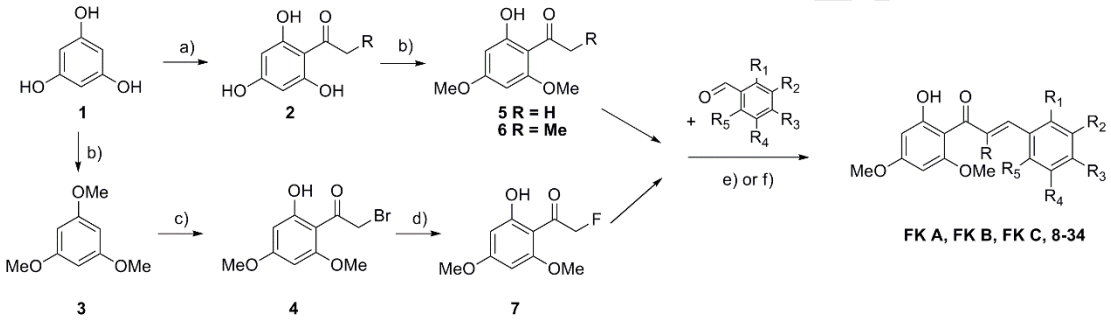
Figure 1. The structures of chalcone and **FK A**, **FK B** and **FK C**.

[Please Insert Figure 1 Here]

2. Results and discussion

2.1 Chemistry

Intermediates **5**, **6**, **7** were prepared from the commercially available reagent *m*-trihydroxybenzene **1** in three steps. The target compounds were synthesized via Claisen-Schmidt condensation between compounds **5**, **6**, **7**, respectively, with appropriate benzaldehydes (including purchased and self-prepared ones) under suitable basic conditions with 30 % to 80 % isolated yields[22]. The general synthetic route of **FK A**, **FK B**, **FK C** and **8 - 34** was illustrated in **Scheme 1**.



Series 1 R = H

FK A: R1 = R2 = R4 = R5 = H, R3 = OMe
FK B: R1 = R2 = R3 = R4 = R5 = H
FK C: R1 = R2 = R4 = R5 = H, R3 = OH
8: R1 = R3 = R4 = R5 = H, R2 = OMe
9: R1 = R2 = R4 = R5 = H, R3 = Me
10: R1 = R2 = R4 = R5 = H, R3 = Br
11: R1 = R2 = R4 = R5 = H, R3 = NO_2
12: B ring = pyridin-3-yl
13: R1 = R4 = R5 = H, R2 = OH, R3 = OMe
14: R1 = R4 = R5 = H, R2 = R3 = OMe
15: R1 = R4 = R5 = H, R2+R3 = methylene-dioxy
16: R1 = R3 = R4 = H, R2 = R5 = OMe
17: R1 = R2 = R5 = H, R3 = R5 = OMe
18: R1 = R5 = H, R2 = R3 = R4 = OMe
19: R1 = R4 = H, R2 = R3 = R5 = OMe
20: R1 = R4 = R5 = H, R2 = Br, R3 = OMe
21: R1 = R5 = H, R2 = R3 = OMe, R4 = NO2
22: R1 = R2 = R4 = R5 = H, R3 = hydroxyethyl
23: R1 = R2 = R4 = R5 = H, R3 = hydrooxypropyl

Series 2 R = Me

24: R1 = R2 = R4 = R5 = H, R3 = OMe
25: R1 = R3 = R4 = R5 = H, R2 = OMe
26: R1 = R2 = R4 = R5 = H, R3 = Me
27: R1 = R2 = R3 = R4 = R5 = H

Series 3 R = F

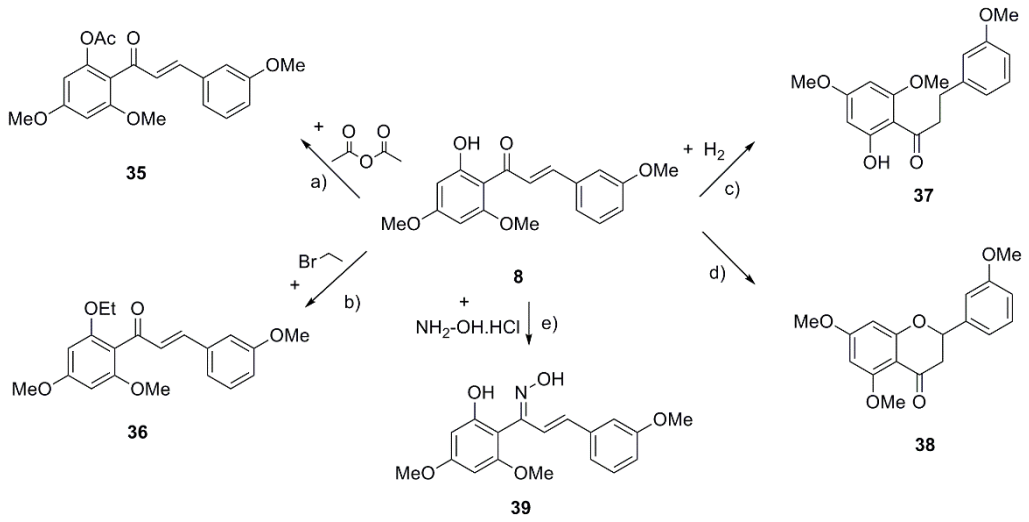
28: R1 = R2 = R4 = R5 = H, R3 = OMe
29: R1 = R3 = R4 = R5 = H, R2 = OMe
30: R1 = R2 = R3 = R4 = R5 = H
31: R1 = R2 = R4 = R5 = H, R3 = OH
32: R1 = R2 = R4 = R5 = H, R3 = Me
33: R1 = R4 = R5 = H, R2 = R3 = OMe
34: R1 = R3 = R4 = H, R2 = R5 = OMe

Scheme 1. The synthetic route of **FK A**, **FK B**, **FK C**, **8 - 34**. Reagents and conditions: a) Acetic anhydride or Propionic anhydride, $BF_3.OEt_2$, N_2, 50 ^oC; b) Acetone, Me_2SO_4, K_2CO_3, reflux; c) dry CS_2, Bromoacetyl bromide, $AlCl_3$, N_2, reflux; d) dry MeCN, KF, TMBA(cat. amount), reflux; e) R = H or Me: MeOH, 40 % NaOH, N_2, 0 ^oC - r.t.; f) R = F: MeOH, Piperidine(cat. amount), r.t..

[Please Insert Scheme 1 Here]

In view of the preliminary activity data of series 1 - 3, compounds **35 - 39** were designed and synthesized from compound **8** in various yields, shown as **Scheme 2**, to

check the importance of 2'-hydroxy group of A ring and α,β unsaturated carbonyl bridge.



Scheme 2. The synthetic routes of **35** - **39**. Reagents and conditions: a) reflux; b) dry THF, NaH, N_2, 0 ^oC; c) MeOH, PtO_2, r.t.; d) MeOH, c.c. HCl, reflux; e) MeOH, pyridine, r.t..

By using the same method described above to synthesize **FK A** and so on, three A ring simplified chalcones were obtained from 2'-hydroxy-4'-methoxyacetophenone and 2'-hydroxyacetophenone with 4-methoxybenzaldehyde or 3-methoxybenzaldehyde. Their structures were outlined as the following **Figure 2**:

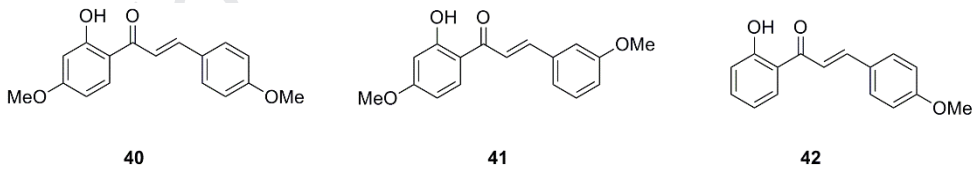
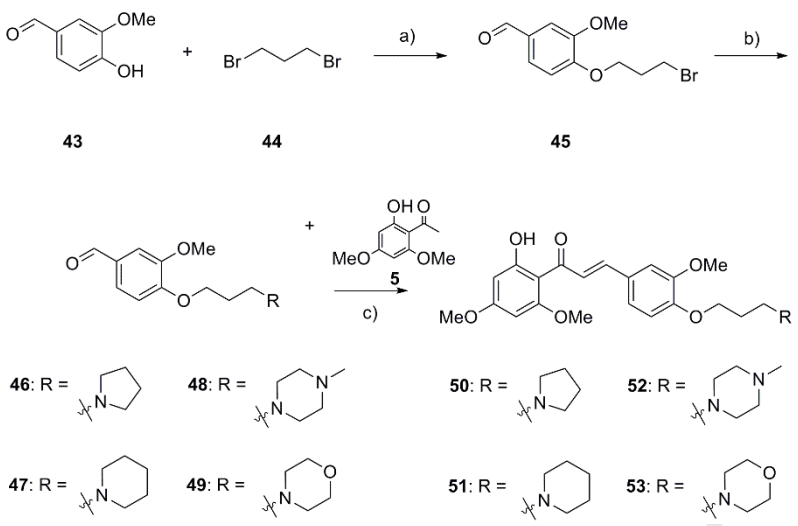


Figure 2. The structures of **40** - **42**.

[Please Insert Figure 2 Here]

Taking the significance of a methoxy group at 3-position and potential helpful contribution for the NO production inhibitive activity of a linker at 4-position on the B ring into consideration, compounds **50** - **53** were designed and synthesized, the preparative route was shown as **Scheme 3**.



Scheme 3. The synthetic route of **50 - 53**. Reagents and conditions: a) MeCN, K_2CO_3, reflux; b) MeCN, pyrrolidine or piperidine or N-methyl piperazine or morpholine, K_2CO_3, r.t.; c) MeOH, 40 % NaOH, N_2, 0 ^oC - r.t..

[Please Insert Scheme 3 Here]

2.2 Biological evaluation

2.2.1 Inhibition of NO production in LPS-induced RAW 264.7 cells

RAW 264.7 macrophages were treated with the target compounds at the concentration of 10 μM and LPS (1 μg/mL) for 24 hours. Inhibitory effects of tested compounds on NO production were indirectly measured by the analysis of nitrite concentration based on the Griess reaction[35, 38]. A naturally occurring anti-inflammatory flavone **Luteolin** (**Lut.**) was used as a positive control[39-42]. The result was summarized in **Table 1**.

Table 1. Inhibitory effect of compounds **FK A**, **FK B**, **FK C** and **8 - 42**, **50 - 53** on the production of NO in LPS-induced RAW 264.7 macrophages.

Cpd.	NO inhibition rate (%) ± SD[a]	Cpd.	NO inhibition rate (%) ± SD[a]
FK A	79.5 ± 2.6	**28**[b]	50.3 ± 3.3
FK B	80.6 ± 2.4	**29**[b]	58.1 ± 4.2
FK C	66.8 ± 1.3	**30**[b]	47.4 ± 1.9
8	86.9 ± 2.2	**31**[b]	70.0 ± 2.8
9	82.1 ± 3.7	**32**[b]	50.8 ± 3.1

10	70.2 ± 3.1	**33**[b]	62.6 ± 3.8
11	35.4 ± 1.9	**34**[b]	42.6 ± 4.1
12	78.0 ± 2.4	**35**[b]	84.6 ± 3.2
13	70.6 ± 3.5	**36**[b]	85.6 ± 4.4
14	78.7 ± 3.6	**37**[b]	52.2 ± 1.9
15	70.0 ± 2.8	**38**	57.3 ± 2.8
16	73.7 ± 4.5	**39**[b]	46.4 ± 3.7
17	67.6 ± 2.9	**40**	70.0 ± 2.6
18	77.4 ± 4.5	**41**	82.5 ± 1.8
19	78.4 ± 2.2	**42**	76.7 ± 2.2
20	73.8 ± 1.0	**50**[b]	83.1 ± 2.2
21[b]	30.8 ± 3.4	**51**[b]	74.8 ± 3.6
22[b]	73.6 ± 2.4	**52**[b]	70.4 ± 3.1
23[b]	78.2 ± 4.1	**53**[b]	84.0 ± 2.5
24	41.6 ± 3.9	**Lut.**	54.6 ± 2.9
25[b]	68.3 ± 1.5	**Control**	100.0
26[b]	56.8 ± 2.7	**LPS**	0.0
27	57.5 ± 3.3		

[a] The cells were pretreated with **FK A**, **FK B**, **FK C** and **8 - 42**, **50 - 53**, **Lut.** at a concentration of 10 μM and LPS (1 μg/mL) for 24 hours. The inhibition of the LPS-treated group was set as 0.0 %, and the control group was set as 100.0 %. Inhibition rate (%) = 100.0 % – [Compound (OD_{540}) – Control (OD_{540})] / [LPS (OD_{540}) – Control (OD_{540})] × 100.0 %. The results were shown as the means ± SD (n = 3). [b] Novel compound.

2.2.2 IC_{50} of NO production and cytotoxicity of the selected compounds

To investigate the inhibitory effect on NO production in LPS stimulated RAW 264.7 macrophages of the derivatives in a dose-dependent manner, eight compounds with the positive control **Lut.**, including **FK B**, were chosen as excellent ones whose NO inhibition rate at 10 μM was > 80.0 % in the previous study. The result expressed in IC_{50} was shown as following:

Table 2. IC_{50} values of selected compounds on NO production in RAW 264.7 cells.

Cpd.	IC_{50} of NO production (μM)[a]	Cpd.	IC_{50} of NO production (μM)[a]
FK B	4.2	**41**	3.2
8	2.4	**50**	6.5
9	6.3	**53**	6.4
35	3.6	**Lut.**	9.2
36	2.7		

[a] IC_{50} value is defined as the concentration that results in the reduction of 50.0 % production of nitric oxide. All values are the mean of three independent experiments with the same patterns.

To check whether the inhibitory effect on the NO production was related to cell viability or not, a MTT assay was adopted to determine the cytotoxicity of tested compounds **FK B**, **8**, **9**, **35**, **36**, **41**, **50**, **53** and **Lut.**[35, 38]. The cytotoxicity of these compounds was summarized in **Table 3**.

Table 3. Cytotoxicity of selected compounds in RAW 264.7 cells.

Cpd.	IC_{50} of cytotoxicity (μM)[a]	Cpd.	IC_{50} of cytotoxicity (μM)[a]
FK B	26.5	**41**	29.7
8	34.0	**50**	>40.0
9	>40.0	**53**	>80.0
35	30.7	**Lut.**	>40.0
36	34.0		

[a] All values are the mean of three independent experiments with the same patterns.

*2.2.3 Compound **53** inhibits iNOS expression in LPS-stimulated RAW 264.7 cell*

Among the molecules tested, compound **53** was the most potent inhibitor of NO production in LPS-induced macrophages with much lower cytotoxicity than any other compound. Because the high level of NO is mainly generated by iNOS, and in order to investigate the signaling pathway affected by compound **53**[43], we studied the inhibitory effects of compound **53** on iNOS expression in LPS-induced RAW 264.7 in detail. Western blotting analysis indicated that the level of iNOS protein was significantly decreased by compound **53** in a dose-dependent manner (**Figure 3**). Therefore, compound **53** inhibited NO production through the down-regulation of

LPS-induced iNOS expression at the transcription level.

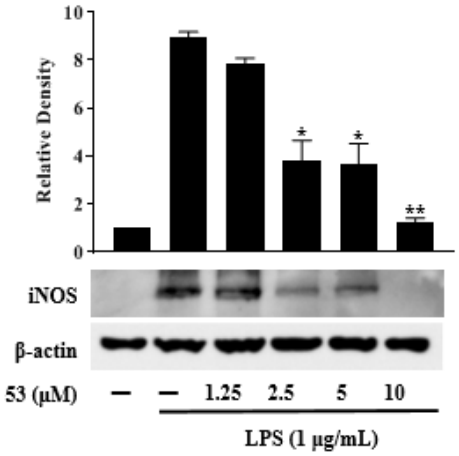


Figure 3. Effect of compound **53** on LPS-induced iNOS protein expression in RAW 264.7 cells. The RAW 264.7 cells were treated with LPS and different concentrations of **53** for 24hr. Equal amounts of total protein were separated on 10 % SDS-PAGE and blotted with antibodies (iNOS) as described in methods. Relative protein levels were quantified by scanning densitometry. The values are expressed as means ± SD of triplicate tests. (*$p < 0.05$, **$p < 0.01$)

[Please Insert Figure 3 Here]

*2.2.4 Docking study of compound **53** with murine iNOS*

In order to gain a better understanding of the binding mode between compound **53** with iNOS (PDB_ID: 3E67), a molecular docking study was carried out with the Libdock program (Discovery Studio), and the result was shown in **Figure 4**. Compound **53** could be divided into four parts, namely, A ring, B ring, propenone linker and side chain with morpholine ring. According to the docking result, side chain with morpholine ring occupied the polar domain and A ring dropped into the non-polar part of iNOS. Both the oxygen atom as receptor and H atoms of methylenes as a donor of morpholine ring formed a hydrogen bond with GLY365 and PRO344, respectively. It was also noted that there was a Pi-alkyl interaction between the morpholine ring with four pyrrole rings of the built-in ligand porphyrin ring of iNOS. Moreover, a Pi-anion interaction between A ring with C-terminal of GLU488 and a Pi-lone pair electron interaction between A ring with carbonyl group on ASN348 were also observed. In summary, this computational result may help us to confirm our

hypothesis that compound **53** was a potent iNOS inhibitor through interaction with the active site of iNOS.

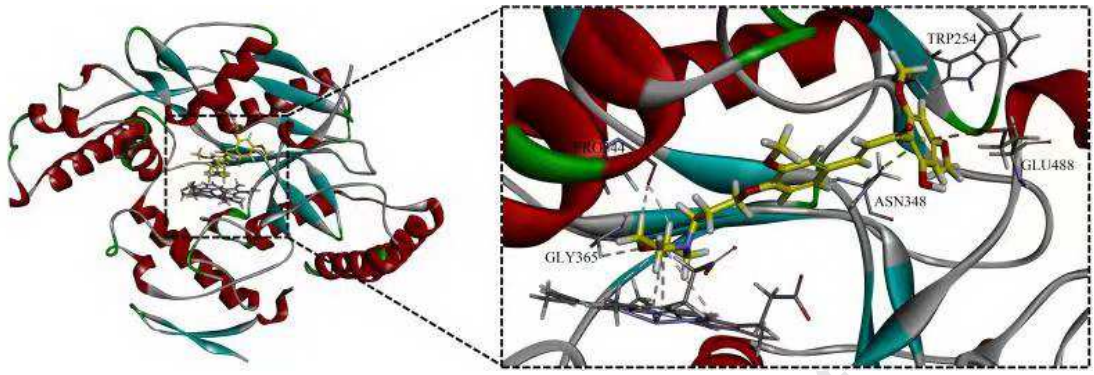


Figure 4. Docking study of compound **53** with iNOS.

[Please Insert Figure 4 Here]

*2.2.5. Anti-arthritic effects of compound **53** on CIA models*

Collagen-induced arthritis (CIA) is a form of chronic arthritis and commonly used to test agents for anti-inflammatory activity[44]. As the most potent molecule, compound **53** was chosen to examine the ability of anti-inflammation on CIA models. CIA mice were treated orally with compound **53** at a dose of 10 mg/kg/day following the appearance of first CIA signs.

Results showed that the severity of CIA was alleviated in compound **53** treated animal models (**Figure 5**). The performance of the arthritis score was significantly reduced during the treatment (**Figure 5A**). Macroscopic observation of the ankle joint and joint histology showed that compound **53** significantly reduced the paw redness compared with the vehicle group, further confirming the therapeutic effect. Histological evaluation by H&E staining and Safranin O staining revealed that a broken joint structure was observed in the vehicle group with a large amount of inflammatory cell infiltration and cartilage destruction (**Figure 5B**). However, mice treated with compound **53** significantly inhibited these changes with few signs of inflammation, and the histological state of the joint was intact and the cartilage was almost completely preserved.

To explore whether compound **53** possesses the ability to reduce the expression of pro-inflammatory cytokines, joint tissues were collected and the relative mRNA levels of TNF-α, IL-lβ and IL-6 were measured by RT-PCR. It was found that the

levels of these pro-inflammatory cytokines in joint tissues were significantly reduced in the compound **53** treated groups (**Figure 5C**).

According to reports, CD4 and CD8 double-positive T cells (DPT) in patients with rheumatoid arthritis (RA) play an important role in the pathogenesis and progression of RA[45, 46]. To analyze the expression level of DPT in CIA mice, the flow cytometry method was used. The results showed that the expression of DPT in compound **53** treated group mice was significantly decreased (**Figure 5D**). These observations suggested that compound **53** might be an effective agent for the management of RA.

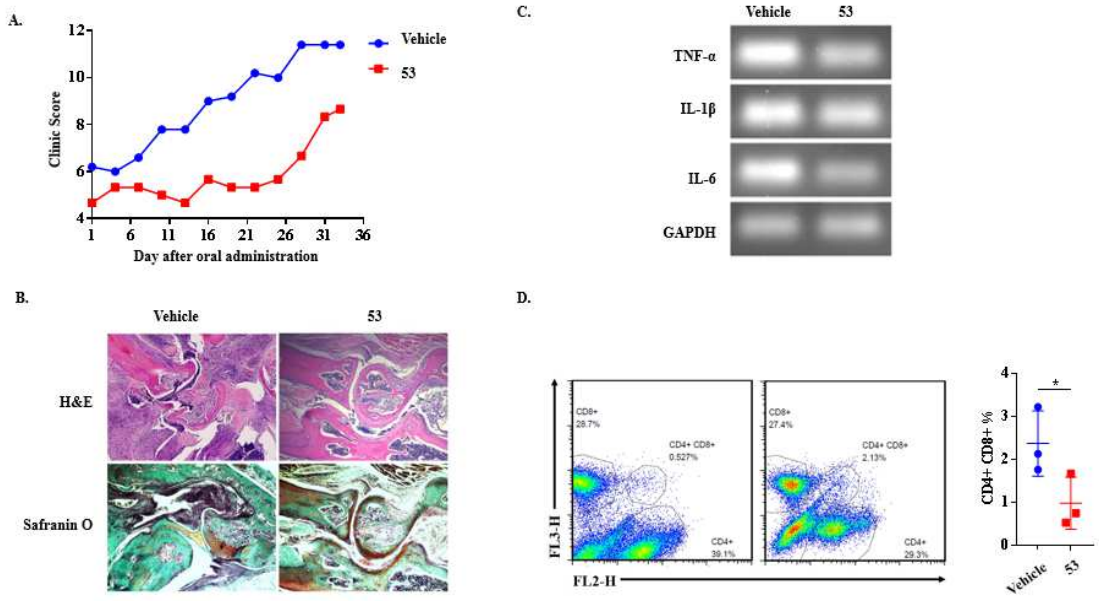


Figure 5. Anti-arthritic effects of compound **53** on CIA models. Compound **53** was orally administered once daily at a dose of 10 mg/kg. (A) Clinical score of the compound **53** group. The results were expressed as the means (n = 5). (B) Representative histopathologies of the knee joints stained with H&E and Safranin O fast green (× 40). (C) Inhibitory effects on the mRNA levels of TNF-α, IL-1β and IL-6 in joint tissues. The mRNA levels were measured by RT-PCR. (D) Lymphocytes from spleen and lymph nodes were collected after dissection. Cells were stained for CD4 and CD8. The percentage/mean fluorescence intensity of CD4+ and CD8+ cells were presented in the upper right quadrant. One representative experiment of three independent experiments was shown.

[Please Insert Figure 5 Here]

*2.2.6 Anti-arthritic effects of compound **53** on AIA models*

In most cases, adjuvant-induced arthritis (AIA) used for RA was also applied to agents testing for anti-inflammatory activity. Compound **53** was chosen to verify the anti-arthritis effects on AIA model for its effective inhibition on CIA model. Almost all of the rats developed arthritis within 13 - 15 days after the adjuvant injection. It was then treated with compound **53** at a dose of 10 mg/kg/day.

As shown in **Figure 6**, compound **53** treated rats did not show significant arthritic symptoms, and arthritis scores were significantly reduced during the treatment (**Figure 6A**). According to the results of H&E staining (**Figure 6B**), signs of severe arthritis were shown in the vehicle group, while no obvious histopathological changes were observed in the group treated with compound **53** at a dose of 10 mg/kg/day. This result indicated compound **53** also possesses potential immunomodulatory activity.

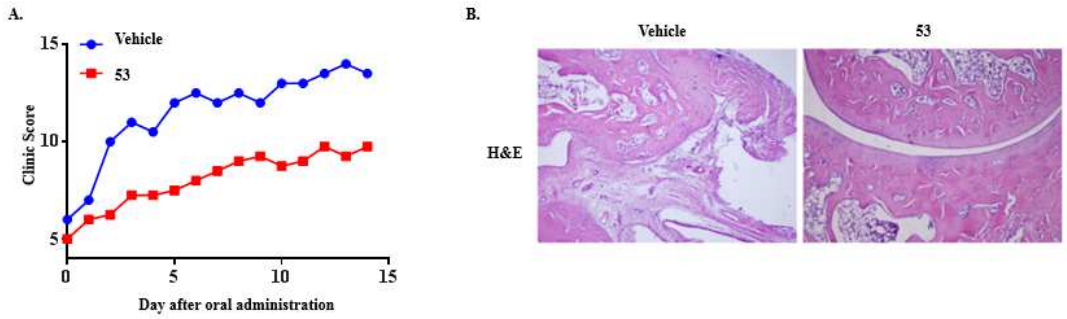


Figure 6. Anti-arthritic effects of compound **53** on AIA models. Compound **53** was orally administered once daily at a dose of 10 mg/kg. (A) Clinical disease activity score of compound **53**. The result was expressed as the means (n = 5, ***p < 0.001). (B) Representative histopathologies of the knee joints stained with H&E (× 40).

[Please Insert Figure 6 Here]

3. Structure-activity relationship (SAR) analysis

According to the results in **Table 1**, among kava chalcones, **FK A** and **FK B** showed a better inhibitory effect on NO production than **FK C** (**FK B** 80.6 % ≈ **FK A** 79.5 % > **FK C** 66.8 %), indicated that the inhibitory effect was related to the hydrogen bond formation ability or acidity of the B ring at 4-position. The introduction of a single methoxyl group on the B ring at 3-position slightly increased

the inhibitory effect (**8** 86.9 % > **FK B** 80.6 % ≈ **FK A** 79.5 %). The modification of the hydroxyl group on the A ring at 2'-position or removal of the methoxyl group at 6'-position of **8** did not decrease the effect distinctly (**35** 84.6 %, **36** 85.6 %, **41** 82.5 %). However, the removal of the methoxyl group on the A ring at 6'-position of **FK A**, decreased the inhibitory effect slightly (**FK A** 79.5 % > **40** 70.0 %). These results indicated that A ring was slightly tolerant to simple modifications at specific positions, and the methoxyl group at 3-position of the B ring was very critical to maintain or increase the activity. The addition of one or two methoxyl groups or other electron donating groups at different positions on the B ring did not show improved inhibitory effects (**FK A** 79.5 % VS **14** 78.7 %, **16** 73.7 %, **17** 67.6 %, **18** 77.4 %, **19** 78.4 %). The addition of electron withdrawing groups on the B ring decreased the inhibitory effect obviously (**FK B** 80.6 % VS **10** 70.2 %, **11** 35.4 %; **14** 78.7 % VS **20** 73.8 %, **21** 38.0 %). These results suggested that electron density on the B ring played a crucial role. The replacement of benzene ring by heterocycle failed to increase the inhibitory effect (**FK B** 80.6 % VS **12** 78.0 %). Depressingly, the addition of a methyl group or a fluorine atom at the α-position of the double bond decreased the inhibitory effect dramatically (**FK B** 80.6 % VS **24** 41.6 %, **28** 50.3 %; **8** 86.9 % VS **25** 68.3 %, **29** 58.1 %). Saturation of α, β-double bond (**8** 86.9 % VS **37** 52.2 %), formation of flavanone (**8** 86.9 % VS **38** 57.3 %) or oxime (**8** 86.9 % VS **39** 46.4 %) also have obvious negative effects on the inhibitory activity. These results proved that the main structure of chalcone with an unsaturated three carbon bridge was significant for maintaining the anti-inflammatory activity. N.K. Sahu and co-workers also pointed out the common unsaturated ketone moiety is likely to be responsible for the versatile biological activities observed, as removal of this structural feature results in loss of bioactivity[47]. At the early stage of our study, undesirable cytotoxicity of several compounds including **FK B**, **8**, **35**, **36**, **41** were detected according to the MTT assay results. So, a further modification of these compounds should be carried out to reduce the toxicity. The introduction of a hydroxyethyl or hydroxypropyl group to 4-position of **FK C** increased the activity (**23** 78.2 % > **22** 73.6 % > **FK C** 66.8 %) slightly indicated that further modification on this position could be acceptable. Taking the

importance of a methoxyl group at the 3-position of B ring into consideration, compounds **50**, **51**, **52**, **53** were designed and synthesized. Fortunately, compound **53** showed a better activity (84.0 %) with much lower cytotoxicity than any other compound. The structure-activity relationship of the kava chalcones analogs was depicted in **Figure 7**.

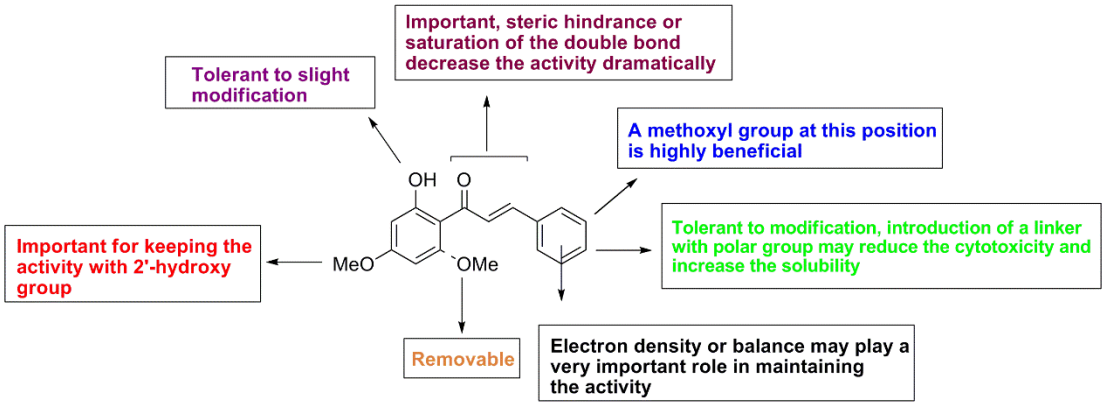


Figure 7. Structure-activity relationship analysis.

[Please Insert Figure 7 Here]

H. ur Rashid and co-workers recently summarized the naturally occurring and synthetic chalcones with various substitutions on the A ring and B ring as NO production inhibitors in detail[14]. As is shown in **Table 4**, these chalcones bring new structural elements that will help us in the design of more novel potential chalcones and clarify the SAR of kava chalcones in future.

Table 4. Selected chalcones from different sources as NO inhibitor and their IC_{50} values.

Name of chalcone	**source**	**IC 50 value (μM)**
1-[6-(3,7-dimethyl-octa-2,6-dienyl)-5,7-dihydroxy-2,2-dimethyl-2*H*-chromen-8-yl]-3-(4-hydroxy-phenyl)-propenone	*Mallotus philippinensis*	7.6

3-(3,4-dihydroxy-phenyl)-1-[6-(3,7-dimethyl-octa-2,6-dienyl)-5,7-dihydroxy-2,2-dimethyl-2*H*-chromen-8-yl]-propenone	*Mallotus philippinensis*	9.5
1-[5,7-dihydroxy-2-methyl-6-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-2*H*-chromen-8-yl]-3-(3,4-dihydroxy-phenyl)-propenone	*Mallotus philippinensis*	38.6
Broussochalcone A	*Broussonetia papyrifera* (L.)	11.3
Isobavachalcone	*Cullen corylifolium*	1.6 ± 0.11
Bavachromene	*Cullen corylifolium*	2.4 ± 0.18
Kanzonol B	*Cullen corylifolium*	2.2 ± 0.21
4-hydroxylonchocarpin	*Psoralea corylifolia*	10.2

(*E*)-1-(2,6-dimethoxyphenyl)-3-(4-(dimethylamino)phenyl)prop-2-en-1-one	Synthetic	0.6
(*E*)-1-(2,5-dimethoxyphenyl)-3-(4-(dimethylamino)phenyl)prop-2-en-1-one	Synthetic	0.7
3,4,5-trimethoxy-4'-fluorochalcone	Synthetic	0.033
(3-(2-Hydroxyphenyl)-1-(thiophene-3-yl)prop-2-en-1-one) (TI-I-174)	Synthetic	5.75
2-(3-(3,4-dimethoxyphenyl)propyl)-5-methoxyphenol	Synthetic	6.5
(*E*)-1-(4-hydroxy-3-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one	Synthetic	4.19
(*E*)-1-(3-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one	Synthetic	2.88
2'-methoxy-3,4-dichlorochalcone	Synthetic	7.1
2'-hydroxy-6'-methoxychalcone	Synthetic	9.6
2'-hydroxy-3-bromo-6'-methoxychalcone	Synthetic	7.8
2',5'-dihydroxy-4-chloro-dihydrochalcone	Synthetic	4.0 ± 1.6

4. **Materials and Methods**

4.1. Chemistry

All the reagents and solvents of analytical grade were purchased from commercial sources and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F-254 thin-layer plates and spots of desired products were located by ultraviolet (UV) lamp and Iodine. Intermediates

and target compounds were recrystallized from MeOH or purified by column chromatography using 300 - 400 mesh silica gel. ^{1}H spectra were recorded on Bruker AV-600, 400 and 300 MHz, ^{13}C spectra were recorded on Bruker AV-100 MHz. HRMS (high resolution) spectra were measured by Waters Vion® IMS QTof mass spectrometer utilizing electrospray ionization (ESI).

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (FK A): yellowish powder, yield 65.0 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.83 (s, 3H), 3.85 (s, 3H), 3.91 (s, 3H), 5.96 (s, 1H), 6.10-6.11 (d, *J* = 3.0 Hz, 1H), 6.91-6.94 (d, *J* = 9.0 Hz, 2H), 7.55-7.58 (d, *J* = 9.0 Hz, 2H), 7.78 (s, 1H), 7.79 (s, 1H), 14.00 (s, 1H). The analytical data were nicely consistent with related reference[48].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-phenylprop-2-en-1-one (FK B): yellowish powder, yield 71 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.84 (s, 3H), 3.92 (s, 3H), 5.97 (d, *J* = 2.3 Hz, 1H), 6.12 (d, *J* = 2.3 Hz, 1H), 7.40-7.41 (m, 3H), 7.60-7.61 (m, 2H), 7.78-7.80 (d, *J* = 18.0 Hz, 1H), 7.89-7.91 (d, *J* = 12.0 Hz, 1H). The analytical data were nicely consistent with related reference[33].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one (FK C): yellowish powder, yield 51 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.84 (s, 3H), 3.92 (s, 3H), 5.96-5.97 (d, *J* = 6.0 Hz, 1H), 6.11 (d, *J* = 6.0 Hz, 1H), 6.86-6.87 (d, *J* = 6.0 Hz, 1H), 7.07-7.08 (d, *J* = 6.0 Hz, 1H), 7.51-7.55 (q, 2H), 7.77-7.80 (q, 2H), 14.39 (s, 1H). The analytical data were nicely consistent with related reference[48].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (8): yellowish powder, yield 74 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.84 (s, 3H), 3.85 (s, 3H), 3.92 (s, 3H), 5.96-5.97 (d, *J* = 6.0 Hz, 1H), 6.11-6.12 (d, *J* = 6.0 Hz, 1H), 6.93-6.95 (q, 1H), 7.13 (s, 1H), 7.20-7.22 (d, *J* = 12.0 Hz, 1H), 7.31-7.34 (t, *J* = 6.0, 12.0 Hz, 1H), 7.73-7.76 (d, *J* = 18.0 Hz, 1H), 7.86-7.89 (d, *J* = 18.0 Hz, 1H). The analytical data were nicely consistent with related reference[49].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-p-tolylprop-2-en-1-one (9): yellowish powder, yield 71 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 2.39 (s, 3H), 3.84 (s, 3H), 3.92 (s, 3H), 5.96-5.97 (d, *J* = 6.0 Hz, 1H), 6.11 (d, *J* = 6.0 Hz, 1H), 7.20-7.22 (d, *J* =

12.0 Hz, 2H), 7.50-7.51 (d, *J* = 6.0 Hz, 2H), 7.76-7.78 (d, *J* = 12.0 Hz, 1H), 7.86-7.88 (d, *J* = 12.0 Hz, 1H). The analytical data were nicely consistent with related reference[50].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(4-bromophenyl)prop-2-en-1-one (10): yellowish powder, yield 45 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.84 (s, 3H), 3.92 (s, 3H), 5.95-5.97 (d, *J* = 6.0 Hz, 1H), 6.10-6.12 (d, *J* = 6.0 Hz, 1H), 7.44-7.47 (d, *J* = 9.0 Hz, 2H), 7.52-7.55 (d, *J* = 9.0 Hz, 2H), 7.67-7.72 (d, *J* = 15.0 Hz, 1H), 7.65-7.90 (d, *J* = 15.0 Hz, 1H), 14.21 (s, 1H). The analytical data were nicely consistent with related reference[21].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(4-nitrophenyl)prop-2-en-1-one (11): brown powder, yield 57 %. ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 3.90 (s, 3H), 3.93 (s, 3H), 6.37-6.38 (d, *J* = 6.0 Hz, 1H), 6.70-6.70 (d, 1H), 6.83 (s, 1H), 8.13-8.15 (d, *J* = 12.0 Hz, 2H), 8.28-8.30 (d, *J* = 12.0 Hz, 2H). The analytical data were nicely consistent with related reference[51].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(pyridin-3-yl)prop-2-en-1-one (12): brown powder, yield 44 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.87 (s, 3H), 3.94 (s, 3H), 5.99-6.00 (d, *J* = 6.0 Hz, 1H), 6.14 (d, 1H), 7.37-7.38 (t, 1H), 7.74-7.77 (d, *J* = 18.0 Hz, 1H), 7.89-7.90 (d, *J* = 6.0 Hz, 1H), 7.96-7.99 (d, *J* = 18.0 Hz, 1H), 8.63 (s, 1H), 8.87 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 191.84, 182.51, 165.81, 165.18, 161.88, 136.95, 134.90, 130.91, 125.37, 110.56, 106.36, 93.85, 91.14, 62.16, 56.28, 55.74. ESI-HRMS calculated for $C_{16}H_{16}NO_4$ $[M+H]^+$: 286.1001, found: 286.1070.

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)prop-2-en-1-one (13): light yellowish powder, yield 66 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.84 (s, 3H), 3.92 (s, 3H), 3.94 (s, 3H), 5.96-5.97 (d, *J* = 6.0 Hz, 1H), 6.11 (d, 1H), 6.86-6.88 (d, *J* = 12.0 Hz, 1H), 7.09-7.11 (q, *J* = 6.0, 6.0 Hz, 1H), 7.24-7.26 (d, *J* = 12.0 Hz, 1H), 7.71-7.74 (d, *J* = 18.0 Hz, 1H), 7.76-7.79 (d, *J* = 18.0 Hz, 1H). The analytical data were nicely consistent with related reference[52].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one (14): yellowish powder, yield 82 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.84 (s, 3H), 3.91 (s, 3H), 3.93 (s, 3H), 3.94 (s, 3H), 5.97-5.97 (d, *J* = 6.0 Hz, 1H),

6.10-6.12 (d, *J* = 6.0 Hz, 1H), 6.91 (s, 1H), 7.13 (s, 1H), 7.24 (s, 1H), 7.77-7.79 (q, 2H). The analytical data were nicely consistent with related references[53, 54].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(benzo[d][1,3]dioxol-5-yl)prop-2-en-1-one (15): yellowish powder, yield 49 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.84 (s, 3H), 3.92 (s, 3H), 5.96-5.97 (d, *J* = 6.0 Hz, 1H), 6.02 (s, 2H), 6.11-6.11 (d, *J* = 6.0 Hz, 1H), 6.83-6.84 (d, *J* = 6.0 Hz, 1H), 7.09-7.10 (d, *J* = 6.0 Hz, 1H), 7.12 (s, 1H), 7.74-7.74 (d, 2H). The analytical data were nicely consistent with related reference[49].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(2,5-dimethoxyphenyl)prop-2-en-1-one (16): yellowish powder, yield 59 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.82 (s, 3H), 3.84 (s, 3H), 3.87 (s, 3H), 3.90 (s, 3H), 5.96 (d, *J* = 2.2 Hz, 1H), 6.11 (d, *J* = 2.2 Hz, 1H), 6.87 (d, *J* = 9.0 Hz, 1H), 6.92 (dd, *J* = 9.0, 3.0 Hz, 1H), 7.15 (d, *J* = 2.9 Hz, 1H), 7.93 (d, *J* = 18.0 Hz, 1H), 8.09 (d, *J* = 18.0 Hz, 1H). The analytical data were nicely consistent with related reference[55].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(2,4-dimethoxyphenyl)prop-2-en-1-one (17): yellowish powder, yield 68 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.83-3.91 (s, 12H), 5.96 (d, *J* = 3.0 Hz, 1H), 6.11 (d, *J* = 3.0 Hz, 1H), 6.47 (d, *J* = 3.0 Hz, 1H), 6.53-6.54 (dd, *J* = 3.0, 3.0 Hz, 1H), 7.54 (d, *J* = 12.0 Hz), 7.90 (d, *J* = 18.0 Hz, 1H), 8.11 (d, *J* = 18.0 Hz, 1H). The analytical data were nicely consistent with related reference[55].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (18): brown powder, yield 78 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.84-3.91 (s, 15H), 5.97 (s, 1H), 6.12 (s, 1H), 6.84 (s, 2H), 7.70-7.72 (t, *J* = 6.0, 6.0 Hz, 1H), 7.77-7.81 (q, *J* = 6.0, 6.0 Hz, 1H). The analytical data were nicely consistent with related references[56, 57].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(2,4,5-trimethoxyphenyl)prop-2-en-1-one (19): dark yellowish powder, yield 81 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.82-3.94 (s, 15H), 5.95 (d, *J* = 3.0 Hz, 1H), 6.10 (d, *J* = 3.0 Hz, 1H), 6.51 (s, 1H), 7.11 (s, 1H), 7.85 (d, *J* = 15.5 Hz, 1H), 8.13 (d, *J* = 15.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.81, 168.38, 165.82, 162.38, 154.52, 152.24, 143.19, 138.01,

125.28, 116.19, 111.44, 106.44, 96.87, 93.82, 91.20, 56.48, 56.37, 56.08, 55.73, 55.58. The analytical data were nicely consistent with related references [58].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-bromo-4-methoxyphenyl)prop-2-en-1-one (20): dark yellowish powder, yield 29 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.84 (s, 3H), 3.92 (s, 3H), 3.94 (s, 3H), 5.96-5.97 (d, *J* = 3.0 Hz, 1H), 6.10-6.11 (d, *J* = 3.0 Hz, 1H), 6.90-6.93 (d, *J* = 9.0 Hz, 1H), 7.48-7.51 (m, 1H), 7.52-7.82 (m, 3H), 14.30 (s, 1H). The analytical data were nicely consistent with related reference[59].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3,4-dimethoxy-5-nitrophenyl)prop-2-en-1-one (21): brown powder, yield 68 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.91 (s, 3H), 3.96 (s, 3H), 3.99 (s, 3H), 4.05 (s, 3H), 6.15 (s, 1H), 6.28 (s, 1H), 7.32 (s, 2H), 7.60 (s, 1H), 7.64 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.80, 169.22, 168.87, 159.65, 152.46, 149.01, 148.68, 142.12, 121.87, 113.25, 108.12, 105.22, 105.05, 94.22, 89.31, 56.51, 56.46, 56.29, 56.25.

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(4-(2-hydroxyethoxy)phenyl)prop-2-en-1-one (22): pale yellowish powder, yield 78 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.81 (s, 3H), 3.83 (s, 3H), 3.97-4.01 (m, 2H), 4.12-4.15 (t, *J* = 3.0, 6.0 Hz, 2H), 5.96-5.97 (d, *J* = 3.0 Hz, 1H), 6.10-6.11 (d, *J* = 3.0 Hz, 1H), 6.93-6.96 (d, *J* = 9.0 Hz, 2H), 7.55-7.58 (d, *J* = 9.0 Hz, 2H), 7.79-7.80 (d, *J* = 3.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.57, 168.38, 166.06, 162.45, 142.28, 130.14, 128.73, 125.38, 114.91, 93.80, 91.26, 69.29, 61.39, 55.87, 55.60. ESI-HRMS calculated for $C_{19}H_{21}O_6$ $[M+H]^+$: 345.1260, found: 345.1330.

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(4-(3-hydroxypropoxy)phenyl)prop-2-en-1-one (23): pale yellowish powder, yield 77 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 2.06-2.08 (t, *J* = 6.0, 6.0 Hz, 2H), 3.84 (s, 3H), 3.87-3.89 (t, *J* = 6.0, 6.0 Hz, 2H), 3.92 (s, 3H), 4.17-4.19 (t, *J* = 6.0, 6.0 Hz, 2H), 5.97-5.97 (d, *J* = 6.0 Hz, 1H), 6.11-6.11 (d, *J* = 6.0 Hz, 1H), 6.93-6.94 (d, *J* = 6.0 Hz, 2H), 7.55-7.56 (d, *J* = 6.0 Hz, 2H), 7.79 (d, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.60, 168.38, 168.01, 166.04, 162.46, 160.58, 142.45, 130.15, 128.43, 125.18, 119.53, 114.86, 110.49, 106.35, 103.88, 93.80, 91.25, 87.50, 65.66, 60.21, 55.87, 55.61, 31.92. ESI-HRMS calculated

for $C_{20}H_{22}O_6Na$ $[M+Na]^+$: 381.1314, found: 381.1306.

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-2-methyl-3-(4-methoxyphenyl)prop-2-en-1-one (24): light yellowish powder, yield 78 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 2.15 (s, 3H), 3.71 (s, 3H), 3.83 (s, 6H), 5.95 (s, 1H), 6.14 (s, 1H), 6.76 (s, 1H), 6.91-6.92 (d, *J* = 12.0 Hz, 2H), 7.34-7.36 (d, *J* = 12.0 Hz, 2H), 13.56 (s, 1H). The analytical data were nicely consistent with related reference[60].

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-2-methyl-3-(3-methoxyphenyl)prop-2-en-1-one (25): light yellowish powder, yield 76 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 2.14 (s, 3H), 3.74 (s, 3H), 3.82 (s, 3H), 3.84 (s, 3H), 5.94-5.95 (d, *J* = 3.0 Hz, 1H), 6.13-6.14 (d, *J* = 3.0 Hz, 1H), 6.68 (s, 1H), 6.82 (d, *J* = 3.0 Hz, 1H), 6.85 (d, *J* = 3.0 Hz, 1H), 6.91 (s, 1H), 7.30 (d, *J* = 6.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.83, 165.85, 165.56, 161.70, 159.20, 138.15, 132.73, 130.99, 129.21, 113.83, 105.51, 93.64, 91.28, 55.70, 55.60, 55.31, 15.41. ESI-HRMS calculated for $C_{19}H_{21}O_5$ $[M+H]^+$: 329.1311, found: 329.1386.

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-2-methyl-3-p-tolylprop-2-en-1-one (26): light yellowish powder, yield 69 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 2.15 (s, 3H), 2.32 (s, 3H), 3.71 (s, 3H), 3.83 (s, 3H), 5.94-5.95 (d, *J* = 6.0 Hz, 1H), 6.13-6.14 (d, *J* = 6.0 Hz, 1H), 6.73 (s, 1H), 7.18-7.20 (d, *J* = 12.0 Hz, 2H), 7.28-7.29 (d, *J* = 6.0 Hz, 2H), 12.10 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.01, 166.02, 165.81, 161.84, 139.37, 137.67, 133.76, 132.25, 129.40, 129.10, 105.42, 93.62, 91.29, 55.70, 55.61, 21.32, 15.40. ESI-HRMS calculated for $C_{19}H_{20}O_4$ $[M+H]^+$: 313.1362, found: 313.1432.

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-2-methyl-3-phenylprop-2-en-1-one (27): light yellowish powder, yield 66 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 2.15 (s, 3H), 3.73 (s, 3H), 3.84 (s, 3H), 5.95 (d, *J* = 3.0 Hz, 1H), 6.13 (d, *J* = 3.0 Hz, 1H), 6.73 (s, 1H), 7.27-7.39 (m, 5H), 12.23 (s, 1H). The analytical data were nicely consistent with related reference[61].

(*Z*)-2-fluoro-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (28): yellowish powder, yield 67 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.86 (s, 3H), 3.91 (s, 3H), 3.96 (s, 3H), 6.13-6.13 (d, *J* = 2.0 Hz, 1H), 6.38-6.38 (d, *J*

= 2.0 Hz, 1H), 6.76 (s, 1H), 6.94-6.96 (d, *J* = 12.0 Hz, 2H), 7.82-7.84 (d, *J* = 12.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.67, 168.81, 168.71, 160.56, 159.31, 146.78, 132.89, 125.31, 114.34, 111.03, 105.45, 93.92, 89.14, 56.21, 56.10, 55.37. ESI-HRMS calculated for $C_{18}H_{17}O_5$ $[M\text{-}HF+H]^+$: 313.1076, found: 313.1068.

(*Z*)-2-fluoro-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (29): yellowish powder, yield 71 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.87 (s, 3H), 3.90 (s, 3H), 3.95 (s, 3H), 6.14-6.14 (d, *J* = 3.0 Hz, 1H), 6.38-6.38 (d, *J* = 3.0 Hz, 1H), 6.74 (s, 1H), 6.91-6.93 (dd, *J* = 6.0, 6.0 Hz, 1H), 7.32-7.36 (t, *J* = 6.0, 9.0 Hz, 1H), 7.44-7.45 (d, *J* = 9.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.68, 169.07, 169.03, 159.68, 159.42, 147.97, 133.79, 129.72, 123.87, 116.20, 115.09, 110.66, 105.17, 94.09, 89.26, 56.24, 56.16, 55.33. ESI-HRMS calculated for $C_{18}H_{17}O_5$ $[M\text{-}HF+H]^+$: 313.1076, found: 313.1069.

(*Z*)-2-fluoro-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-phenylprop-2-en-1-one (30): yellowish powder, yield 75 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.92 (s, 3H), 3.96 (s, 3H), 6.14-6.15 (d, *J* = 6.0 Hz, 1H), 6.40-6.40 (d, *J* = 6.0 Hz, 1H), 6.78 (s, 1H), 7.36-7.38 (t, *J* = 12.0 Hz, 1H), 7.42-7.44 (t, *J* = 6.0, 6.0 Hz, 2H), 7.86-7.88 (d, *J* = 12.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.73, 169.10, 169.00, 159.44, 147.88, 132.59, 131.12, 129.33, 128.79, 110.79, 105.24, 94.07, 89.25, 56.25, 56.15. ESI-HRMS calculated for $C_{17}H_{15}O_4$ $[M\text{-}HF+H]^+$: 283.0970, found: 283.0963.

(*Z*)-2-fluoro-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one (31): yellowish powder, yield 49 %. ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 3.89 (s, 3H), 3.91 (s, 3H), 6.33-6.33 (d, *J* = 2.0 Hz, 1H), 6.65 (s, 1H), 6.68-6.68 (d, *J* = 2.0 Hz, 1H), 6.86-6.88 (d, *J* = 12.0 Hz, 2H), 7.78-6.80 (d, *J* = 12.0 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.85, 168.55, 167.89, 159.08, 158.74, 133.01, 123.05, 115.97, 110.40, 104.26, 94.23, 89.73, 56.42, 56.06. ESI-HRMS calculated for $C_{17}H_{15}O_5$ $[M\text{-}HF+H]^+$: 299.0920, found: 299.0910.

(*Z*)-2-fluoro-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-p-tolylprop-2-en-1-one (32): yellowish powder, yield 56 %. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 2.39 (s, 3H), 3.91 (s, 3H), 3.96 (s, 3H), 6.13-6.14 (d, *J* = 6.0 Hz, 1H), 6.39-6.40 (d, *J* = 6.0 Hz, 1H), 6.76 (s, 1H), 7.22-7.24 (d, *J* = 12.0 Hz, 2H), 7.75-7.77 (d, *J* = 12.0 Hz, 2H). ^{13}C NMR

(100 MHz, $CDCl_3$) δ 180.76, 168.99, 168.87, 159.37, 147.46, 139.78, 131.14, 129.78, 129.58, 111.07, 105.35, 93.99, 89.20, 56.23, 56.13, 21.60. ESI-HRMS calculated for $C_{18}H_{17}O_4$ $[M\text{-}HF+H]^+$: 297.1127, found: 297.1120.

(*Z*)-2-fluoro-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one (33): yellowish powder, yield 63 %. ^1H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.92 (s, 3H), 3.94 (s, 3H), 3.96 (s, 3H), 3.97 (s, 3H), 6.15-6.15 (d, *J* = 6.0 Hz, 1H), 6.36-6.36 (d, *J* = 6.0 Hz, 1H), 6.75 (s, 1H), 6.92-6.93 (d, *J* = 9.0 Hz, 1H), 7.45-7.47 (d, *J* = 6.0, 9.0 Hz, 2H).

(*Z*)-2-fluoro-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(2,5-dimethoxyphenyl)prop-2-en-1-one (34): yellowish powder, yield 62 %. ^1H NMR (600 MHz, $CDCl_3$): δ (ppm) = 3.86 (s, 6H), 3.90 (s, 3H), 3.95 (s, 3H), 6.13 (s, 1H), 6.36-6.36 (d, *J* = 2.0 Hz, 1H), 6.84-6.85 (d, *J* = 6.0 Hz, 1H), 6.88-6.90 (dd, *J* = 6.0, 3.0 Hz, 1H), 7.28 (s, 1H), 7.81-7.81 (d, *J* = 3.0 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.98, 166.04, 152.42, 149.28, 147.36, 143.38, 112.38, 99.50, 94.70, 90.03, 56.58, 56.15, 55.51. ESI-HRMS calculated for $C_{19}H_{19}O_6$ $[M\text{-}HF+H]^+$: 343.1182, found: 343.1178.

(*E*)-1-(2'-acetoxy-4',6'-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (35): 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one 94.3 mg (0.3 mmol, 1eq) was dissolved into 3 ml Ac_2O and refluxed for 3 hours, after work-up, the crude product was purified by Si-gel chromatography to afford 84.4 mg yellowish oil, yield 79 %. ^1H NMR (300 MHz, $CDCl_3$): δ (ppm) = 2.18 (s, 3H), 3.80 (s, 3H), 3.82 (s, 3H), 3.84 (s, 3H), 6.30-6.30 (d, *J* = 3.0 Hz, 1H), 6.40-6.41 (d, *J* = 3.0 Hz, 1H), 6.91-6.95 (m, 2H), 7.00-7.04 (d, *J* = 12.0 Hz, 1H), 7.11-7.14 (d, *J* = 12.0 Hz, 1H), 7.28-7.31 (d, *J* = 9.0 Hz, 1H), 7.37-7.42 (d, *J* = 9.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.68, 169.21, 162.15, 159.87, 159.19, 150.08, 144.25, 136.16, 129.88, 128.27, 121.12, 116.35, 115.80, 113.16, 100.26, 96.71, 56.06, 55.66, 55.30, 20.89. ESI-HRMS calculated for $C_{20}H_{20}O_6Na$ $[M+Na]^+$: 379.1158, found: 379.1156.

(*E*)-1-(2'-ethoxy-4',6'-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (36): 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one 94.3 mg (0.3 mmol, 1eq) was dissolved into 10 ml acetone, K_2CO_3 (3 eq.) was added, the mixture was stirred for a while, then bromoethane 46 mg (0.39 mmol, 1.3 eq) was

added. The mixture was refluxed until the starting material was consumed over. The solvent was removed under reduced pressure, and the crude product was purified by Si-gel chromatography （PE/EA=6/1）to afford 84 mg yellowish oil, yield 82 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.28-1.31 (t, J = 6.0, 3.0 Hz, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 3.92 (s, 3H), 5.97 (d, *J* = 6.0 Hz, 1H), 6.12 (d, *J* = 6.0 Hz, 1H), 6.94 (q, *J* = 6.0, 6.0 Hz, 1H), 7.12 (s, 1H), 7.21 (d, *J* = 6.0 Hz, 1H), 7.32 (d, *J* = 6.0 Hz, 1H), 7.75 (d, *J* = 9.0 Hz, 1H), 7.87 (d, *J* = 9.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.30, 162.40, 159.85, 158.91, 158.31, 143.76, 136.50, 129.80, 129.40, 121.06, 116.07, 113.04, 112.06, 91.57, 90.75, 64.38, 55.93, 55.46, 55.30, 14.67. ESI-HRMS calculated for $C_{20}H_{22}O_5Na$ $[M+Na]^+$: 365.1365, found: 365.1355.

1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxyphenyl)propan-1-one (37): 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one 94.3 mg (0.3 mmol, 1eq) was dissolved into 5 ml MeOH, catalytic amount PtO2 was added. The air was exchanged by H_2 for three times, the mixture was stirred for 24 hours at room temperature. The solvent was removed and the crude product was purified by Si-gel column chromatography (PE/EA=8/1) to afford desired compound, white powder, 53 mg, yield 56 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 2.97-3.02 (t, *J* = 9.0 Hz, 2H), 3.32-3.37 (t, *J* = 9.0 Hz, 2H), 3.84 (s, 3H), 3.85 (s, 3H), 3.92 (s, 3H), 5.94 (d, *J* = 3.0 Hz, 1H), 6.10 (d, *J* = 3.0 Hz, 1H), 6.77-7.22 (m, 3H), 7.22-7.28 (m, 1H), 14.07 (s, 1H).

5,7-dimethoxy-2-(3-methoxyphenyl)chroman-4-one (38): 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one 94.3 mg (0.3 mmol, 1eq) was dissolved into 5 ml MeOH, followed by the addition of 3 ml 5N aq. HCl solution, the mixture was refluxed for overnight, after the starting material was consumed completely and cooled to room temperature, 20 ml water was added. Extracted with ethyl acetate for three times, the organic layer was combined and dried, the solvent was removed under reduced pressure. The residue was purified by Si-gel chromatography (PE/EA=6/1) to afford desired compound, 59 mg, white solid, yield 62 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 2.73 (dd, *J* = 3.0, 3.0 Hz, 1H), 2.94 (dd, *J* = 12.0, 12.0 Hz, 1H), 3.75 (s, 3H), 3.76 (s, 3H), 3.82 (s, 3H), 5.28 (dd, *J* = 3.0, 3.0

Hz, 1H), 6.02 (d, *J* = 3.0 Hz, 1H), 6.09 (d, *J* = 3.0 Hz, 1H), 6.83 (t, 1H), 7.19 (s, 2H), 7.23-7.29 (m, 1H). The analytical data were nicely consistent with related reference[62].

(2*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one oxime (39): 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one 94.3 mg (0.3 mmol, 1eq) was dissolved into followed by the addition of hydroxylamine hydrochloride 41.7 mg (0.6 mmol, 2eq) and piperidine (3.0 eq). The mixture was stirred for 12 hours. After the starting material was consumed completely, the solvent was removed. The residue was purified by Si-gel column chromatography (PE/EA=6/1) to afford desired compound, 41.5 mg, white solid, yield 42 %. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 3.64 (s, 3H), 3.74 (s, 3H), 3.75 (s, 3H), 6.10-6.13 (d, *J* = 3.0, 6.0 Hz, 2H), 6.26-6.31 (d, *J* = 15.0 Hz, 1H), 6.83-6.86 (q, *J* = 3.0, 3.0 Hz, 1H), 6.95 (s, 1H), 7.01-7.04 (d, *J* = 9.0 Hz, 1H), 7.21-7.27 (t, *J* = 9.0, 9.0 Hz, 1H), 7.55-7.60 (d, *J* = 15.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.16, 159.80, 156.81, 138.32, 137.67, 129.64, 120.35, 116.83, 115.06, 112.45, 111.87, 102.40, 99.98, 93.54, 91.91, 55.73, 55.43, 55.31. ESI-HRMS calculated for $C_{18}H_{20}NO_5$ $[M+H]^+$: 330.1263, found: 330.1339.

(*E*)-1-(2'-hydroxy-4'-methoxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (40): yellowish solid, yield 78 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.83-3.86 (s, 6H), 6.47-6.50 (t, *J* = 3.0, 6.0 Hz, 2H), 6.93-6.96 (d, *J* = 9.0 Hz, 2H), 7.44-7.49 (d, *J* = 15.0 Hz, 1H), 7.60-7.63 (d, *J* = 9.0 Hz, 2H), 7.81-7.89 (t, *J* = 9.0, 15.0 Hz, 2H), 13.54 (s, 1H). The analytical data were nicely consistent with related reference[63].

(*E*)-1-(2'-hydroxy-4'-methoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (41): yellowish solid, yield 79 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.85-3.86 (s, 6H), 6.48-6.51 (t, *J* = 6.0, 3.0 Hz, 2H), 6.96-6.99 (m, 1H), 7.15-7.16 (d, *J* = 3.0 Hz, 1H), 7.32-7.37 (t, *J* = 9.0, 6.0 Hz, 1H), 7.53-7.59 (d, *J* = 18.0 Hz, 1H), 7.82-7.87 (t, *J* = 6.0, 9.0 Hz, 2H), 13.41 (s, 1H). The analytical data were nicely consistent with related reference[64].

(*E*)-1-(2'-hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (42): yellowish

solid, yield 81 %. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 3.96 (s, 3H), 6.91-7.04 (m, 4H), 7.48-7.84 (m, 4H), 7.88-7.93 (m, *J* = 6.0, 6.0 Hz, 2H), 12.93 (s, 1H). The analytical data were nicely consistent with related reference[65].

4-(3-bromopropoxy)-3-methoxybenzaldehyde (45): 4-hydroxy-3-methoxybenzaldehyde (1.52 g, 10.0 mmol, 1.0 eq) was dissolved into 20 ml MeCN, followed by the addition of K_2CO_3 (2.07 g, 15.0 mmol, 1.5 eq). After stirred for a while, 1,3-dibromopropane (6.06 g, 30.0 mmol, 3.0 eq) was added into the solution and refluxed at 85 ^{o}C for about 4 hours. When the starting material was consumed over, the solution was cooled to r.t. and the diluted with 120 ml water. The mixture was extracted with EA (40 ml) for three times, the organic layer was combined and dried with Na_2SO_4, filtered and the solvent was removed to give oil residue. The residue was purified by Si-gel column chromatography to afford compound **45** as white solid 2.46 g, yield 90.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 2.41-2.44 (m, 2H), 3.63-3.66 (t, *J* = 4.0, 8.0 Hz, 2H), 3.93 (s, 3H), 4.24-4.27 (t, *J* = 8.0, 4.0 Hz, 2H), 7.00-7.02 (d, *J* = 8.0 Hz, 1H), 7.42 (d, 1H), 7.44-7.47 (dd, *J* = 4.0, 8.0 Hz, 1H), 9.86 (s, 1H).

3-methoxy-4-(3-(pyrrolidin-1-yl)propoxy)benzaldehyde (46): compound **45** (546.24 mg, 2.0 mmol, 1.0 eq) was added into 10 ml MeCN, followed by the addition of K_2CO_3 (414.63 mg, 3.0 mmol, 1.5 eq) and pyrrolidine (284.48 mg, 4.0 mmol, 2.0 eq). The mixture was refluxed at 85 ^{o}C for about 5 hours until the starting material **45** was consumed over. The solvent was removed under reduced pressure, and the residue was purified by Si-gel column chromatography (DCM/MeOH = 15/1) to afford compound **46** as pale white oil 442.4 mg, yield 84.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.80 (s, 4H), 2.10-2.15 (m, 2H), 2.57 (t, 4H), 2.66-2.69 (t, *J* = 8.0, 4.0 Hz, 2H), 3.92 (s, 3H), 4.17-4.21 (t, *J* = 8.0, 8.0 Hz, 2H), 7.00-7.02 (d, *J* = 8.0 Hz, 1H), 7.40 (d, 1H), 7.42-7.45 (dd, *J* = 4.0, 4.0 Hz, 1H), 9.84 (s, 1H).

3-methoxy-4-(3-(piperidin-1-yl)propoxy)benzaldehyde (47): compound **45** (546.24 mg, 2.0 mmol, 1.0 eq) was added into 10 ml MeCN, followed by the addition of K_2CO_3 (414.63 mg, 3.0 mmol, 1.5 eq) and piperidine (340.44 mg, 4.0 mmol, 2.0 eq). The mixture was refluxed at 85 ^{o}C for about 5 hours until the starting material **45** was

consumed over. The solvent was removed under reduced pressure, and the residue was purified by Si-gel column chromatography (DCM/MeOH = 15/1) to afford compound **47** as pale white oil 504.8 mg, yield 91.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.46-1.47 (brd, *J* = 4.0 Hz, 2H), 1.60-1.65 (m, 4H), 2.07-2.14 (m, 2H), 2.46 (br, 4H), 2.53-2.56 (t, *J* = 4.0, 8.0 Hz, 2H), 3.92 (s, 3H), 4.16-4.20 (t, *J* = 8.0, 8.0 Hz, 2H), 7.00-7.02 (d, *J* = 8.0 Hz, 1H), 7.40-7.41 (d, *J* = 4.0 Hz, 1H), 7.43-7.45 (dd, 1H), 9.85 (s, 1H).

3-methoxy-4-(3-(4-methylpiperazin-1-yl)propoxy)benzaldehyde (48): compound **45** (546.24 mg, 2.0 mmol, 1.0 eq) was added into 10 ml MeCN, followed by the addition of K_2CO_3 (414.63 mg, 3.0 mmol, 1.5 eq) and N-methyl piperazine (400.64 mg, 4.0 mmol, 2.0 eq). The mixture was refluxed at 85 ^{o}C for about 5 hours until the starting material **45** was consumed over. The solvent was removed under reduced pressure, and the residue was purified by Si-gel column chromatography (DCM/MeOH = 15/1) to afford compound **48** as pale yellowish solid 514.5 mg, yield 88.0%.^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 2.05-2.08 (m, 2H), 2.22 (s, 1H), 2.29 (s, 3H), 2.46-2.56 (m, 9H), 3.92 (s, 3H), 4.16-4.20 (t, *J* = 8.0, 8.0 Hz, 2H), 7.00-7.02 (d, *J* = 8.0 Hz, 1H), 7.41 (d, 1H), 7.43-7.45 (dd, 1H), 9.85 (s, 1H).

3-methoxy-4-(3-morpholinopropoxy)benzaldehyde (49): compound **45** (546.24 mg, 2.0 mmol, 1.0 eq) was added into 10 ml MeCN, followed by the addition of K_2CO_3 (414.63 mg, 3.0 mmol, 1.5 eq) and morpholine (348.48 mg, 4.0 mmol, 2.0 eq). The mixture was refluxed at 85 ^{o}C for about 5 hours until the starting material **45** was consumed over. The solvent was removed under reduced pressure, and the residue was purified by Si-gel column chromatography (DCM/MeOH = 15/1) to afford compound **49** as pale white oil 519.6 mg, yield 93.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) = 2.03-2.10 (m, 2H), 2.47 (br., 2H), 2.52-2.56 (t, *J* = 8.0, 8.0 Hz, 2H), 3.70-3.73 (t, *J* = 4.0, 8.0 Hz, 4H), 3.92 (s, 3H), 4.17-4.21 (t, *J* = 8.0, 8.0 Hz, 2H), 7.00-7.02 (d, *J* = 8.0 Hz, 1H), 7.40-7.41 (d, *J* = 4.0 Hz, 1H), 7.43-7.45 (dd, 1H), 9.85 (s, 1H).

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxy-4-(3-(pyrrolidin-1-yl)propoxy)phenyl)prop-2-en-1-one (50): yellowish solid, yield 63 %. ^{1}H NMR (600

MHz, DMSO-d_6): δ (ppm) = 1.66 (s, 4H), 1.87-1.89 (t, *J* = 6.0, 6.0 Hz, 2H), 2.41 (s, 6H), 3.80 (s, 3H), 3.81 (s, 3H), 3.87 (s, 3H), 4.04-4.05 (d, *J* = 6.0 Hz, 2H), 6.10-6.10 (d, *J* = 6.0 Hz, 1H), 6.14-6.14 (d, *J* = 6.0 Hz, 1H), 6.99-7.00 (d, *J* = 6.0 Hz, 1H), 7.24-7.26 (d, *J* = 12.0 Hz, 1H), 7.27 (s, 1H), 7.57-7.64 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.47, 168.38, 166.01, 162.40, 150.67, 149.41, 142.77, 128.48, 125.29, 122.65, 112.64, 111.05, 106.34, 93.82, 91.26, 67.55, 56.02, 55.82, 55.60, 54.26, 52.99, 28.69, 23.48. ESI-HRMS calculated for $C_{25}H_{32}NO_6$ $[M+H]^+$: 442.2151, found: 442.2222.

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxy-4-(3-(piperidin-1-yl)propoxy)phenyl)prop-2-en-1-one (51): yellowish solid, yield 57 %. ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 1.36 (s, 2H), 1.46-1.48 (t, *J* = 6.0, 6.0 Hz, 4H), 1.84-1.86 (t, *J* = 6.0, 6.0 Hz, 2H), 2.30 (s, 4H), 2.34-2.36 (t, *J* = 6.0, 6.0 Hz, 2H), 3.80 (s, 3H), 3.81 (s, 3H), 3.87 (s, 3H), 4.02-4.04 (t, *J* = 6.0, 6.0 Hz, 2H), 6.10-6.10 (d, *J* = 6.0 Hz, 1H), 6.14-6.14 (d, *J* = 6.0 Hz, 1H), 6.99-7.01 (d, *J* = 12.0 Hz, 1H), 7.25-7.25 (d, *J* = 6.0 Hz, 1H), 7.27 (s, 1H), 7.57-7.64 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.46, 168.38, 166.01, 162.40, 150.68, 149.42, 142.77, 128.46, 125.27, 122.65, 112.67, 111.04, 106.33, 93.81, 91.26, 67.63, 56.00, 55.81, 55.76, 55.59, 54.62, 26.63, 26.02, 24.46. ESI-HRMS calculated for $C_{26}H_{34}NO_6$ $[M+H]^+$: 456.2308, found: 456.2382.

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxy-4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl)prop-2-en-1-one (52): yellowish powder, yield 57 %. ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 1.84-1.89 (m, 2H), 2.13 (s, 3H), 2.37-2.42 (m, 10H), 3.81 (s, 3H), 3.82 (s, 3H), 3.89 (s, 3H), 4.01-4.05 (t, 2H), 6.12 (s, 1H), 6.15 (s, 1H), 76.99-7.02 (d, *J* = 18.0 Hz, 1H), 7.26-7.29 (d, *J* = 18.0 Hz, 2H), 7.62-6.62 (d, *J* = 6.0 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 192.31, 166.11, 165.90, 165.09, 164.92, 162.73, 161.57, 149.89, 149.07, 142.93, 127.97, 125.44, 122.61, 113.08, 111.04, 106.53, 105.46, 93.85, 93.60, 90.96, 90.79, 56.11, 56.03, 55.68, 55.61, 32.68. ESI-HRMS calculated for $C_{26}H_{35}N_2O_6$ $[M+H]^+$: 471.2417, found: 471.2492.

(*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxy-4-(3-morpholinopropoxy)phenyl)prop-2-en-1-one (53): yellowish powder, yield 62 %. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) = 2.23 (s, 2H), 2.48-2.52 (m, 2H), 3.06-3.13 (m, 2H), 3.24 (s,

2H), 3.45 -3.49 (d, *J* = 12.0 Hz, 2H), 3.81 (s, 3H), 3.84 (s, 3H), 3.88 (s, 3H), 3.92-3.98 (d, *J* = 18.0, 18.0 Hz, 2H), 4.10-4.14 (t, *J* = 12.0, 12.0 Hz, 2H), 6.13-6.13 (d, *J* = 6.0 Hz, 1H), 6.15-6.16 (d, *J* = 6.0 Hz, 1H), 7.05-7.06 (d, *J* = 6.0 Hz, 1H), 7.28-7.32 (m, 2H), 7.62-7.68 (d, *J* = 3.0, 15.0 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.45, 168.37, 166.04, 162.39, 150.54, 149.41, 142.69, 128.59, 125.37, 122.61, 112.63, 110.97, 106.32, 93.82, 91.28, 67.21, 66.98, 55.96, 55.82, 55.61, 55.37, 53.71, 26.22. ESI-HRMS calculated for $C_{25}H_{32}NO_7$ $[M+H]^+$: 458.2101, found: 458.2170.

4.2 Cell culture

RAW 264.7 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 medium containing 10 % FBS in a 95 % air, 5 % CO_2 humidified atmosphere at 37 °C. Cells were treated in the presence or absence of compounds with LPS (1 μg/mL, Escherichia coli serotype 0111:B4) stimulation for 24 hours, the supernatants were collected for Nitric Oxide analysis by Nitric Oxide Assay Kit and expressed as IC_{50}[66].

4.3 Cell cytotoxicity

Cell cytotoxicity was evaluated by MTT assay and expressed as IC_{50}. RAW 264.7 cells were treated with or without compounds for 24 hours. Cells were incubated in 0.5 mg/mL MTT reagent dissolved in H_2O for 4 hours, and the formazan product dissolved in 150 μL of DMSO. The optical density was measured at 570 nm.

4.4 Western blot[67]

RAW 264.7 cells were treated with or without various concentrations of compound **53** and LPS in a manner as described for examining NO production. Whole cells were collected from treated and untreated cells. Equal amounts of protein were loaded by 8 % gradient gels and resolved by 10 % SDS-PAGE for iNOS at 350 mA for 90 min. The size-separated proteins were transferred onto the immobile polyvinylidene difluoride membrane (PVDF) membrane (Millipore, Bedford, MA) at 90 V for 60 min with transfer buffer composed of 25 mM Tris-HCl (pH = 8.9), 192 mM glycine, and 20 % methanol. The membrane was incubated in blocking buffer (5 % w/v skim milk in TBST buffer) for 1 hr at room temperature and then incubated with anti-iNOS (1:1000) (Cayman Chemicals) overnight at 4 ^{o}C. After hybridization with

primary antibodies, the membrane was washed with 0.1 % TBST (TBS containing 0.1 % Tween 20) three times, incubated with anti-rabbit secondary antibodies conjugated with horseradish peroxidase 2 hr at room temperature, and then washed with TBST three times. Finally, the membrane was detected by the enhanced chemiluminescence reagents (ECL, Pierce). The densities of the bands were quantitated with a computer densitometer (IS-100 Digital Imaging System). β-actin levels were measured as a loading control.

4.5 Induction of collagen-induced arthritis[44]

Male DBA/1 (6-8 weeks old, Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, P. R. China) were injected intracutaneously with 1.5 mg bovine type II collagen (Chondrex, 20021) emulsified in 0.1 mL complete Freund's adjuvant (Sigma, F5581) at the base of the tail on day 1. On day 21, the mice were injected of bovine type II collagen emulsified in incomplete Freund's adjuvant (Sigma, F5506) to a second injection. Compound **53** was orally administered once daily at a dose of 10 mg/kg started from day 30. All rats were dissected on day 63.

4.6 Induction of adjuvant-induced arthritis[11]

Female Lewis rats (6 - 8 weeks old, Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, P. R. China) were injected subcutaneously at the base of the tail with 0.5 mg heat-killed inactivated mycobacterium tuberculosis emulsified in 0.1 mL incomplete Freund's adjuvant (Sigma, F5506) on day 1. Compound **53** was administered orally once a day at a dose of 10 mg/kg started from day 14. All rats were dissected on day 28.

4.7 Clinical evaluation of arthritis[46]

Clinical parameters were measured by an articular index score. The articular index score was performed using a 0 - 4 scale system: 0, normal; 1, red and swelling of toe joint; 2, swelling of the toe joint and ankle; 3, swelling of the paws; 4, swelling of all the feet including the ankle joint. The sum of the scores of the four joints was expressed by the clinic score with a maximum of 16 points.

4.8 Histological Examination[46]

For histological analysis, paws were skinned and fixed in 4 % paraformaldehyde,

decalcified in EDTA buffer for 15 days, and then embedded in paraffin. The tissues were stained with hematoxylin-eosin and Safranin O-fast green. The histological changes were examined under the microscope.

4.9 RNA extraction and semiquantitative RT-PCR[68]

The hind paw joints were collected and the fine powder was obtained by rapid freezing and pulverization in liquid nitrogen. Total RNA was prepared by extracting the powder with Trizol (Ambion, LOT.36008). The total RNA was dissolved in water and the concentration was measured spectrophotometrically. The cDNA was reverse transcribed using the Premix Ex Taq kit (TAKARA, D332A). PCR reactions were performed under the following conditions: denaturation at 94 ^{o}C for 5 seconds, primer annealing at 60 ^{o}C for 30 seconds, and primer extension at 72 ^{o}C for 30 seconds. Primer sequences are shown in **Table 5**. The PCR products were run on a 1 % agarose gel. For each data point, tissues from three mice were pooled.

Table 5. The primer sequences

Gene	Forward primer	Reverse primer
TNF-α	5‘-ATCCGCGACGTGGAACTG-3’	5‘-ACCGCCTGGAGTTCTGGAA-3’
IL-1β	5‘-TCAAGGCATAACAGGCTCATC-3’	5‘-CCACGGGCAAGACATAGGTAG-3’
IL-6	5‘-CTTGGGACTGATGTTGTTGAC-3’	5‘-GAAGTTGGGGTAGGAAGGAC-3’

4.10 Flow cytometry[46]

For CD4 and CD8 staining ex vivo, lymphocytes were collected and isolated from spleens or draining LNs after mice dissection. Cells were then stained with APC-conjugated anti-mouse CD4 (ebioscience, 17-0041-83) and FITC-conjugated anti-mouse CD8 (TONBO, 35-0081-U500). Flow cytometry analysis was performed using BD LSRII.

4.11 Docking study

One chain of murine iNOS (dimer) was chosen as the target receptor of compound **53**. The 3D structure of the receptor was retrieved from the Protein Data Bank (PDB_ID: 3E67). Compound **53** was docked into the active site of iNOS by using a protein-ligand docking program Libdock (Discovery Studio). Scoring

functions Chemgauss version two, shapegauss and screenscore were employed for exhaustive searching, solid body optimization and interaction scoring.

5. Conclusions

Inflammation is a response of the host to inside and outside stimuli. Endogenous free radical nitric oxide (NO), a pro-inflammation cytokine mainly produced by activated macrophages and lymphocytes, plays a very important role in the inflammatory cascades, and inhibition of NO production serves as an effective therapy for inflammatory diseases.

In this study, thirty-nine chalcones and related compounds based on bioactive kava chalcones were designed, synthesized and their inhibitory effects on the NO production in RAW 264.7 cells were evaluated. The novel compound **53** showed a better activity (inhibitory rate 84.0 %) at 10 μM (IC_{50} = 6.4 μM) with the lowest cytotoxicity. Besides, compound **53** also showed an inhibitory effect on the expression of iNOS protein by western blot analysis and a binding ability to the iNOS active site by docking study. Furthermore, compound **53** was chosen to investigate its anti-inflammation effect in two rheumatoid arthritis models of collagen-induced arthritis and adjuvant-induced arthritis. In these models, oral administration (10 mg/kg/day) of compound **53** reduced foot paw swelling and improvements of pathology from joints of animals and resulted in the inhibition of arthritic inflammation were observed. All these data suggested that compound **53** might be an effective agent for the treatment of chronic inflammation and further the development of this compound may be of interest.

SAR analysis indicated that: (1) 2’-hydroxyl and 4’-methoxy group on the A ring is required for maintaining the activity; 2’-hydroxyl is also tolerant to slight modification; the introduction of electron withdrawing groups reduces the activity. (2) the main structure of chalcone with an unsaturated three carbon bridge was significant for maintaining the anti-inflammatory activity. The common unsaturated ketone moiety is likely to be responsible for the observed versatile biological activities. (3) electron cloud density on the B ring is very important for bioactivity; the introduction

of a linker at 4-position to a polar cyclic part would not lower the activity dramatically but may reduce the toxicity. Further mechanism and pharmacological studies of compound **53** will be reported in due course.

Abbreviations:

NO: nitric oxide; TNF-α: tumor necrosis factors-α; PGE2: prostaglandin E2; IL-1β: interleukin-1β; iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; CIA: collagen-induced arthritis; AIA: adjuvant-induced arthritis; SAR: structure-activity relationship; RA: rheumatoid arthritis; DPT cells: double-positive T cells; TLC: thin-layer chromatography; UV: ultraviolet; HRMS: high resolution mass spectrum; PVDF: polyvinylidene difluoride;

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Appendix A. Supplementary data:

The analytical data and spectra of intermediates and target compounds are available online.

Declarations of interest:

None, all the authors declare no conflict of interest.

#: these authors contributed to this work equally.

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Highlights

1. The novel compound (*E*)-1-(2'-hydroxy-4',6'-dimethoxyphenyl)-3-(3-methoxy-4-(3-morpholinopropoxy)phenyl)prop-2-en-1-one (**53**) showed a better inhibitory effect on NO production with much lower cytotoxicity than any other compound;
2. Compound **53** could down-regulate the expression of iNOS and dock into the active site of iNOS;
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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: