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Synthesis and biological evaluation of 3′,4′,5′-trimethoxychalcone analogues as inhibitors of nitric oxide production and tumor cell proliferation

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ABSTRACT

A series of 23 3′,4′,5′-trimethoxychalcone analogues was synthesized and their inhibitory effects on nitric oxide (NO) production in LPS/IFN-γ-treated macrophages, and tumor cell proliferation has been investigated. 4-Hydroxy-3,3′,4′,5′-tetramethoxychalcone (**7**), 3,4-dihydroxy-3′,4′,5′-trimethoxychalcone (**11**), 3-hydroxy-3′,4,4′,5′-tetramethoxychalcone (**14**), and 3,3′,4′,5′-tetramethoxychalcone (**15**) were the most potent growth inhibitory agents on NO production, with an IC_{50} value of 0.3, 1.5, 1.3 and 0.3 μM, respectively. The tumor cells proliferation assay results revealed that several compounds exhibited potent inhibition activity against different cancer cell lines. The chalcone **15** was the most potent anti-proliferative compound in the series with IC_{50} values of 1.8 and 2.2 μM toward liver cancer Hep G2 and colon cancer Colon 205 cell lines, respectively. 2,3,3′,4′,5′-Pentamethoxychalcone (**1**), 3,3′,4,4′,5,5′-hexamethoxychalcone (**3**), 2,3′,4,4′,5,5′-hexamethoxychalcone (**5**), 2-hydroxy-3,3′,4′,5′-tetramethoxychalcone (**10**), **11** and **14** showed significant anti-proliferation actions in Hep G2 and Colon 205 cells with an IC_{50} values ranging between 10 and 20 μM. Among the tested agents, compound **7** showed selective NO production inhibition (IC_{50} = 0.3 μM), while has no effect on tumor cell proliferation (IC_{50} >100 μM). 3,3′,4,4′,5′-Pentamethoxychalcone (**2**) showed selective anti-proliferation effect in Hep G2 cells, in addition to its potent NO inhibition, however has no such response in Colon 205 cells. In contrast, 3-formyl-3′,4′,5′-trimethoxychalcone (**22**) showed moderate growth inhibition in Colon 205 cells, while has no such effect on NO production and Hep G2 cells proliferation. These results provide insight into the correlation between some structural properties of 3′,4′,5′-trimethoxychalcones and their in vitro anti-inflammatory and anti-cancer differentiation activity.

1. Introduction

Chalcones, constitute an important group of natural products and serve as precursors for the synthesis of different classes of flavonoids, which are common substances in plants.[1] Chalcones are open-chain flavonoids in which two aromatic rings are joined by a three carbon α,β-unsaturated carbonyl system (1,3-diphenyl-2-propen-1-ones).[1] Chalcone derivatives have received a great deal of attention due to their relatively simple structures, and wide variety of pharmacological activities reported for these compounds include anti-inflammatory,[2] anti-bacterial,[1] anti-fungal,[3–5] and anti-tumor activities.[6–9] These activities are largely attributed due to the α,β-unsaturated ketone moiety. Introduction of various substituents into the two aryl rings is also a subject of interest because it leads to useful structure–activity relationship (SAR) conclusions and thus helps to synthesize pharmacologically active chalcones.[6]

In previous communications the authors of this study reported the preparation of 2′-hydroxychalcone derivatives and corresponding flavones, and evaluation of their biological activities.[10,11] Research into the anti-tumor properties of chalcones has received significant attention over the last few years, particularly with the discovery that these compounds possess a similar mode of action to the structurally related combretastatins. Combretastatin A-4 (CA-4, Fig. 1), a natural *cis*-stilbene product has received major attention owing to its strong inhibition of tubulin polymerization and selective targeting of tumor vascular systems (cancer vascular disrupting), which cuts off the tumor blood flow, leading to hemorrhagic necrosis as well as cancer antiangiogenesis.[12] A reported critical structural requirement for the activity of these compounds is the cis-configuration of the double bond and the 3,4,5-trimethoxyphenyl ring (ring A).[13] Taking into account of these reports, and an attempt to discover a potent compound that suppresses both the inflammation and cancer was an attractive idea.[14] It is reasonable that the dual inhibitors of both inflammatory mediators such as NO and tumor cell proliferation were more effective than single inhibitors as blocking multiple signaling pathways in cancer

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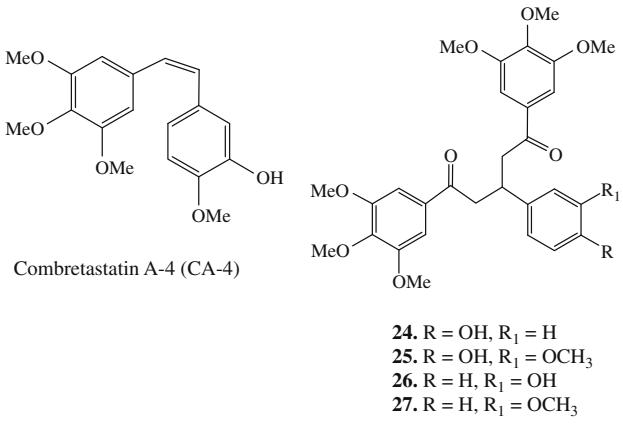


Figure 1. The Michael adducts (**24**–**27**) obtained from the Claisene Schmidt condensation between 3,4,5-trimethoxyacetophenone and corresponding benzaldehyde under reflux.

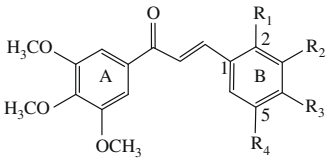
therapy and could be beneficial to overcome drug resistance. With the goal of identifying such a new anti-inflammatory and anti-cancer strategy, therefore, we intended to synthesize 3′,4′,5′-trimethoxychalcone analogues with different substituent on B-ring. We now present the synthesis of 3′,4′,5′-trimethoxychalcones analogues using a classical base catalyzed condensation reaction and their inhibitory effects on nitric oxide production and tumor cell proliferation.

2. Results and discussion

2.1. Chemistry

The preparation of the 3′,4′,5′-trimethoxychalcone analogues (Table 1) was carried out via Claisen–Schmidt condensation (Scheme 1).[10] Thus, an appropriate commercially available aryl aldehydes were reacted with inexpensive 3,4,5-trimethoxyacetophenone in ethanol/KOH at 0–5 °C. Upon completion, the cooled reaction mixture was poured into ice water and treated with HCl yielded the desired 3′,4′,5′-trimethoxychalcone analogues (**1**–**23**) with an average yield of 57–92% (Table 1).[10] When needed, the crude product was purified by silica gel column chromatography. The structure of compounds **1**–**23** were established with NMR and mass spectrometry measurements. Coupling constants (J) from the proton nuclear magnetic resonance (^{1}H NMR) spectra clearly indicated that compounds **1**–**23** were geometrically pure and were exclusively trans (E) isomers ($J_{transC=C}$ = 15–16 Hz). This aldol condensation via a one-step reaction produced five new substituted 3′,4′,5′-trimethoxychalcones (**1**, **5**, **6**, **10** and **11**) and eighteen known derivatives (**2**–**4**, **7**–**9** and **12**–**23**) as listed in Table 1. Our attempt to shorten the reaction time by refluxing a reaction mixture of 3,4,5-trimethoxyacetophenone and *p*-hydroxybenzaldehyde for 20 min resulted in only one product different from the expected chalcone. Its ^{1}H NMR spectrum showed no characteristic signals of the α,β-olefinic protons and eight aromatic protons instead of the six expected ones. In addition, two more signals were observed—two doublets of doublets for four protons at δ 3.47 and 3.18, and a pentet for one proton at δ 3.90. This information showed that at heating the reaction runs fast to receive the chalcone, which was further attacked by acetophenone to give the Michael adduct **24** (Fig. 1).[15] The compounds **25**–**27** were also prepared in this same manner from the corresponding substituted benzaldehydes, and their structures (Fig. 1) were additionally confirmed by the ^{13}C NMR and mass spectral analyses. To our knowledge these compounds has not been described in the literature up till now. Their spectral data are presented herein.

Table 1
3′,4′,5′-Trimethoxychalcone analogues **1**–**23** prepared via Aldol condensation reaction



Compound	R_1	R_2	R_3	R_4	Formula	Yield[a] (%)	Ref.
1	OMe	OMe	H	H	$C_{20}H_{22}O_6$	88	N
2	H	OMe	OMe	H	$C_{20}H_{22}O_6$	90	15
3	H	OMe	OMe	OMe	$C_{21}H_{24}O_7$	86	15
4	H	H	$N(CH_3)_2$	H	$C_{20}H_{23}NO_4$	78	24
5	OMe	H	OMe	OMe	$C_{21}H_{24}O_7$	87	N
6	H	H	OH	H	$C_{18}H_{18}O_5$	75	N
7	H	OMe	OH	H	$C_{19}H_{20}O_6$	83	15
8	OMe	H	OMe	H	$C_{20}H_{22}O_6$	88	25
9	OH	H	H	OH	$C_{18}H_{18}O_6$	58	26
10	OH	OMe	H	H	$C_{19}H_{20}O_6$	72	N
11	H	OH	OH	H	$C_{18}H_{18}O_6$	57	N
12	H	OH	H	H	$C_{18}H_{18}O_5$	70	15
13	H	H	OMe	H	$C_{19}H_{20}O_5$	89	24
14	H	OH	OMe	H	$C_{19}H_{20}O_6$	85	15
15	H	OMe	H	H	$C_{19}H_{20}O_5$	92	25
16	H	H	F	H	$C_{18}H_{17}FO_4$	77	24
17	H	H	Br	H	$C_{18}H_{17}BrO_4$	75	24
18	H	H	NO_2	H	$C_{18}H_{17}NO_6$	68	15
19	H	H	Me	H	$C_{19}H_{20}O_4$	82	25
20	OH	H	H	NO_2	$C_{18}H_{17}NO_7$	66	25
21	CHO	H	H	H	$C_{19}H_{18}O_5$	70	24
22	H	CHO	H	H	$C_{19}H_{18}O_5$	72	24
23	H	H	H	CHO	$C_{19}H_{18}O_5$	75	24

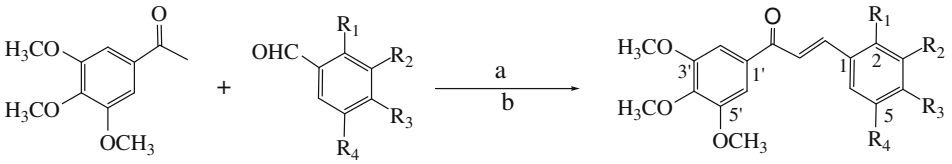
[a] Isolated yield including recovered starting material. N, new compound.

2.2. Biological evaluation

2.2.1. Inhibition of nitric oxide (NO) production

Macrophages are the main pro-inflammatory cells responsible for invading pathogens by releasing many pro-inflammatory molecules, including the free radical nitric oxide (NO) which is produced endogenously by the nitric oxide synthase (NOS).[16] Activated macrophages transcriptionally express inducible nitric oxide synthase (iNOS), which is responsible for the prolonged and profound production of NO. The aberrant release of NO can lead to amplification of inflammation as well as tissue injury.[16] Therefore, inhibition of neutrophils and/or macrophages activation and the following release of inflammatory mediator NO provide a promising strategy for the development of potential anti-inflammatory agents.

A number of synthetic and naturally occurring chalcone derivatives exhibit potent anti-inflammatory activities,[2,17] although the mechanism of action is not yet fully understood. Anti-inflammatory activity of chalcones are associated with suppression of inflammatory mediators including NO.[17] In this study, all synthesized 3′,4′,5′-trimethoxychalcone analogues were screened for their activities on NO production in the mouse macrophages, stimulated by lipopolysaccharide (LPS, 2 μg/mL) plus interferon (IFN)-γ (10 U/mL). As shown in Table 2, among 23 synthesized chalcones, four compounds including 4-hydroxy-3,3′,4′,5′-tetramethoxychalcone (**7**), 3,4-dihydroxy-3′,4′,5′-trimethoxychalcone (**11**), 3-hydroxy-3′,4,4′,5′-tetramethoxychalcone (**14**), and 3,3′,4′,5′-tetramethoxychalcone (**15**) showed most potent inhibitory effect on NO production with an IC_{50} values of 0.3, 1.5, 1.3 and 0.3 μM, respectively (Table 2). A similar trend was observed for chalcones **1**, **3**, and **10** with an IC_{50} value of 2.4, 2.8 and 3.0 μM, respectively (Table 2). Furthermore, the chalcones **2**, **5**, **6**, **19** and **20** also exhibited potent NO growth inhibition with an IC_{50} values ranging between 4 and 5 μM



a. 50% KOH, EtOH, r.t., 24 h; b. 2N HCl

Scheme 1. General synthetic route for the synthesis of 3′,4′,5′-trimethoxychalcone analogues.

Table 2
Growth inhibition of nitric oxide production and tumor cell proliferation by 3′,4′,5′-trimethoxychalcone analogues **1**–**23** as calculated from dose–response curves[a]

Compound	IC_{50}[b] (μM)[c]		
	NO	Hep G2	Colon 205
1	2.4 ± 0.3	11.5 ± 1.4	13.2 ± 2.0
2	4.5 ± 0.5	20.3 ± 1.9	>100
3	2.8 ± 0.2	19.6 ± 2.1	19.2 ± 1.7
4	27.0 ± 4.0	30.0 ± 3.8	75.0 ± 5.5
5	4.6 ± 1.1	16.1 ± 2.9	18.6 ± 3.5
6	5.0 ± 0.7	16.0 ± 1.8	29.7 ± 4.3
7	0.3 ± 0.1	>100	>100
8	>50	>100	82.5 ± 5.0
9	>50	>100	>100
10	3.0 ± 0.2	13.5 ± 0.8	13.0 ± 1.2
11	1.5 ± 0.4	14.9 ± 2.1	19.8 ± 2.7
12	13.5 ± 1.2	94.0 ± 4.6	49.0 ± 3.2
13	7.6 ± 1.9	49.5 ± 3.6	46.4 ± 5.2
14	1.3 ± 0.3	10.6 ± 1.3	11.2 ± 2.4
15	0.3 ± 0.1	1.8 ± 0.3	2.2 ± 0.7
16	17.6 ± 3.6	22.0 ± 2.5	25.7 ± 2.8
17	20.0 ± 4.1	80.0 ± 4.9	22.5 ± 3.2
18	30.0 ± 4.5	>100	43.0 ± 5.0
19	4.5 ± 1.2	52.0 ± 4.1	41.5 ± 3.2
20	4.4 ± 0.7	>100	76.0 ± 4.7
21	27.0 ± 2.5	>100	88.0 ± 5.1
22	>50	>100	22.5 ± 2.3
23	>50	>100	68.8 ± 5.4

[a] Sigmoidal dose–response curves (variable slopes) were generated using GRAPHPAD PRISM V. 4.02 (GRAPHPAD Software Inc.).
[b] Tested compound concentration required to inhibit cell proliferation by 50% after 48 h of treatment compared with vehicle control DMSO.
[c] Values are the mean of triplicate of at least three independent experiments.

(Table 2). The opposite situation was found in compounds **8**, **9**, **22** and **23** with the IC_{50} values greater than 50 μM (Table 2). The new compounds **1**, **5**, **6**, **10** and **11** with a methoxyl and/or hydroxyl groups at C-2 and/or C-3 and/or C-4 showed potent inhibition activity against NO production with an IC_{50} values ranging 1.5–5.0 μM.

With respect to different substituted groups on B-ring, oxygenated groups on B ring in which one group substituted at 3-position and the other substituted at 2- or 4-position played an important role in the different IC_{50} trends against the NO production. As shown in Table 2, a strong electron withdrawal group of F, Br, NO_2 and CHO in **16**, **17**, **18** and **23**, respectively at C-4 position significantly decreased NO production inhibition. However, the presence of electron donating group of OH, OCH_3 and CH_3 in **6**, **13** and **19**, respectively at the same position exhibited a stronger activity than that of electron withdrawal group containing compounds (**16**–**18** and **23**). This result indicated that the hydroxyl group at the *para*-position of the benzene ring is enhanced to delocalize the electron density based on a resonance effect and, generally showed increased suppression of NO production.[18] Interestingly, compound **15** with a methoxyl group at C-3 showed the best activity with an IC_{50} value of 0.3 μM and was 45-times more potent than that of **12** with a hydroxyl group at C-3 (IC_{50} = 13.5 μM). The inhibitory effect of tested compounds was not due to cytotoxicity, since tested treatment did not affect cell viability under these experimental conditions, as assessed by MTT assay (data not shown). Although the small number of examples in this study precludes us from discerning a clear SAR, these encouraging results have motivated the synthesis and biological evaluation of an expanded series of analogs for further structural optimization of these chalcones as anti-inflammatory agents.

2.2.2. Inhibition of tumor cell proliferation

We next evaluated the chalcones **1**–**23** for their in vitro anti-proliferation screening using a 72-h continuous exposure MTT assay. Concentrations of compounds that inhibited tumor cell growth by 50% relative to an untreated control (DMSO), or IC_{50} (μM) values for both human cancer cell lines HepG2 (human hepatoma cancer cell line), and Colon 205 (human colonic cancer cell line) are shown in Table 2. The IC_{50} values were determined by interpolation from dose–response curves. A total of 100 μM was chosen as maximal concentration because much higher doses do not normally reach the blood plasma,[19] and we are looking for compounds with high activity (low IC_{50}) which could eventually be used in the future as a drug for chemotherapy with very low to no side effects.

Anti-proliferation results for Hep G2 cells treated with each compound indicate that chalcone **15** (3,3′,4′,5′-tetramethoxychalcone) showed the highest activity with IC_{50} value of 1.8 μM. The compounds displaying the potent anti-proliferation effect in Hep G2 cells were **1** (2,3-di-OMe), **2** (3,4-di-OMe), **3** (3,4,5-tri-OMe), **5** (2,4,5-tri-OMe), **6** (4,-OH), **10** (2-OH, 3-OMe), **11** (3,4-di-OH) and **14** (3-OH, 4-OMe), with IC_{50} value of 11.5, 20.3, 19.6, 16.1, 16.0, 13.5, 14.9 and 10.6 μM, respectively (Table 2). Compound **2** or **7** with an additional methoxyl or hydroxyl group at C-4 showed less potency (IC_{50} = 20.3 μM) or inactive (IC_{50} >100 μM), respectively than that of **15** with C-3 methoxyl group on ring-B (Table 2). Replacement the 3-methoxy group of ring-B with hydroxyl group (**6**), also displayed a decrease in the anti-proliferation activity by ten orders of magnitude against Hep G2 cells. Chalcones **7**–**9**, **18** and **20**–**23** were found to exhibit negligible Hep G2 cell growth inhibitory activity with an IC_{50} value greater than 100 μM. This result implies that the methoxyl group at the C-3 position of ring-B is important for the anti-proliferation activity profile in the tested compounds. Shifting the methoxyl group at R_2 from the *meta*-position (**15**) to *para*-position (**13**) on the ring-B decreased the anti-proliferation activity. Introduction of a weak electron-withdrawing group, a *para*-fluorine atom (**16**), and increasing the size of the halogen from fluorine to bromine (**17**) decreased the anti-proliferative activity. The replacement of the *para*-fluoro group with a nitro moiety, to give **18**, eliminated anti-proliferative activity (IC_{50} >100 μM).[13] Turning to the effects of an electron releasing group on the *para*-position, we found that replacement of *p*-methoxyl (**13**) with a *p*-methyl (**19**) group caused only a minor decrease in anti-proliferative activity, while the trimethoxy derivatives **3** and **5** were more active than **13**. Future investigation of chalcone **15** and the other structural modifications noted herein as Hep G2 cell growth inhibitors may provide further SAR insights.

In the Colon 205 cells, the results followed a similar trend; compound **15** was a good substrate for these cells inhibition, being provided with the lowest IC_{50} value (2.2 μM) among the prepared compounds. However, replacement of methoxyl group of **15** to hydroxyl group of **12** and aldehyde group of **22** resulted in the decrease of the anti-proliferative effect (Table 2). This might be due to disfavored interactions between the hydrogen-bond acceptor of **12** and **22,** and their molecular targets that counteract the overall activity in Colon 205 cells.[20] Compounds **1**, **10** and **14** showed moderate anti-proliferation activity in Colon 205 cells with an IC_{50} value of 13.2, 13.0 and 11.2 μM, respectively (Table 2). Particularly, the chalcone **14** which has substituent similar to that of CA-4 showed moderate anti-proliferation activity in both Hep G2 and Colon 205 cells, but the presence of a methoxyl group (in **2**) instead of hydroxyl (in **14**) at C-4 of ring-B is detrimental to activity in Colon 205 cells (Table 2). When a methoxyl group is present in the *meta*-position of the B-ring, increased activity is seen, in both Hep G2 and Colon 205 cell lines, compared with the same group in the *para*-position for the 3′,4′,5′-trimethoxychalcones (e.g., **13** vs **15**). However, this effect was in opposite when a hydroxyl group is present in these positions (**6** vs **12**, Table 2). Interestingly, C-4-substituted analog with either electron-withdrawing groups such as 4-fluoro (**16**), 4-bromo (**17**), and 4-nitro (**18**) or an electron donating group like 4-methoxyl (**13**) and 4-methyl (**19**) exhibited moderate activities with IC_{50} values of 25.7, 22.5, 43.0, 46.4 and 41.5 μM, respectively. Furthermore, compound **7** bearing 3-methoxy-4-hydroxy group and **9** bearing 2,5-dihydroxy group exhibited negligible anti-proliferation response in both the cell lines Hep G2 and Colon 205. These results are in consistent with previous reports on SAR studies on chalcone derivatives, which showed that even a subtle modification of chalcone chemical structure may produce significant changes in their biological potency.[18] Together, these results indicated plausible correlations between structural characteristics of the tested compounds with their anti-proliferative effects on the Colon 205 cells. Similar to data indicated in the Hep G2 cells, compound **15** (IC_{50} = 1.8 μM) demonstrated most potent activity against Colon 205 cells among modified ring-B-3′,4′,5′-trimethoxychalcone analogs. The most prevalent form of cancer among Taiwanese people is colorectal cancer, topping the list of the five most common cancers in Taiwan according to a 2006 cancer report released by the Department of Health. About 15.03 per 100,000 individuals die per year of colorectal cancer, and the current clinical therapies are limited to surgery, radiotherapy, general chemotherapy, and gene therapy.[21] So that there is a need to identify new therapeutic agents for the treatment of colon cancer. These preliminary results for our novel compounds may useful to discover the lead derivatives. Taking into account both anti-inflammatory and anti-proliferative data from this study, the tested chalcones might decrease tumor cells proliferation through inhibition of the inflammatory mediators such as NO. This is consistent with results from other studies that tested the anti-tumor activity of anti-inflammatory chalcones.[14,22,23]

3. Conclusions

The synthesis and biological evaluation of 3′,4′,5′-trimethoxychalcone analogues **1**–**23** has been described. The synthetic approach involves Aldol condensation of 3,4,5-trimethoxyacetophenone and aldehydes in the presence of KOH to generate 3′,4′,5′-trimethoxychalcone analogues in high yields, in addition to the novel Michael adducts. The prepared compounds **1**–**23** were evaluated for suppression of LPS/IFN-γ-induced NO generation, and human tumor cell proliferation in vitro. Compound **15** exhibited potent inhibition NO production as well as tumor cell proliferation, and may provide a template to design new novels with a dual activity of anti-inflammation and anti-cancer. Compound **2** selectively inhibited NO production and Hep G2 proliferation; while **22** showed selective anti-proliferation affect in colon cancer Colon 205 cells. Taken together, these results indicates that 3′,4′,5′-trimethoxychalcone analogues may consider as potential anti-inflammatory and anti-cancer agents. Further studies will be required to better understand signaling mechanisms of the subject compounds, and needs to be generalized to a larger series bearing other substitution patterns on the ring-B. On the other hand, the most active compounds should be analyzed for effects on normal human cells, in order to determine potential therapeutic windows supporting further studies on experimental tumor-bearing animals finalized to verify possible in vivo anti-cancer activity.

4. Experimental section

4.1. General

Melting points (mp) were determined on a Buchi B-540 apparatus and were uncorrected. NMR spectra were measured on Bruker Avance 500 MHz spectrometer, using TMS as an internal standard. Abbreviations for signal coupling are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants *J* are reported (Hz). Silica gel for column chromatography (CC), (0.063–0.200 mm), was a product of Merck Company. TLC was performed on Merck TLC plates (0.23 mm thickness), with compounds visualized by spraying with 8% (v/v) H_2SO_4 in EtOH and then heating on a hot plate.

Lipopolysaccharide (LPS, *Escherichia coli* 055: B5), bovine serum albumin (BSA), phosphate-buffered solution (PBS), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and Griess reagent were purchased from Sigma Chemical (St. Louis, MO, USA). Recombinant interferon-γ (IFN-γ) was purchased from PeproTech (Margravine, London, England). RPMI-1640 medium, Hank's balanced salt solution (HBSS), penicillin, streptomycin, L-glutamine and fetal calf serum were purchased from Gibco BRL (Grand Island, NY, USA). Colon 205 (human colon cancer cell line), and HepG2 (human hepatoma cancer cell line) were obtained from American Type Culture Collection (Rockville, MD), and cultured with complete RPMI-1640 medium (Gibco BRL) supplemented with 10% fetal calf serum, antibiotics, L-glutamine.

4.2. General procedures for the preparation of the aldol condensation products of 3,4,5-trimethoxyacetophenone with aldehydes (1–23)

A stirred solution of 3,4,5-trimethoxyacetophenone (6.0 mmol) in ethanol (30 mL) was added to KOH (1.2 mmol), and the mixture was stirred at 0–5 °C temperature for 1 h. The aldehydes (6.0 mmol) were added to the mixture, and the resulting mixture was stirred at room temperature for 24 h. The mixture was concentrated under reduced pressure, and the residue was treated with water (35 mL). The aqueous mixture was neutralized by the addition of aqueous 10% HCl solution and extracted with ethylacetate (2 × 30 mL). The organic phase was washed with aqueous saturated NH_4Cl solution (30 mL) and brine (30 mL). The organic layer was separated and dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure to give the crude product, which was purified by flash silica chromatography to produce the pure substituted 3′,4′,5′-trimethoxychalcone analogues **1**–**23**. For the known compounds spectral data, see the Supplementary data and Table 1.[15,24–26]

4.2.1. 2,3,3′,4′,5′-Pentamethoxychalcone (1)

Pale yellow solid, mp 98–99 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.02 (d, *J* = 15.5, 1H), 7.51 (d, *J* = 15.5, 1H), 7.24 (s, 2H), 7.22 (d,

J = 8.0, 1H), 7.06 (t, J = 8.0, 1H), 6.94 (d, J = 8.0 Hz, 1H), 3.90 (s, 6H, 2 × –OCH_3), 3.88 (s, 3H, –OCH_3), 3.86 (s, 3H, –OCH_3), 3.84 (s, 3H, –OCH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 190.0 (C=O), 153.4, 153.3, 149.1, 142.6, 139.9, 133.8, 129.3, 124.5, 123.8, 120.1, 114.4, 106.4, 61.5, 61.2, 56.6, 56.1. MS (ESI): 359.1 (M^++1).

4.2.2. 2,3′,4,4′,5,5′-Hexamethoxychalcone (5)

Orange yellow solid, mp 135–136 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.04 (d, J = 16.0, 1H), 7.36 (d, J = 16.0, 1H), 7.24 (s, 2H), 7.10 (s, 1H), 6.51 (s, 1H), 3.93 (s, 3H, –OCH_3), 3.92 (s, 6H, 2 × –OCH_3), 3.91 (s, 3H, –OCH_3), 3.89 (s, 3H, –OCH_3), 3.87 (s, 3H, –OCH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 190.5 (C=O), 154.9, 153.3, 152.8, 143.5, 142.3, 140.5, 134.4, 120.7, 115.7, 112.1, 106.4, 61.2, 56.6, 56.5, 56.3, 56.2. MS (ESI): 389.1 (M^++1).

4.2.3. 4-Hydroxy-3′,4′,5′-trimethoxychalcone (6)

Orange solid, mp 198–199 °C; ^{1}H NMR (CD_3OD, 500 MHz): δ 7.77 (d, J = 15.5 Hz, 1H), 7.65 (d, J = 8.0 Hz, 2H), 7.57 (d, J = 15.5 Hz, 1H), 7.38 (s, 2H), 6.84 (d, J = 8.0 Hz, 2H), 3.93 (s, 6H, 2 × –OCH_3), 3.85 (s, 3H, –OCH_3). ^{13}C NMR (CD_3OD, 125 MHz): δ 191.5 (C=O), 162.2, 154.7, 147.0, 144.0, 135.3, 132.1, 127.9, 119.5, 117.1, 107.5, 61.4, 57.0. MS (ESI): 315.1 (M^++1).

4.2.4. 2-Hydroxy-3,3′,4′,5′-tetramethoxychalcone (10)

Yellow solid, mp 178–179 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.13 (d, J = 15.5, 1H), 7.81 (d, J = 15.5, 1H), 7.54 (d, J = 8.0, 1H) 7.39 (s, 2H), 7.04 (d, J = 8.0, 1H), 6.85 (t, J = 8.0 Hz, 1H), 3.89 (s, 6H, 2 × –OCH_3), 3.84 (s, 3H, –OCH_3), 3.76 (s, 3H, –OCH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 188.1 (C=O), 152.9, 148.0, 146.5, 141.8, 138.9, 133.3, 121.8, 120.8, 119.6, 119.0, 113.6, 106.0, 60.1, 56.2, 56.0, MS (ESI): 345.1 (M^++1).

4.2.5. 3,4-Dihydroxy-3′,4′,5′-trimethoxychalcone (11)

Pale yellow solid, mp 158–159 °C; ^{1}H NMR ((CD_3)$_2$CO, 500 MHz): δ 7.68 (d, J = 15.5, 1H), 7.62 (d, J = 15.5, 1H), 7.43 (s, 2H), 7.31 (d, J = 2.0, 1H), 7.17 (dd, J = 8.0, 2.0, 1H), 6.90 (d, J = 8.0 Hz, 1H), 3.94 (s, 6H, 2 × –OCH_3), 3.82 (s, 3H, –OCH_3). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 188.7 (C=O), 154.4, 149.0, 146.4, 145.3, 143.4, 134.9, 128.5, 123.6, 119.8, 116.4, 115.8, 107.0, 60.7, 56.7 MS (ESI): 331.1 (M^++1).

4.3. General procedure for the preparation of Michael adducts (24–27)

3′,4′,5′-Trimethoxyacetophenone (1 mmol) and aryl benzaldehyde (1 mmol) were dissolved in 10 mL ethanol and to this solution 10 mL of 50% KOH was added. The reaction mixture was then refluxed for 20 min, and then cooled in an ice-water bath. The solution was diluted with distilled water (100 mL) and acidified with 10 mL concd HCl. The product obtained was purified with column chromatography over silica using n-hexane/ethylacetate increasing polarity to yield the corresponding Michael adducts.

4.3.1. 3-(4-Hydroxyphenyl)-1,5-di-(3,4,5-trimethoxyphenyl)pentane-1,5-dione (24)

Amorphous powder, yield 76%, mp 163–164 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.24 (s, 4H), 7.08 (d, J = 8.0 Hz, 2H), 6.67 (d, J = 8.0 Hz, 2H), 3.90 (p, 1H), 3.89 (s, 18H, 6 × –OCH_3), 3.47 (dd, J = 16.0, 7.0 Hz, 2H), 3.18 (dd, J = 16.0, 7.0 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 198.2 (C=O), 154.9, 153.3, 142.9, 135.3, 132.2, 128.8, 115.8, 106.0, 61.2, 56.6, 45.4, 37.9. MS (ESI): 513.1 (M^++1).

4.3.2. 3-(4-Hydroxy-3-methoxyphenyl)-1,5-di-(3,4,5-trimethoxyphenyl)pentane-1,5-dione (25)

Amorphous powder, yield 78%, mp 170–171 °C; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.23 (s, 4H), 6.87 (d, J = 1.5 Hz, 1H), 6.68 (dd, J = 8.5, 1.5 Hz, 1H), 6.60 (d, J = 8.5 Hz, 1H), 3.84 (s, 12H, 4 × –OCH_3), 3.80 (p, 1H), 3.73 (s, 6H, 2 × –OCH_3), 3.70 (s, 3H, –OCH_3), 3.40 (m, 4H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 197.8 (C=O), 152.7, 147.2, 144.8, 141.8, 135.0, 132.2, 119.7, 115.1, 112.1, 105.6, 60.1, 56.0, 55.6, 44.4, 37.0. MS (ESI): 543.1 (M^++1).

4.3.3. 3-(3-Hydroxyphenyl)-1,5-di-(3,4,5-trimethoxyphenyl)-pentane-1,5-dione (26)

Amorphous powder, yield 71%, mp 132–133 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.27 (s, 4H), 7.15 (t, J = 7.5 Hz, 1H), 6.83 (d, J = 7.5 Hz, 1H), 6.79 (s, 1H), 5.39 (s, 1H), 4.05 (p, 1H), 3.92 (s, 18H, 6 × –OCH_3), 3.47 (dd, J = 16.0, 7.0 Hz, 2H), 3.25 (dd, J = 16.0, 7.0 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 197.8 (C=O), 156.2, 153.3, 145.7, 142.9, 132.2, 130.1, 119.9, 114.7, 114.1, 106.0, 61.2, 56.6, 44.9, 38.1. MS (ESI): 513.1 (M^++1).

4.3.4. 3-(3-Methoxyphenyl)-1,5-di-(3,4,5-trimethoxyphenyl)-pentane-1,5-dione (27)

Amorphous powder, yield 69%, mp 210–211 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.24 (s, 4H), 7.03 (t, J = 8.0 Hz, 1H), 6.82 (d, J = 7.5 Hz, 1H), 6.77 (s, 1H), 5.19 (s, 1H), 4.06 (p, 1H), 3.87 (s, 12H, 4 × –OCH_3), 3.77 (s, 6H, 2 × –OCH_3), 3.73 (S, 3H, –OCH_3), 3.28 (m, 4H). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 206.1 (C=O), 159.7, 153.2, 152.6, 143.7, 141.2, 136.5, 134.4, 129.6, 121.5, 114.9, 112.8, 106.3, 61.0, 56.6, 55.3, 42.6, 38.5. MS (ESI): 527.1 (M^++1).

4.4. Macrophage cultures and nitric oxide determination

The inhibitory assay for inflammatory mediator NO induced by LPS/IFN-γ stimulants in murine peritoneal macrophage cells was performed by the methods as we described previously.[27,28]

4.5. Tumor cell growth inhibition assay

The anti-proliferation effect of compounds **1**–**23** against human hepatoma cancer Hep G2 and human colonic cancer Colon 205 cells obtained from American Type Culture Collection (ATCC, Rockville, MD), were performed by the method as we described previously.[29–31]

4.6. Statistical analysis

Each experiment was performed in triplicate and repeated three times (n = 9). The results were expressed as means ± SD. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.022.

References and notes

1. Avila, H. P.; Smania, E. F.; Monache, F. D.; Smania, A., Jr. *Bioorg. Med. Chem.* **2008**, *16*, 9790.
2. Cheng, J. H.; Hung, C. F.; Yang, S. C.; Wang, J. P.; Won, S. J.; Lin, C. N. *Bioorg. Med. Chem.* **2008**, *16*, 7270.
3. Sortino, M.; Delgado, P.; Juarez, S.; Quiroga, J.; Abonia, R.; Insuasty, B.; Nogueras, M.; Rodero, L.; Garibotto, F. M.; Enriz, R. D.; Zacchino, S. A. *Bioorg. Med. Chem.* **2007**, *15*, 484.
4. Vargas, M. L. Y.; Castelli, M. V.; Kouznetsov, V. V.; Urbina, G. J. M.; Lopez, S. N.; Sortino, M.; Enriz, R. D.; Ribas, J. C.; Zacchino, S. *Bioorg. Med. Chem.* **2003**, *11*, 1531.

5. Lopez, S. N.; Castelli, M. V.; Zacchino, S. A.; Dominguez, J. N.; Lobo, G.; Charris-Charris, J.; Cortes, J. C.; Ribas, J. C.; Devia, C.; Rodriguez, A. M.; Enriz, R. D. *Bioorg. Med. Chem.* **2001**, *8*, 1999.
6. Katsori, A. M.; Hadjipavlou-Litina, D. *Curr. Med. Chem.* **2009**, *16*, 1062.
7. Achanta, G.; Modzelewska, A.; Feng, L.; Khan, S. R.; Huang, P. *Mol. Pharmacol.* **2006**, *70*, 426.
8. Modzelewska, A.; Pettit, C.; Achanta, G.; Davidson, N. E.; Huang, P.; Khan, S. R. *Bioorg. Med. Chem.* **2006**, *14*, 3491.
9. Kumar, S. K.; Hager, E.; Pettit, C.; Gurulingappa, H.; Davidson, N. E.; Khan, S. R. *J. Med. Chem.* **2003**, *46*, 2813.
10. Rao, Y. K.; Fang, S. H.; Tzeng, Y. M. *Bioorg. Med. Chem.* **2004**, *12*, 2679.
11. Rao, Y. K.; Fang, S. H.; Tzeng, Y. M. *Bioorg. Med. Chem.* **2005**, *13*, 6850.
12. Alloatti, D.; Giannini, G.; Cabri, W.; Lustrati, I.; Marzi, M.; Ciacci, A.; Gallo, G.; Tinti, M. O.; Marcellini, M.; Riccioni, T.; Guglielmi, M. B.; Carminati, P.; Pisano, C. *J. Med. Chem.* **2008**, *51*, 2708.
13. Tron, G. C.; Pagliai, F.; Del Grosso, E.; Genazzani, A. A.; Sorba, G. *J. Med. Chem.* **2005**, *48*, 3260.
14. Won, S. J.; Liu, C. T.; Tsao, L. T.; Weng, J. R.; Ko, H. H.; Wang, J. P.; Lin, C. N. *Eur. J. Med. Chem.* **2005**, *40*, 103.
15. Batovska, D.; Parushev, S.; Stamboliyska, B.; Tsvetkova, I.; Ninova, M.; Najdenski, H. *Eur. J. Med. Chem.* **2009**, *44*, 2211.
16. Tripathi, P.; Tripathi, P.; Kashyap, L.; Singh, V. *FEMS Immunol. Med. Microbiol.* **2007**, *51*, 443.
17. Ko, H. H.; Tsao, L. T.; Yu, K. L.; Liu, C. T.; Wang, J. P.; Lin, C. N. *Bioorg. Med. Chem.* **2003**, *11*, 105.
18. Nowakowska, Z. *Eur. J. Med. Chem.* **2007**, *42*, 125.
19. Dupuis, C.; Mercier, C.; Yang, C.; Monjanel-Mouterde, S.; Ciccolini, J.; Fanciullino, R.; Pourroy, B.; Deville, J. L.; Duffaud, F.; Bagarry-Liegey, D.; Durand, A.; Iliadis, A.; Favre, R. *Anticancer Drugs* **2008**, *19*, 267.
20. Shaw, A. Y.; Chang, C. Y.; Liau, H. H.; Lu, P. J.; Chen, H. L.; Yang, C. N.; Li, H. Y. *Eur. J. Med. Chem.* **2009**, *44*, 2552.
21. Durai, R.; Yang, S. Y.; Seifalian, A. M.; Winslet, M. C. *J. Gastrointest. Liver Dis.* **2008**, *17*, 59.
22. Epifano, F.; Genovese, S.; Menghini, L.; Curini, M. *Phytochemistry* **2007**, *68*, 939.
23. Cabrera, M.; Simoens, M.; Falchi, G.; Lavaggi, M. L.; Piro, O. E.; Castellano, E. E.; Vidal, A.; Azqueta, A.; Monge, A.; Lopez de Cerain, A.; Sagrera, G.; Seoane, G.; Cerecetto, H.; Gonzalez, M. *Bioorg. Med. Chem.* **2007**, *15*, 3356.
24. Edwards, M. L.; Stemerick, D. M.; Sunkara, P. S. *J. Med. Chem.* **1990**, *33*, 1948.
25. Batovska, D.; Parushev, S.; Slavova, A.; Bankova, V.; Tsvetkova, I.; Ninova, M.; Najdenski, H. *Eur. J. Med. Chem.* **2007**, *42*, 87.
26. Nam, N. H.; Hong, D. H.; You, Y. J.; Kim, Y.; Bang, S. C.; Kim, H. M.; Ahn, B. Z. *Arch. Pharm. Res.* **2004**, *27*, 581.
27. Fang, S. H.; Rao, Y. K.; Tzeng, Y. M. *Bioorg. Med. Chem.* **2005**, *13*, 2381.
28. Rao, Y. K.; Fang, S. H.; Hsieh, S. C.; Yeh, T. H.; Tzeng, Y. M. *J. Ethnopharmacol.* **2009**, *121*, 292.
29. Rao, Y. K.; Fang, S. H.; Tzeng, Y. M. *J. Ethnopharmacol.* **2007**, *114*, 78.
30. Geethangili, M.; Rao, Y. K.; Fang, S. H.; Tzeng, Y. M. *Phytother. Res.* **2008**, *22*, 1336.
31. Yeh, C. T.; Rao, Y. K.; Yao, C. J.; Yeh, C. F.; Li, C. H.; Chuang, S. E.; Luong, J. H.; Lai, G. M.; Tzeng, Y. M. *Cancer Lett.* **2009**, *285*, 73.