**Design, Synthesis and Biological Evaluation of Ligustrazine-Flavonoid Derivatives as Potential Anti-Tumor Agents**

Hui Wang 1, Wenxi Zhang 1, Yatao Cheng 2, Xinyu Zhang 1, Nannan Xue 1, Gaorong Wu 1, Meng Chen 1, Kang Fang 1, Wenbo Guo 1, Fei Zhou 1, Herong Cui 1,*, Tao Ma 1,*, Penglong Wang 1,*, and Haimin Lei 1,*,

1 School of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing 100102, China; 15652387323@163.com (H.W.); 20170931820@bucm.edu.cn (W.Z.); 20160931837@bucm.edu.cn (X.Z.); 18810612758@163.com (N.X.); gaorongwu09@163.com (G.W.); 18702267252@163.com (M.C.); 18364169946@163.com (K.F.); wb_guo@126.com (W.G.); zf116318@163.com (F.Z.); zhenzhu7696@163.com (H.C.)

2 International Cooperation Division, China Sinopharm International Corporation, Beijing 100102, China; chengyatao@sinopharm.com

* Correspondence: mosesmatao@163.com (T.M.); wppl581@126.com (P.W.); hm_lei@126.com (H.L.); Tel.: +86-10-8473-8640 (P.W.); +86-10-8473-8645 (H.L.)

Received: 16 July 2018; Accepted: 21 August 2018; Published: 30 August 2018

**Abstract:** In the clinic some anti-tumor drugs have shown damage to normal blood vessels, which could lead to vascular diseases. Therefore, it is necessary to evaluate the effects of anti-tumor drugs on normal blood vessels at the beginning of the drug design process. In this study, ligustrazine (TMP) and flavonoids were selected as raw materials. Sixteen novel TMP-flavonoid derivatives were designed and synthesized. Interestingly, compounds 14 and 16 were obtained by hydrolysis of a dihydroflavone to a chalcone under alkaline conditions. The cytotoxicity of the TMP-flavonoid derivatives was evaluated on five human tumor cell lines and one classical type of normal endothelial cell lines (HUVEC-12) by an MTT assay. Part of the derivatives showed better anti-tumor activities than the corresponding raw materials. Among them, compound 14 exhibited the closest activity to the positive control against the Bel-7402 cell line (IC₅₀ = 10.74 ± 1.12 µM; DDP IC₅₀ = 6.73 ± 0.37 µM) and had no toxicity on HUVEC-12 (IC₅₀ > 40 µM). Subsequently, fluorescence staining and flow cytometry analysis indicated that compound 14 could induce apoptosis of Bel-7402 cell lines. Moreover, the structure-activity relationships of these derivatives were briefly discussed.

**Keywords:** TMP-flavonoid derivatives; anti-tumor; human tumor cell lines; HUVEC-12; fluorescence staining; flow cytometry

1. **Introduction**

As the world’s population ages and grows, the global burden of cancer continues to increase. Cancer is the leading cause of death and a major public health problem around the world [1,2]. Chemotherapy is commonly used for cancer treatment. In recent years, many chemotherapeutic drugs with high cytotoxicity have been discovered and used as first-line clinical drugs [3,4]. However, many anti-tumor drugs, such as cisplatin, paclitaxel and bleomycin [5–7], have serious side effects, including lethal hemoptysis and anemia [8,9]. The reasons are that the new tumor blood vessels were usually produced by an existing vascular system and considered to be the same as normal blood vessels. When drugs work, they have the same inhibition on normal endothelial cells (NECs) and tumor endothelial cells (TECs) [10–12]. Therefore, it is necessary to evaluate the effects of drugs on...
NECs while evaluating their anti-tumor activities. Compared with other drugs, Traditional Chinese Medicines (TCMs) have the advantages of lower toxicity and more targets. Therefore, it is possible to obtain high efficiency and low toxicity anti-tumor leading compounds by combining the anti-tumor ingredients from TCM.

In the clinic, TCMs with similar activities are often used together to realize synergistic effects. In the same way, it was possible to obtain more active compounds by synthesis of ingredients with similar effects [13,14]. TMP and flavonoids are two such ingredients [15]. Ligustrazine (TMP, 2,3,5,6-tetramethylpyrazine) is one of the active ingredients of Ligusticum Chuanxiong Hort, and it is widely used in occlusive cerebrovascular diseases. Previous studies have found that TMP and its derivatives could not only inhibit growth, invasion and metastasis of tumor cells, but also could reverse the multi-drug resistance and act as potential reversal drugs on P-gp [16–19]. It is widely used in the synthesis of leading compounds and a lot of compounds with significant effects have been obtained [20–22]. Flavonoids are a class of polyphenolic compounds widely distributed in the plant kingdom and abundantly consumed in the daily diet. They display potential therapeutic benefits in cancer treatment and may be considered as candidates for tumor therapeutic agents [23–25]. Flavonoids have been reported to be correlated to G1 and G2 cell cycle arrest and apoptosis promotion. Moreover, the flavonoids can reverse the multidrug resistance (MDR) of tumors and affect the expression and function of efflux transporters [26–28]. The nine flavonoids selected in this study are shown in Table 1. The main types of flavones were chosen, including flavones, flavonols, isoflavones and dihydroflavones.

Table 1. The structures of the nine flavonoid compounds.

| Classification | Flavonoids’ Structure          | Flavonoid Compounds (1–9) |
|----------------|--------------------------------|---------------------------|
| **Flavone**    |                                |                           |
|                | Luteolin (1)                   | 5, 7, 3', 4'—OH           |
|                | Baicalein (2)                  | 5, 6, 7—OH                |
|                | Apigenin (3)                   | 5, 7, 3'—OH               |
|                | Chrysin (4)                    | 5, 7—OH                   |
|                | Quercetin (5)                  | 5, 7, 3', 4'—OH           |
|                | Fisetin (6)                    | 7, 3', 4'—OH              |
| **Flavonol**   |                                |                           |
|                | Genistein (7)                  | 5, 7, 4'—OH               |
|                | Daidzein (8)                   | 7, 4'—OH                  |
| **Isoflavone** |                                |                           |
|                | Naringenin (9)                 | 5, 7, 4'—OH               |
In this study, we conjugated TMP and flavonoids, the main components of blood-activating drugs with anti-tumor activities, into the one molecule via ether bonds, constructing a number of new TMP-flavonoid derivatives. A variety of tumor cell lines were selected to evaluate the anti-tumor activities of these compounds, and human umbilical vein endothelial cells (HUVEC-12) as the NECs in vitro toxicity measurement model.

2. Results

2.1. Chemical Synthesis

As shown in Scheme 1, we first synthesized the intermediate TMP-Br via a free radical reaction. Then, TMP-Br was linked to flavonoids via ether bonds to obtain TMP-flavonoid derivatives. Finally, end-products were obtained by crystallization. All TMP-flavonoid derivatives were synthesized by adopting a similar alkaline catalyst strength, which should be strictly controlled, as the flavanone structure is unstable under strong alkaline conditions. The C-O bond of C ring of naringenin derivatives was disconnected to form a chalcone structure (compounds 14, 16). Treatment of TMP-Br with daidzein in dry acetone at 75 °C for 2 h, afforded compound 10. We had tried to synthesize more compounds, but the yield was too low. We could not obtain more compounds except these 16 derivatives (Table 2) and they are new compounds that had not been previously reported. All TMP-flavonoid derivatives were characterized by \(^{1}\)H-NMR, \(^{13}\)C-NMR and HRMS.

![Scheme 1. Synthesis routes to ligustrazine-flavonoid derivatives (10-25).](image)

| No. | Structure | No. | Structure |
|-----|-----------|-----|-----------|
| 10  | ![Structure 10](image) | 11 | ![Structure 11](image) |
| No. | Structure | No. | Structure |
|-----|-----------|-----|-----------|
| 12  | ![Image](image1.png) | 13 | ![Image](image2.png) |
| 14  | ![Image](image3.png) | 15 | ![Image](image4.png) |
| 16  | ![Image](image5.png) | 17 | ![Image](image6.png) |
| 18  | ![Image](image7.png) | 19 | ![Image](image8.png) |
| 20  | ![Image](image9.png) | 21 | ![Image](image10.png) |
| 22  | ![Image](image11.png) | 23 | ![Image](image12.png) |
Table 2. Cont.

| No. | Structure | No. | Structure |
|-----|-----------|-----|-----------|
| 24  | ![Structure 24](image) | 25  | ![Structure 25](image) |

2.2. Biological Activities

2.2.1. Cytotoxicity Assay Using Five Tumor Cell Lines

All the synthesized compounds were tested for their cytotoxicities on five tumor cell lines (HepG-2, Bel-7402, HT-29, MCF-7 and HeLa) using the standard MTT assay with DDP as positive control. The IC$_{50}$ values of these compounds are summarized in Table 3. After combination, the activity of most synthesized compounds had not been improved significantly compared to the parent flavonoids. Among them, compounds $14$ and $19$ displayed lower IC$_{50}$ values than the rest of compounds against Bel-7402, and MCF-7, respectively. As shown in Figure 1, the activity of compound $14$ was significantly better than that of naringenin on all cell lines. Furthermore, compound $14$ had the strongest inhibitory effect on Bel-7402 and slightly weaker than that of DDP. In the same way, the activity of compound $19$ was superior to that of daidzein, and was shown to be slightly weaker than that of DDP against MCF-7.

Table 3. IC$_{50}$ values of TMP-flavonoid derivatives and raw materials on HepG-2, Bel-7402, HT-29, MCF-7 and HeLa for 72 h. Data were expressed as inhibitory ratio ± SD based on three independent experiments ($n = 3$).

| Compound   | HepG-2 IC$_{50}$ (µM) | Bel-7402 IC$_{50}$ (µM) | HT-29 IC$_{50}$ (µM) | MCF-7 IC$_{50}$ (µM) | HeLa IC$_{50}$ (µM) |
|------------|------------------------|--------------------------|----------------------|----------------------|---------------------|
| Luteolin   | 11.83 ± 0.44           | 14.51 ± 0.68             | 19.24 ± 1.17         | 16.99 ± 0.31         | 21.28 ± 1.12        |
| Baicalein  | 20.35 ± 2.07           | 21.59 ± 3.68             | 19.20 ± 2.72         | 37.88 ± 1.52         | 22.76 ± 3.54        |
| Apigenin   | 16.11 ± 1.52           | 28.22 ± 0.38             | 23.44 ± 1.89         | 29.71 ± 1.43         | 30.58 ± 0.71        |
| Chrysin    | 15.56 ± 0.30           | 35.00 ± 1.46             | 30.31 ± 1.96         | 32.06 ± 2.78         | >40                 |
| Quercetin  | 13.57 ± 0.73           | 30.39 ± 2.51             | 27.00 ± 0.47         | >40                  | 20.83 ± 4.04        |
| Fisetin    | 17.39 ± 1.04           | 25.46 ± 1.54             | 23.88 ± 0.81         | 38.10 ± 1.83         | 20.88 ± 1.65        |
| Genistein  | 19.76 ± 1.31           | 34.66 ± 0.63             | 31.13 ± 0.26         | 35.62 ± 1.38         | 34.61 ± 2.52        |
| Daidzein   | 21.81 ± 1.53           | >40                      | >40                  | 36.82 ± 1.48         | >40                 |
| 10         | >40                    | >40                      | >40                  | >40                  | 26.67 ± 0.34        |
| 13         | 13.37 ± 2.50           | 26.13 ± 0.11             | 22.42 ± 0.35         | >40                  | 17.31 ± 1.07        |
| 14         | 17.31 ± 0.47           | 10.74 ± 1.12             | 31.88 ± 1.96         | 29.79 ± 2.18         | 25.11 ± 1.80        |
| 16         | 20.62 ± 1.39           | >40                      | 34.39 ± 2.32         | 16.54 ± 0.45         | 30.50 ± 2.62        |
| 17         | >40                    | >40                      | 10.67 ± 1.35         | >40                  | >40                 |
| 19         | 14.49 ± 0.48           | 28.87 ± 0.49             | 11.72 ± 1.29         | 10.43 ± 1.23         | 14.31 ± 1.17        |
| 20         | >40                    | 25.16 ± 1.72             | 10.90 ± 2.30         | >40                  | 16.48 ± 1.79        |
| DDP        | 4.14 ± 0.16            | 6.73 ± 0.37              | 5.83 ± 0.59          | 6.75 ± 0.57          | 4.76 ± 0.41         |

IC$_{50}$ values: when the IC$_{50}$ values of the compounds were higher than 40 for all cells, the groups of data were not listed in the table.

In addition, it was observed that different types of flavonoids could lead to different cytotoxicities. The activity of derivatives with flavones, isoflavones and chalcone as mother nucleus was better than other derivatives, such as compound $14$ (IC$_{50}$ was 17.31 ± 0.47 µM, 10.74 ± 1.12 µM, 31.88 ± 1.96 µM, 29.79 ± 2.18 µM, 25.11 ± 1.80 µM against HepG-2, Bel-7402, HT-29, MCF-7 and HeLa respectively)
and compound 19 (IC50 was 14.49 ± 0.48 µM, 28.87 ± 0.49 µM, 11.72 ± 1.29 µM, 10.43 ± 1.23 µM, 14.31 ± 1.17 µM against HepG-2, Bel-7402, HT-29, MCF-7 and HeLa respectively). Because of the limited number of derivatives, the relationships between the number and position of introduced TMP moieties and the activities of TMP-flavonoid derivatives need to be further explored.

![Figure 1](image_url)

**Figure 1.** The IC50 values against HepG-2, Bel-7402, HT-29, MCF-7, HeLa. (a) Naringenin, compound 14, DDP. (b) Daidzein, compound 19, DDP. **p < 0.001, ***p < 0.001, vs. Naringenin, Daidzein. The red mark indicated that the IC50 value of the compounds to the cells were closest to that of the DDP.

2.2.2. Cytotoxicity Assay Using HUVEC-12 Cell

In order to further investigate the effects of compounds with clear anti-tumor activities on NECs, MTT colorimetry was applied using human umbilical vein endothelial (HUVEC-12) cells. As shown in Table 4, when the concentration was 40 µM, compound 14 presented a little cytotoxicity. However, it showed a certain role in promoting cell proliferation and a concentration dependence at most concentrations. On the contrary, compound 19 appeared to have weak damaging effects and DDP had definite cytotoxic effects on cells (compounds 14 and 19 IC50 > 40 µM; DDP IC50 = 9.11 ± 0.54 µM) [29,30]. The results of compound 14 were consistent with expectations that the compounds had no effect on NECs. Therefore, compound 14 with the greater difference was selected for further mechanistic study.

### Table 4. Proliferation rates of TMP-flavonoid derivatives on HUVEC-12 for 24 h. Data were expressed as inhibitory ratio ± SD based on three independent experiments (n = 3).

| Compound | Proliferation Rate (%) | IC50 (µM) |
|----------|------------------------|-----------|
|          | 2.5 µM | 5 µM | 10 µM | 20 µM | 40 µM |          |
| 14       | 1.62 ± 0.75 | 1.99 ± 0.23 | 14.84 ± 1.41 | 16.71 ± 0.29 | −2.32 ± 1.37 | >40 |
| 19       | −8.75 ± 0.38 | −19.35 ± 0.35 | −19.49 ± 2.17 | −24.22 ± 3.19 | −25.07 ± 1.02 | >40 |
| DDP      | −23.93 ± 0.75 | −31.38 ± 1.46 | −58.24 ± 0.82 | −66.58 ± 1.75 | −82.69 ± 2.19 | 9.11 ± 0.54 |

2.2.3. Morphological Analysis Using Giemsa and DAPI Staining

To confirm the apoptotic morphological changes induced by compound 14, Bel-7402 cells were treated with 0, 10, 20 and 40 µM of compound 14 for 72 h and then Giemsa staining and DAPI staining were performed. As shown in Figure 2I, the number and morphological changes of Bel-7402 cells significantly increased with increasing concentrations of compound 14. When the dose reached 40 µM, there were mainly cell fragments rather than complete cells. In the same way, as we can see from Figure 2II, the cells treated with compound 14 showed morphological features of apoptosis, including a number decrease, nuclear fragmentation, cytoplasmic shrinkage, and the formation of apoptotic bodies with irregular shape while untreated cells displayed normal.
Figure 2. Gimesa (I) and DAPI (II) staining on Bel-7402 cells induced by compound 14 with different concentrations: (100×): (a) control group; (b) 10 µM; (c) 20 µM; (d) 40 µM. The cell morphology was observed under the fluorescence microscope. The most representative fields are shown.

2.2.4. Apoptosis Analysis by Flow Cytometric Using Annexin V-FITC/Propidium Iodide (PI) Staining

The effects of compound 14 on apoptosis of Bel-7402 cells were further determined by flow cytometric analysis. The cells were treated with compound 14 at four concentrations of 0, 5, 10, 20 µM and then stained with both annexin Annexin V-FITC and PI. The flow cytometry observed four quadrant images: necrotic (Q1; Annexin−/PI+), late apoptotic (Q2; Annexin+/PI+), intact (Q3; Annexin−/PI−), and early apoptotic (Q4; Annexin+/PI−) cells. The results are shown in Figure 3, where the apoptosis ratios (including the early and late apoptosis ratios) increased to 33.7% (5 µM), 45.5% (10 µM), 54.6% (20 µM) while that of control was 5.4%. Furthermore, the results indicated that compound 14 could induce Bel-7402 cells apoptosis in a concentration-dependent manner.

Figure 3. Apoptosis analysis of Bel-7402 cells induced by compound 14 using AnnexinV-FITC/PI staining: (a) control group; (b) 5 µM; (c) 10 µM; (d) 20 µM.
3. Discussion

In this study, although chalcone was not selected, compounds 14 and 16 with chalcone as the mother nucleus were obtained via synthesis. This was due to the fact the dihydroflavonones hydrolyzed back into chalcone under alkaline conditions [31]. In addition, we analyzed the effects of the TMP-flavonoid derivatives on five tumor cell lines. The flavonoids selected showed clear anti-tumor activity, especially luteolin (IC₅₀ was 11.83 ± 0.44 µM, 14.51 ± 0.68 µM, 19.24 ± 1.17 µM, 16.99 ± 0.31 µM, 21.28 ± 1.12 µM against HepG-2, Bel-7402, HT-29, MCF-7 and HeLa, respectively). The results confirmed the anti-tumor potential of luteolin in tumor therapy [32]. Most of the flavonoids had better inhibition on HepG-2 than other cell lines, which was consistent with previous studies [27,33]. After TMP was introduced, the selectivity of the derivatives changed. For example, compound 14 showed the strongest inhibitory effect on Bel-7402. Among all compounds, the compounds with isoflavones and chalcone as the mother nucleus had better anti-tumor activities than other compounds. Furthermore, the retention of 5, 7-OH could increase the inhibition of the compounds on tumor cells, such as compound 10 > 11, compound 17 > 18, compound 19 > 20. That is to say, isoflavones, chalcone and its derivatives with 5, 7-OH substituents have great potential in tumor therapy [26]. Since vascular diseases caused by anti-tumor drugs are common, it is necessary to consider the effect of antitumor drugs on normal blood vessels at the beginning of the design plan. In this study, we considered not only the inhibition of drugs on tumor cell lines, but also the effects of drugs on normal vascular cells. The design idea of this study was from the perspective of clinical application. While compound 14 had a definite inhibition on Bel-7402, it also had a little proliferation on HUVEC-12. The selectivity was not detected for other samples, including positive ones. The results of staining and flow cytometric also demonstrated that compound 14 promoted the apoptosis of Bel-7402. The results are consistent with previous studies [26]. The flavonoids selected have been shown to be correlated to a G1 and G2 cell cycle arrest [27,28], so it is worth performing further studies on the effect of TMP-flavonoid derivatives in cell cycle determination. Based on the above, compound 14 showed bright prospects and should be further studied.

4. Materials and Methods

4.1. Materials and Instruments

Ligustrazine, luteolin, baicalein, apigenin, chrysins, quercetin, fisetin, genistein, daidzein, naringenin, and DDP were purchased from Aladdin Bio-Chem Technology Co., Ltd., (Shanghai, China) and Alfa Aesar Chemical Co., Ltd. (Tianjin, China). The purity of all the materials was more than 98%. All reagents were used without any further purification. Reagents of analytical reagent grade were provided by Beijing Chemical Plant (Beijing, China). ¹H-NMR and ¹³C-NMR spectra were recorded on a DRX-500 spectrometer at 500/125 MHz (Bruker, Fällanden, Switzerland). ESI–MS were recorded on a Thermo Scientific TM LTQ Orbitrap XL hybrid FTMS instrument (Thermo Technologies, New York, NY, USA). Reactions were monitored by thin-layer chromatography (TLC) on precoated silica gel G-254 plates, which visualized in UV light (254 nm). Flash column chromatography was performed using 200–300 mesh silica gel (Qingdao Haiyang Chemical Co., Qingdao, China). Cellular morphologies were observed using an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan). The yields were calculated based on the last step reaction.

Fetal bovine serum (FBS) and RPMI 1640 (DMEM) medium, Penicillin and streptomycin were obtained from Thermo Technologies. Gimesa, 6-diamidino-2-phenylindole (DAPI) were obtained from Molecular Probes/Invitrogen Life Technologies (Carlsbad, CA, USA). Five kinds of tumor cell lines were provided by the Institute of Peking Union Medical College: Hepatocellular cancer cell lines (HepG-2, Bel-7402), human breast cancer cell lines (MCF-7), human colon cancer cells (HT-29), human cervical cancer cells (HeLa), human umbilical vein endothelial cells (HUVEC-12).
4.2. Chemical Syntheses

2-(Bromomethyl)-3,5,6-trimethylpyrazine (TMP-Br): TMP-3H$_2$O (0.26 mol) was dissolved in benzene (120 mL) and refluxed for 10 h. After concentrating the solution, white solids (TMP) were obtained. TMP (0.15 mol) was dissolved in carbon tetrachloride (100 mL) and NBS (0.12 mol) was added in portions. The mixture was refluxed and illuminated by four 60 W tungsten light bulbs. After 12 h, the brown liquid was filtered and dried in vacuum. A brown crude oily product was obtained (TMP-Br), which was not purified further, as it caused a strong mucous membrane irritation. Yield: 70%.

5-Hydroxy-2-phenyl-7-((3,5,6-trimethylpyrazin-2-yl)methoxy)-4H-chromen-4-one, (10). TMP-Br (2.81 mmol) and chrysine (1.97 mmol) were dissolved in dry DMF (30 mL), then K$_2$CO$_3$ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere, the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (10:1) as eluent and recrystallized from dichloromethane. White powder, m.p.: 156.2–157.1 °C, yield 58%. $^1$H-NMR (CDCl$_3$) (ppm): $\delta$ 12.72 (s, 1H), 7.88–7.87 (m, 2H), 6.73–7.52 (m, 2H), 6.66 (s, 1H), 5.67 (s, 1H), 5.22 (s, 2H), 2.53 (s, 6H), 2.47 (s, 3H); $^{13}$C-NMR (CDCl$_3$) (ppm) δ 182.61, 164.59, 164.18, 162.35, 157.83, 151.92, 150.09, 148.98, 144.71, 131.99, 131.42, 129.23, 126.42, 106.05, 99.07, 93.72, 70.47, 21.55, 21.33, 20.74. MS (ESI) m/z: [M + H]$^+$ 389.1510, calcd. for C$_{31}$H$_{30}$N$_2$O$_4$ 388.1423.

2-Phenyl-5,7-bis((3,5,6-trimethylpyrazin-2-yl)methoxy)-4H-chromen-4-one, (11). TMP-Br (5.63 mmol) and chrysine (1.97 mmol) were dissolved in dry DMF (30 mL), then K$_2$CO$_3$ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (10:1) as eluent and recrystallized from dichloromethane. White powder, m.p.: 225.3–226.2 °C, yield 60%. $^1$H-NMR (CDCl$_3$) (ppm) δ 7.83–7.81 (m, 2H), 7.47–7.46 (m, 3H), 6.718 (d, $J = 5.0$ Hz, 2H), 6.58 (s, 1H), 5.30 (s, 2H), 5.20 (s, 2H), 2.69 (s, 3H), 2.57 (s, 3H), 2.51 (s, 6H), 2.49 (s, 3H), 2.47 (s, 3H); $^{13}$C-NMR (CDCl$_3$) (ppm) δ 177.14, 162.73, 160.76, 159.75, 159.60, 151.94, 151.24, 150.88, 150.09, 149.02, 148.28, 145.37, 144.77, 131.72, 131.30, 129.07, 128.08, 110.09, 109.21, 98.65, 95.21, 71.30, 70.42, 21.87, 21.69, 21.57, 21.45, 20.82, 20.73. MS (ESI) m/z: [M + H]$^+$ 523.2359, calcd. for C$_{31}$H$_{30}$N$_2$O$_4$ 522.2267.

5-Hydroxy-2-phenyl-6,7-bis((3,5,6-trimethylpyrazin-2-yl)methoxy)-4H-chromen-4-one, (12). TMP-Br (5.29 mmol) and baicalein (1.85 mmol) were dissolved in dry DMF (30 mL), then K$_2$CO$_3$ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with petroleum ether-acetone (8:1) as eluent and recrystallized from dichloromethane. Yellow powder, m.p.: 185.4–186.9 °C, yield 65%. $^1$H-NMR (CDCl$_3$) (ppm) δ 8.03–7.83 (m, 2H), 7.59–7.45 (m, 3H), 6.81 (s, 1H), 6.67 (s, 1H), 5.25 (s, 2H), 5.14 (s, 2H), 2.61 (s, 3H), 2.57 (s, 3H), 2.53 (s, 6H), 2.49 (s, 3H), 2.45 (s, 3H); $^{13}$C-NMR (CDCl$_3$) (ppm) δ 182.83, 164.16, 158.21, 153.89, 153.54, 151.94, 150.87, 150.54, 150.34, 148.81, 148.56, 146.40, 144.56, 132.00, 131.66, 131.47, 129.27, 126.40, 106.70, 105.83, 92.34, 74.26, 71.03, 21.91, 21.73, 21.54, 21.46, 20.83, 20.50. MS (ESI) m/z: [M + H]$^+$ 539.2303, calcd. for C$_{31}$H$_{30}$N$_2$O$_4$ 538.2216.

2-Phenyl-5,6,7-tris((3,5,6-trimethylpyrazin-2-yl)methoxy)-4H-chromen-4-one, (13). TMP-Br (7.93 mmol) and baicalein (1.85 mmol) were dissolved in dry DMF (30 mL), then K$_2$CO$_3$ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated in vacuum. The product was separated by flash chromatography with petroleum ether-acetone (8:1) as eluent and recrystallized from dichloromethane. White powder, m.p.: 131.73, 131.46, 129.16, 126.13, 113.69, 108.54.
(E)-1-(2-Hydroxy-4,6-bis((3,5,6-trimethylpyrazin-2-yl)methoxy)phenyl)-3-(4-((3,5,6-trimethylpyrazin-2-yl)methoxy)phenyl)prop-2-en-1-one (14). TMP-Br (7.88 mmol) and naringenin (1.84 mmol) were dissolved in dry DMSO (30 mL), then K$_2$CO$_3$ (7.24 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (10:1) as eluent and recrystallized from dichloromethane. Yellow powder, mp: 176.4–177.6 °C. White powder, mp: 134.6–135.4 °C.

5,7-bis((3,5,6-Trimethylpyrazin-2-yl)methoxy)-2-(4-((3,5,6-trimethylpyrazin-2-yl)methoxy)phenyl)chroman-4-one (15), TMP-Br (7.88 mmol) and naringenin (1.84 mmol) were dissolved in dry DMSO (30 mL), then K$_2$CO$_3$ (14.49 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (10:1) as eluent and recrystallized from dichloromethane. White powder, mp: 128.1–129.1 °C.

(E)-3-(4-((3,5,6-Trimethylpyrazin-2-yl)methoxy)phenyl)-1-(2,4,6-tris((3,5,6-trimethylpyrazin-2-yl)-methoxy)phenyl)prop-2-en-1-one (16). TMP-Br (10.51 mmol) and naringenin (1.84 mmol) were dissolved in dry DMSO (30 mL), then K$_2$CO$_3$ (14.49 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (10:1) as eluent and recrystallized from dichloromethane. Yellow powder, mp: 61.6–62.3 °C.

5-Hydroxy-7-((3,5,6-Trimethylpyrazin-2-yl)methoxy)-3-((3,5,6-Trimethylpyrazin-2-yl)methoxy)-4H-chromen-4-one (17). TMP-Br (5.29 mmol) and genistein (1.85 mmol) were dissolved in dry DMSO (30 mL), then K$_2$CO$_3$ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (10:1) as eluent and recrystallized from dichloromethane. White powder, mp: 148.94, 148.76, 148.74, 148.64, 148.62, 148.56, 98.39, 76.27, 75.13, 70.92, 21.92, 21.73, 21.53, 21.41, 20.77, 20.36. MS (ESI) m/z: [M + H]$^+$ 673.3163, calcd. for C$_{39}$H$_{40}$N$_{4}$O$_{5}$ 672.3060.
69.99, 40.15, 28.35, 21.71, 21.60, 21.43, 20.57. MS (ESI) m/z: [M + H]⁺ 539.2296, calcd. for C₃₁H₃₀N₄O₅ 538.2216.

5,7-bis(3,5,6-Trimethylpyrazin-2-yl)methoxy)-3-(4-((3,5,6-trimethylpyrazin-2-yl)methoxy)phenyl)-4H-chromen-4-one (18). TMP-Br (7.93 mmol) and genistein (1.85 mmol) were dissolved in dry DMF (30 mL), then K₂CO₃ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated in vacuum. The product was separated by flash chromatography with dichloromethane-acetone (10:1) as eluent and recrystallized from dichloromethane. White powder, m.p.: 188.2–189.1 °C, yield 53%. ¹H-NMR (CDCl₃) (ppm) δ 7.74 (s, 1H), 7.43 (d, J = 8.7 Hz, 2H), 7.05 (d, J = 8.7 Hz, 2H), 6.77 (d, J = 2.1 Hz, 1H), 6.62 (d, J = 2.1 Hz, 1H), 5.31 (s, 2H), 5.23 (s, 2H), 5.18 (s, 2H), 2.67 (s, 3H), 2.61 (s, 4H), 2.60 (s, 3H), 2.56 (s, 7H), 2.54 (s, 6H), 2.51 (s, 3H), 2.50 (s, 3H); ¹³C-NMR (CDCl₃) (ppm) δ 174.88, 162.41, 160.09, 159.65, 158.44, 151.82, 151.21, 150.89, 150.05, 149.97, 148.88, 148.60, 148.08, 145.73, 145.16, 144.63, 130.39, 126.00, 124.91, 115.42, 114.76, 110.50, 98.36, 94.72, 71.20, 70.28, 69.97, 21.76, 21.66, 21.62, 21.45, 21.42, 21.33, 20.85, 20.61. MS (ESI) m/z: [M + H]⁺ 673.3164, calcd. for C₉₉H₆₈N₆O₇ 672.3060.

7-Hydroxy-3-(4-((3,5,6-trimethylpyrazin-2-yl)methoxy)phenyl)-4H-chromen-4-one (19). TMP-Br (2.81 mmol) and daidzein (1.97 mmol) were dissolved in dry acetone (30 mL), then K₂CO₃ (10.87 mmol) was added in portions. The mixture was refluxed for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with petroleum ether-acetone (10:1) as eluent and recrystallized from dichloromethane. White powder, m.p.: 213.1–214.2 °C, yield 51%. ¹H-NMR (DMSO-d₆) (ppm) δ 9.52 (s, 1H), 8.33 (s, 1H), 7.98 (d, J = 8.9 Hz, 1H), 7.35 (d, J = 8.5 Hz, 2H), 7.09 (dd, J = 8.9, 2.3 Hz, 1H), 6.77 (d, J = 8.6 Hz, 2H), 5.27 (s, 2H), 2.46 (s, 3H), 2.40 (s, 6H); ¹³C-NMR (DMSO-d₆) (ppm) δ 175.15, 163.08, 157.72, 153.67, 151.81, 149.86, 148.94, 145.00, 130.54, 127.48, 124.19, 122.80, 118.35, 115.44, 114.92, 102.06, 70.38, 21.76, 21.46, 20.62. MS (ESI) m/z: [M + H]⁺ 389.1506, calcd. for C₂₃H₂₀N₂O₄ 388.1423.

7-(3,5,6-Trimethylpyrazin-2-yl)methoxy)-3-(4-(3,5,6-trimethylpyrazin-2-yl)methoxy)phenyl)-4H-chromen-4-one (20). TMP-Br (5.63 mmol) and daidzein (1.97 mmol) were dissolved in dry DMF (30 mL), then K₂CO₃ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with petroleum ether-acetone (10:1) as eluent and recrystallized from dichloromethane. White solid, m.p.: 185.4–186.9 °C, yield 62%. ¹H NMR (CDCl₃) (ppm) δ 8.223 (d, J = 9 Hz, 1H), 7.94 (s, 1H), 7.51 (d, J = 8.5 Hz, 2H), 7.09 (dd, J = 8.9, 2.3 Hz, 1H), 6.77 (d, J = 8.6 Hz, 2H), 5.27 (s, 2H), 5.21 (s, 2H), 2.62 (s, 3H), 2.61 (s, 3H), 2.56 (s, 6H), 2.55 (s, 6H); ¹³C NMR (CDCl₃) (ppm) δ 175.77, 162.80, 158.57, 157.77, 152.16, 151.83, 151.25, 149.98, 148.90, 148.68, 145.70, 144.61, 130.16, 127.91, 124.84, 124.71, 118.75, 114.93, 114.88, 101.45, 70.38, 69.98, 21.76, 21.66, 21.45, 21.44, 20.62. MS (ESI) m/z: [M + H]⁺ 523.2351, calcd. for C₃₁H₃₀N₄O₄ 522.2267.

2-(3,4-Dihydroxyphenyl)-3,5,7-tris(3,5,6-trimethylpyrazin-2-yl)methoxy)-4H-chromen-4-one (21). TMP-Br (7.09 mmol) and quercetin (1.66 mmol) were dissolved in dry DMF (30 mL), then K₂CO₃ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (15:1) as eluent and recrystallized from dichloromethane. Yellow powder, m.p.: 121.2–122.3 °C, yield 65%. ¹H-NMR (CDCl₃) (ppm) δ 12.63 (s, 1H), 7.48 (s, 1H), 7.44 (d, J = 8.5 Hz, 1H), 7.05 (d, J = 8.5 Hz, 1H), 6.53 (d, J = 1.6 Hz, 1H), 6.45 (d, J = 1.5 Hz, 1H), 5.21 (s, 2H), 5.19 (s, 2H), 5.18 (s, 2H), 2.58 (s, 3H), 2.57 (s, 3H), 2.56 (s, 3H), 2.52 (s, 9H), 2.51 (s, 3H), 2.42 (s, 3H), 2.40 (s, 3H); ¹³C-NMR (CDCl₃) (ppm) δ 178.92, 164.45, 162.18, 156.94, 156.85, 151.92, 151.87, 151.03, 149.95, 149.49, 149.02, 148.82, 148.64, 148.35, 148.24, 145.45, 145.13, 144.73, 137.59, 126.04, 121.31, 117.19, 116.91, 106.50, 98.73, 93.23, 73.15, 72.00, 70.38, 21.85, 21.68, 21.55, 21.37, 21.06, 20.71, 20.59, 20.34. MS (ESI) m/z: [M + H]⁺ 705.3043, calcd. for C₃₉H₄₀N₆O₇ 704.2958.
2-(3,4-bis(3,5,6-Trimethylpyrazin-2-yl)methoxy)phenyl)-3,7-bis((3,5,6-trimethylpyrazin-2-yl)-methoxy)-4H-chromen-4-one (22). TMP-Br (11.83 mmol) and quercetin (1.66 mmol) were dissolved in dry DMF (30 mL), then K₂CO₃ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (15:1) as eluent and recrystallized from dichloromethane. White powder, m.p.: 179.4–180.1 °C, yield 67%.

1H-NMR (CDCl₃) (ppm) δ 7.75 (s, 1H), 7.63 (dd, J = 8.6, 1.7 Hz, 1H), 7.08 (d, J = 8.7 Hz, 1H), 6.74 (s, 1H), 6.70 (d, J = 1.8 Hz, 1H), 5.37 (s, 2H), 5.27 (s, 2H), 5.24 (s, 2H), 5.22 (s, 2H), 5.01 (s, 2H), 2.74 (s, 3H), 2.65 (s, 3H), 2.61 (s, 3H), 2.55 (s, 4H), 2.52 (s, 15H), 2.50 (s, 3H), 2.39 (s, 3H), 2.32 (s, 3H); 13C-NMR (CDCl₃) (ppm) δ 173.50, 162.49, 159.57, 158.53, 153.02, 151.84, 151.31, 151.18, 151.12, 150.77, 150.29, 150.18, 150.07, 149.98, 148.88, 148.49, 148.11, 148.02, 145.98, 145.54, 145.35, 145.19, 144.61, 139.67, 123.86, 122.56, 114.44, 113.34, 110.02, 98.20, 94.63, 72.83, 71.24, 71.05, 70.91, 70.32, 21.78, 21.71, 21.68, 21.52, 21.47, 21.39, 21.37, 21.29, 20.86, 20.73, 20.63, 20.57. MS (ESI) m/z: [M + H]+ 973.4751, calcd. for C₅₅H₄₀N₁₀O₇ 972.4646.

5.7-bis(3,5,6-Trimethylpyrazin-2-yl)methoxy)-2-(4-(3,5,6-trimethylpyrazin-2-yl)methoxy)phenyl)-4H-chromen-4-one (24). TMP-Br (7.93 mmol) and apigenin (1.85 mmol) were dissolved in dry DMF (30 mL), then K₂CO₃ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (10:1) as eluent and recrystallized from dichloromethane. Yellow powder, m.p.: 163.8–164.7 °C, yield 55%.

1H-NMR (CDCl₃) (ppm) δ 12.79 (s, 1H), 7.66 (d, J = 1.9 Hz, 1H), 7.49 (dd, J = 8.5, 2.0 Hz, 1H), 7.17 (d, J = 8.6 Hz, 1H), 6.63 (d, J = 2.1 Hz, 1H), 6.56 (s, 1H), 6.46 (d, J = 2.1 Hz, 1H), 5.28 (s, 2H), 5.26 (s, 2H), 5.22 (s, 2H), 2.59 (s, 3H), 2.57 (s, 3H), 2.57 (s, 3H), 2.53 (s, 9H), 2.51 (s, 3H), 2.51 (s, 3H), 2.50 (s, 3H); 13C-NMR (CDCl₃) (ppm) δ 182.50, 164.45, 163.90, 162.35, 157.72, 151.97, 151.88, 151.83, 151.63, 151.56, 150.36, 150.26, 150.08, 149.00, 148.74, 145.42, 145.18, 144.78, 124.32, 120.58, 114.00, 112.64, 106.02, 104.90, 98.98, 93.65, 71.36, 71.04, 70.48, 21.84, 21.56, 21.53, 20.74, 20.70. MS (ESI) m/z: [M + H]+ 689.3098, calcd. for C₃₉H₄₀N₁₀O₈ 688.3009.

2-(3,4-bis(3,5,6-Trimethylpyrazin-2-yl)methoxy)phenyl)-3,7-bis((3,5,6-trimethylpyrazin-2-yl)-methoxy)-4H-chromen-4-one (25). TMP-Br (9.99 mmol) and fisetin (1.75 mmol) were dissolved in dry DMF (30 mL), then K₂CO₃ (10.87 mmol) was added in portions. The mixture was refluxed and kept at 75 °C for 2 h under a nitrogen atmosphere; the brown solution was filtered and concentrated under vacuum. The product was separated by flash chromatography with dichloromethane-acetone (10:1) as eluent and recrystallized from dichloromethane. White powder, m.p.: 162.7–163.1 °C, yield 48%. 1H-NMR (CDCl₃) (ppm) δ 8.17 (d, J = 8.6 Hz, 1H), 7.81 (s, 1H), 7.69 (d, J = 8.6 Hz, 1H), 7.11 (s, 2H), 7.05 (s, 1H), 5.29 (s, 2H), 5.27 (s, 2H), 5.22 (s, 2H), 2.68 (s, 3H), 2.61 (s, 3H), 2.53 (s, 9H), 2.51 (s, 12H), 2.48 (s, 3H), 2.41 (s, 3H), 2.33 (s, 3H); 13C-NMR (CDCl₃) (ppm) δ 174.55, 163.03, 156.86, 155.57, 151.97, 151.45, 151.25, 151.05, 150.75, 150.28, 150.18, 150.12, 149.03, 148.68, 148.24, 145.97, 145.70, 145.46, 144.74, 139.42, 127.34,
124.11, 123.02, 121.32, 118.52, 117.92, 116.91, 114.88, 114.82, 113.54, 101.45, 72.99, 71.27, 71.04, 70.54, 21.88, 21.81, 21.77, 21.64, 21.57, 21.51, 21.43, 20.74, 20.68. MS (ESI) m/z: [M + H]+ 823.3950, calcd. for C_{47}H_{50}N_{8}O_{8} 822.3853.

4.3. Bio-Evaluation Methods

4.3.1. Drugs

Ligustrazine, luteolin, baicalein, apigenin, chrysin, quercetin, fisetin, genistein, daidzein, naringenin, DDP and TMP-flavonoid derivatives (compounds 10–25). All samples were dissolved in DMSO and prepared to 10^4 µM. The final DMSO concentration did not exceed 0.1% (V/V).

4.3.2. Cell Culture

Five kinds of tumor cell lines and one normal endothelial cell were routinely cultured respectively in RPMI 1640 medium supplemented with 10% (V/V) FBS and 1% (V/V) penicillin-streptomycin, incubated at 37 °C in a 5% CO_2 containing incubator.

4.3.3. Cytotoxicity Assay Using Five Tumor Cell Lines

The five tumor cell lines were plated onto 96-well sterile plates in 100 µL/well of medium at a density of 3.5 × 10^3 cells per well and incubated at 37 °C with 5% CO_2 for 24 h. Then luteolin, baicalein, apigenin, chrysin, quercetin, fisetin, genistein, daidzein, naringenin, DDP and compounds 10–25 were added at various concentrations (5, 10, 20, 40, 50 µM). Each plate contained control group, blank group, drug group and positive group (DDP). After 72 h, 20 µL MTT in phosphate buffered saline (PBS, 5 mg/mL) was added to each well and the plates were incubated at 37 °C for 4 h, then removing the liquid and adding dimethyl sulfoxide (DMSO, 100 µL) to dissolve the MTT formazan. The optical density (OD) for each well was measured on a BIORAD 550 spectrophotometer plate reader (Bio-Gene Technology Ltd., Guangzhou, China) at a wavelength of 490 nm. All tests were carried out three times in parallel. The proliferation inhibition rates of tumor cells were calculated by {1 − [OD_{490} (Drug group) − OD_{490} (Blank group)]/[OD_{490} (Control group) − OD_{490} (Blank group)]} × 100%; the IC_{50} values were defined as the concentration of compounds that produced a 50% proliferation inhibition of surviving cells and calculated using the following equation: logIC_{50} = X_m − log2 × (ΣP − 0.5), Where X_m = logC_{max}, ΣP = sum of proliferation inhibition rates, the number 0.5 is an empirical constant. We first tested the activities of TMP-flavonoid derivatives with concentrations of 10 and 20 µM respectively. When compounds’ inhibition rate was not beyond 35% at a concentration of 20 µM, the compounds would not be considered to study further.

4.3.4. Cytotoxicity Assay Using HUVEC-12 Cell

The HUVEC-12 cell were seeded in 96-well plates (6.0 × 10^3 cells/ well), and incubated at 37 °C in a 5% CO_2 containing incubator. After 24 h, the medium was replaced with fresh medium containing the compounds to be tested. Compounds 14, 19 and DDP were added in final concentrations ranging from 2.5 to 40 µM. Each plate contained control group, blank group and drug group. After 24 h, 20 µL MTT was added to each well and the plates were incubated at 37 °C for 4 h, then removing the liquid and adding dimethyl sulfoxide (DMSO) (100 µL) to dissolve the MTT formazan. The optical density (OD) for each well was measured on a BIORAD 550 spectrophotometer plate reader at a wavelength of 490 nm. All tests were carried out three times in parallel. The proliferation inhibition rates of tumor cells were calculated by {1 − [OD_{490} (Drug group) − OD_{490} (Blank group)]/[OD_{490} (Control group) − OD_{490} (Blank group)]} × 100%.

4.3.5. Morphological Analysis Using Giemsa and DAPI Staining

The Bel-7402 cells were plated onto 12-well sterile plates at a density of 2.4 × 10^4 cells per well and incubated at 37 °C in a 5% CO_2 containing incubator. Then the cells were incubated in the presence
of compound 14 at various concentrations (0, 10, 20, 40 µM) for 72 h. Then cells were washed with PBS twice, fixed with cold ethanol and 4% paraformaldehyde (pH = 7.4) for 10 min respectively and washed with PBS again. Then fixed cells were stained with 6% Giemsa solution or DAPI at the concentration of 1 mg/mL for 5 min in the dark. Finally, the cells washed with water and dried. The cell morphological changes were observed by fluorescent microscopy and images were captured by digital camera [34,35].

4.3.6. Apoptosis Analysis by Flow Cytometric Using Annexin V-FITC/Propidium Iodide (PI) Staining

The Bel-7402 cells were plated onto 6-well sterile plates (4.8 × 10^4 cells/well) and placed at 37 °C with 5% CO_2 for 24 h. Then compound 14 at various concentrations (0, 5, 10, 20 µM) was added. After 72 h, all cells were collected respectively with the right amount of trypsin (without EDTA) digestion. Then the cells were washed with cold PBS twice and centrifuged at 1000 rpm for 5 min. The harvested cells were resuspended in 200 µL binding buffer, which contained 10 µL Annexin V-FITC and PI. After avoided light reaction for 15 min, the cells were analyzed with a flow cytometer [36].

4.4. Statistical Analysis

All data were expressed as the means ± standard deviation (SD) of three replications. The statistical analysis was performed by SPSS software (Version 20.0, International Business Machines Corp. New York, NY, USA) to analyze the variance. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered significant.

5. Conclusions

Using TMP and flavonoids as starting materials, we have designed and synthesized sixteen novel TMP-flavonoid derivatives through conjugation of anti-tumor bioactive compounds via ether bonds. Among them, compounds 14 and 16 were obtained via dihydroflavone hydrolysis to chalcones under alkaline conditions. As the flavonoids and TMP showed broad anti-tumor activities, we chose five different human tumor cell lines to evaluate the TMP-flavonoid derivatives. In vitro chemosensitivity testing showed that most of compounds had certain anti-tumor activity against the HeLa, MCF-7, HT-29, HepG-2, Bel-7402 cell lines. Part of derivatives showed better anti-tumor activities than the raw materials. Besides, compounds 14 and 19 exhibited activities close to that of the positive control (DDP) against the Bel-7402 and MCF-7 cell lines, respectively (IC_{50} = 10.49 ± 1.12, 10.43 ± 1.23 µM; DDP IC_{50} = 6.73 ± 0.37, 6.75 ± 0.57 µM). Then, the effects of compounds 14 and 19 on NECs were determined using HUVEC-12 cells. Compound 14 showed pro-proliferation effects while compound 19 appeared to cause minor damage on cells and DDP had similar cytotoxic effects on HUVEC-12 and tumor cells (compounds 14 and 19 IC_{50} > 40 µM; DDP IC_{50} = 9.11 ± 0.54 µM). Subsequently, the results of fluorescence staining and flow cytometry analysis indicated that compound 14 could induce apoptosis in the Bel-7402 cell line. All results suggested that compound 14 has bright prospects. In addition, the discussion of structure-activity relationships indicated that isoflavones, chalcone and its derivatives with 5, 7-OH substituents have great potential on tumor therapy. Moreover, the attempt to apply structure combination to discover more efficient and multi-effective anti-tumor leading compounds from TCM formulations is viable.

Author Contributions: H.W., T.M., P.W. and H.L. conceived and designed the experiments and discussed the data; H.W. and G.W. performed chemistry experiment; H.W., N.X. and H.C. performed biological activity experiments; X.Z., W.G., Y.C. and F.Z. analyzed the pharmacological data, M.C., K.F. elaborated the cell morphology and conduct data analysis and statistics; H.W., W.Z. wrote the paper and modified the language of the paper. All authors read and approved the final manuscript.

Acknowledgments: This study was financially supported by the National Natural Science Foundation of China (No. 81173519 and 81603256), supported by the Fundamental Research Funds for the Central Universities project of “acid-base complexation” and “structure combination” principle to discover lead compounds from Chinese traditional Chinese medicine (BUCM, 2018–2019), Beijing Key Laboratory for Basic and Development Research on Chinese Medicine (Beijing, 100102), the Fundamental Research Funds for the Central Universities
Conflicts of Interest: The authors declare no conflict of interest.

References
1. Chen, W.; Zheng, R.; Baade, P.D.; Zhang, S.W.; Zeng, H.M.; Bray, F.; Jemal, A.; Yu, X.Q. Cancer statistics in China, 2015. CA Cancer J. Clin. 2016, 66, 115–132. [CrossRef] [PubMed]
2. Jemal, A.; Bray, F.; Center, M.M.; Ferlay, J.J.; Ward, Z.; Forman, D. Global cancer statistics. CA Cancer J. Clin. 2011, 65, 69–90. [CrossRef] [PubMed]
3. Guo, C.Y.; Ma, X.J.; Liu, Q.; Yin, H.J.; Shi, D.Z. Effect of chinese herbal drug-containing serum for activating blood, activating blood and dispelling toxin on tnf-alpha-induced adherence between endothelial cells and neutrophils and the expression of mapk pathway. Chin. J. Integr. Med. 2015, 35, 204–209.
4. Tao, L.; Wang, S.; Zhao, Y.; Wang, A.Y.; Zhang, L.; Ruan, J.S.; Fan, F.T.; Liu, Y.P.; Li, Y.; Yue, Z.Q.; et al. Pleiotropic effects of herbs characterized with blood-activating and stasis-resolving functions on angiogenesis. Chin. J. Integr. Med. 2016, 22, 1–6. [CrossRef] [PubMed]
5. Folkman, J. Angiogenesis: An organizing principle for drug discovery? Nat. Rev. Drug Discov. 2007, 6, 273. [CrossRef] [PubMed]
6. Groselj, A.; Kranjc, S.; Bosnjak, M.; Krzan, M.; Kosjek, T.; Prevc, A.; Cemazar, M.; Sersa, G. Vascularization of the tumours affects the pharmacokinetics of bleomycin and the effectiveness of electrochemotherapy. Basic Clin. Pharmacol. Toxicol. 2018. [CrossRef] [PubMed]
7. Keedy, V.L.; Sandler, A.B. Inhibition of angiogenesis in the treatment of non-small cell lung cancer. Cancer Sci. 2007, 98, 1825–1830. [CrossRef] [PubMed]
8. Saif, M.W.; Elfiky, A.; Salem, R.R. Gastrointestinal perforation due to bevacizumab in colorectal cancer. Ann. Surg. Oncol. 2007, 14, 1860–1869. [CrossRef] [PubMed]
9. Papetti, M.; Herman, I.M. Mechanisms of normal and tumor-derived angiogenesis. Am. J. Physiol. Cell. Physiol. 2002, 282, 947–970. [CrossRef] [PubMed]
10. Akiyama, K.; Maishi, N.; Ohga, N.; Hida, Y.; Ohba, Y.; Alam, M.T.; Taisuke, K.; Hitomi, O.; Kenji, Y.; Chisaho, T.; et al. Inhibition of multidrug transporter in tumor endothelial cells enhances antiangiogenic effects of low-dose metronomic paclitaxel. Am. J. Pathol. 2015, 185, 572–580. [CrossRef] [PubMed]
11. Hida, K.; Maishi, N.; Torii, C.; Hida, Y. Tumor angiogenesis—Characteristics of tumor endothelial cells. Int. J. Clin. Oncol. 2016, 21, 206–212. [CrossRef] [PubMed]
12. Petrik, J.; Lawler, J.; Kortenaar, S.T. Anti-angiogenic therapy and induction of blood vessel normalization in the treatment of ovarian cancer. Curr. Ang. 2014, 3, 203–214.
13. Ren, S.Q.; Yang, J.Z.; Yang, S.M.; Song, Y.Z.; Cao, D.Y. Bacteriostatic activity of combined application of gallic acid, scutellaria baicalensis and rhizoma coptidis against mrsa in vitro. Chin. Pharm. 2010, 21, 198–199.
14. Zhang, C.Z.; Zhao, R.; Yan, W.Q.; Wang, H.; Jia, M.L.; Zhu, N.L.; Zhu, Y.D.; Zhang, Y.Z.; Wang, P.L.; Lei, H.M. Compositions, formation mechanism, and neuroprotective effect of compound precipitation from the traditional chinese prescription huang-lian-jiu-du-tang. Chin. J. Integr. Med. 2003, 28, 295–298.
15. Yang, X.G.; Jiang, C. Ligustrazine as a salvage agent for patients with relapsed or refractory non-hodgkin’s lymphoma. Chin. Med. J. 2010, 123, 3206–3211. [PubMed]
16. Cui, C.; Wang, R.T.; Zhang, Y.H.; Zhi, G.C.; Wang, Z.H.; Deng, Y.Q.; Zhang, Z.Z. Study on the down-regulatory effects of ligustrazine hydrochloride on tumor-induced immunosuppression by Colon26 tumor cells in vitro. Chin. J. Immunol. 2009, 25, 413–416.
20. Zou, J.; Gao, P.; Hao, X.; Xu, H.; Zhan, P.; Liu, X. Recent progress in the structural modification and pharmacological activities of ligustrazine derivatives. *Eur. J. Med. Chem.* 2018, 147, 150–162. [CrossRef] [PubMed]

21. Yong, A.; Zhu, B.; Ren, C.; Kang, F.H.; Li, J.L.; Huang, Z.J.; Lai, Y.S.; Peng, S.X.; Ding, K.; Tian, J.; et al. Discovery of new monocarbonyl ligustrazine-curcumin hybrids for intervention of drug-sensitive and drug-resistant lung cancer. *J. Med. Chem.* 2016, 59, 1747–1760. [CrossRef] [PubMed]

22. Qin, H.L.; Leng, J.; Zhang, C.P.; Jantan, I.; Amjad, M.W.; Sher, M.; Naem-Ul-Hassan, M.; Hussain, M.A.; Bukhari, S.N. Synthesis of α, β-unsaturated carbonyl-based compounds, oxime and oxime ether analogs as potential anticancer agents for overcoming cancer multidrug resistance by modulation of efflux pumps in tumor cells. *J. Med. Chem.* 2016, 59, 3549. [CrossRef] [PubMed]

23. Chang, H.; Xie, Q.; Zhang, Q.Y.; Peng, X.L.; Zhu, J.D.; Mi, M.T. Flavonoids, flavonoid subclasses and breast cancer risk: A meta-analysis of epidemiologic studies. *PLoS ONE* 2013, 8, e54318.

24. Guerrero, L.; Castillo, J.; Quiñones, M.; García-Vallvé, S.; Arola, L.; Pujadas, G.; Muguerza, B. Inhibition of Angiotensin-converting enzyme activity by flavonoids: Structure-activity relationship studies. *PLoS ONE* 2012, 7, e49493. [CrossRef] [PubMed]

25. Casagrande, F.; Darbon, J.M. Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: Regulation of cyclin-dependent kinases CDK2 and CDK1. *Biochem. Pharmacol.* 2001, 61, 1205–1215. [CrossRef]

26. Shi, X.F.; Liu, D.Y.; Zhang, J.M.; Hu, P.B.; Shen, W.; Fan, B.; Ma, Q.H.; Wang, X.D. Extraction and purification of total flavonoids from pine needles of cedrus deodara contribute to anti-tumor in vitro. *BMC Complement. Altern. Med.* 2016, 16, 245. [CrossRef] [PubMed]

27. Han, J.H.; Zhu, S.Q.; Xie, Y. Mechanism of flavonoids against p-glycoprotein mediated tumor multidrug resistance: Research advances. *J. Int. Pharm. Res.* 2016, 43, 818–823.

28. Haddad, A.Q.; Venkateswaran, V.; Viswanathan, L.; Teahan, S.J.; Fleshner, N.E.; Klotz, L.H. Novel anti proliferative flavonoids induce cell cycle arrest in human prostate cancer cell lines. *Prostate Cancer Prostatic Dis.* 2006, 9, 68–76. [CrossRef] [PubMed]

29. Zhao, Y.; Zhao, K.; Jiang, K.; Tao, S.S.; Li, Y.X.; Chen, W.W.; Kou, S.M.; Gu, C.R.; Li, Z.R.; Guo, L.S.; et al. A review of flavonoids from cassia species and their biological activity. *Curr. Pharm. Biotechnol.* 2016, 17, 1134–1146. [CrossRef] [PubMed]

30. Sun, J.; Shao, J.; Sun, C.; Song, Y.; Li, Q.; Lu, L.; Hu, Y.F.; Gui, C.; Zhang, H.; Ju, J.H. Borrelidins F-I, cytotoxic and cell migration inhibiting agents from mangrove-derived streptomyces rochei SCSIO ZJ89. *Bioorg. Med. Chem.* 2018, 26, 8. [CrossRef] [PubMed]

31. Nakatsuka, A.; Izumi, Y.; Yamagishi, M. Spatial and temporal expression of chalcone synthase and dihydroflavonol 4-reductase genes in the asiatic hybrid lily. *Plant Sci.* 2003, 165, 759–767. [CrossRef]

32. Shi, R. Anti-Tumor Mechanisms of Luteolin, a Major Flavonoid of Chrysanthemum Morifolium. Ph.D. Thesis, National University of Singapore, Singapore, 2006.

33. Pótor, M.; Zrínyi, Z.; Köszegi, T. Structure related effects of flavonoid aglycones on cell cycle progression of HepG2 cells: Metabolic activation of fisetin and quercetin by catechol-O-methyltransferase (COMT). *Biomed. Pharm.* 2016, 83, 998–1005. [CrossRef] [PubMed]

34. Chu, F.H.; Xu, X.; Li, G.L.; Gu, S.; Xu, K.; Gong, Y.; Xu, B.; Wang, M.N.; Zhang, H.Z.; Zhang, Y.Z.; et al. Amino acid derivatives of ligustrazine-oleanolic acid as new cytotoxic agents. *Molecules* 2014, 19, 18215–18231. [CrossRef] [PubMed]

35. Guo, W.B.; Yan, M.N.; Xu, B.; Chu, F.H.; Wang, W.; Zhang, C.Z.; Jia, X.H.; Han, Y.T.; Xiang, H.J.; Zhang, Y.Z.; et al. Design, synthesis, and biological evaluation of the novel glycyrrhetinic acid-cinnamoyl hybrids as anti-tumor agents. *Chem. Cent. J.* 2016, 10, 78. [CrossRef]

36. Chen, S.; Cheng, A.C.; Wang, M.S.; Peng, X. Detection of apoptosis induced by new type gosling viral enteritis virus in vitro through fluorescein annexin V FITC/PI double labeling. *World J. Gastroenterol.* 2008, 14, 2174–2178. [CrossRef] [PubMed]

**Sample Availability:** Samples of the compounds 10–25 are available from the authors.