Magnolol–Coumarin–Phenylbutyric acid Conjugates: An Anticancer Prodrug via multiple targets

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Abstract: We present here, the design and synthesis of a phenylbutyric acid-magnolol-coumarin conjugates (5). Compound 5 is a multifunctional molecule composed of magnolol, coumarin, phenylbutyric acid as model active drugs via ester. Upon entering tumor cells, ester bond cleavage occurs as well as active drug magnolol, coumarin and phenylbutyric acid release to attack cancer cells. In vitro anti-proliferation experiments showed that the inhibitory activity of compound 5 against HepG2, MCF-7, A431 and A549 cells was significantly higher than that of its single parent compound. Moreover, compound 5 had a long-term effect. Due to fluorescence of compound 5, it can serve as a fluorescence imaging agent and its uptake can be monitored. Compound 5 may provide a reference for the design of new derivatives of magnolol.

1. Introduction
Magnolol, a small-molecule polyphenol, was isolated from the barks of Magnolia officinalis [1] and displayed a wide range of biological activities, such as anti-oxidative effects[2-6], antimicrobial activity[7-10], antiviral activity[11,12], anticancer activity[13,14]. Because of its diverse biological activities, magnolol receives more attention, especially its anticancer activity. Magnolol can inhibit the proliferation and differentiation of tumor cell[15-18], induce apoptosis of tumor cell[19-22], and even inhibit tumor metastasis[23,24] and angiogenesis[25-27], meaning it and combat cancer cell via multiple targets. However, anti-cancer activity of magnolol is weak, so structure modification of magnolol is an inevitable for improvement of its anticancer activity. Coumarins Obtained from nature and synthetic origin, coumarins are another important class of compounds and also possess diverse biological activities such as antitumor[28], anticoagulant[29] and antifungal activities[30]. In the field of antitumor activity, 7-hydroxy coumarin derivatives received more attention[13]. As one of 7-hydroxy coumarin, 7-hydroxy-4-methylcoumarin (4-MU) derivatives can functions through DNA binding to inhibit proliferation of cancer cell, just like novo biocin, owing to the coplanar coumarin ring[31]. Histones exist in most of chromatin proteins, and have positive charges on the surface. After the lysine in the end of histones is acetylated, the positive charges of histones are neutralized, which reduces the interaction between the negative charges of phosphoryl groups of DNA and the positive charges of histones, thus making chromatin looser, promoting DNA transcription and cell proliferation. Histone deacetylase inhibitors effectively inhibit deacetylation activity of histone deacetylase to reduce acetylation of histone and prevent cell division[32-36]. Phenylbutyric acid is one of histone...
deacetylase inhibitors. Cancer in treatment of chemotherapeutic drug alone often acquire resistance to cause chemotherapy failure. Therefore, combination of different drugs is effective treatment of cancer, via multiple targets[37,38]. In this work, we designed and synthesized a magnolol-coumarin-benzene butanoic acid conjugates (5) through asymmetric acylation. On entry into a cell, compound 5, as a prodrug, can be hydrolyzed to release magnolol, 7-hydroxy-4-methylcoumarin derivative and benzene butanoic acid for synergistic antitumor. Diesterification of phenolic hydroxyl groups of magnolol increased the difficulty of hydrolysis due to the steric effect and prolonged the release of compound 5 to achieve a long-term effectiveness.

2. Results and discussion

2.1. Synthesis of target compounds

As illustrated in scheme 1, compound 3 was synthesized from 4-Methylumbelliferone as previous reports [16] and reacted with magnolol under conditions of EDCI, as condensation agent, and DMAP, as catalyst to obtain a monoesterification of magnolol, compound 4, in 83% yields. In the step, the three times mole ratio of magnolol to compound 3 is necessary for monoesterification of magnolol. Hence, silica gel column chromatography need to be carried out to purify the product. Compound 5, an asymmetric diesterified derivative of magnolol, could be prepared from compound 4 and phenylbutyric acid under the same conditions of compound 4 with a yields of 67%.

2.2. Synthesis of related compounds

The synthesis of compound 8 is similar to that of compound 4. In brief, phenylbutyric acid reacted with excessive magnolol to ensure the monoesterification of magnolol hydroxyl group, in the presence of EDCI and DMAP to generate a monoesterified derivative of magnolol, compound 8 in 76% yields. Another control compound 7 was synthesized by above method in 53% yield. Compounds 7, 8 are all colorless oil compounds.

Figure1 Synthetic route of compounds 3-8
2.3. In vitro antiproliferative assay

The cytotoxicity of target compound and related compounds were tested against HepG2, Hela, A549 and A431 cell lines by MTT assay using 5-fluorouracil as a positive control (table 1). All compounds exhibited a moderate cytotoxicity, except for 5-fluorouracil. The cytotoxicity of compounds 5 against A549, A431, and Hela was stronger than that of 8, 4 and 1, but weaker than that of mixture of 1,3 and 6, which show that compounds 1,3 and 6 had significantly synergetic anti-tumor effect; The steric effect of diesterification of phenolic hydroxyl groups of magnolol prolonged the hydrolysis.

Table 1 Antitumor activity of compounds 1, 3-8

| Compounds   | IC50(μM) | A549   | HepG2  | A431   | Hela   |
|-------------|----------|--------|--------|--------|--------|
| 1           | 36.12 ± 2.59| 35.55 ± 1.19| 55.72 ± 3.73| 45.96 ± 2.32|
| 3           | >400     | >400   | >400   | >400   |
| 4           | 37.54 ± 0.17| 33.64 ± 4.03| 22.19 ± 4.32| 56.05 ± 7.46|
| 5           | 34.85 ± 0.91| 23.34 ± 2.66| 23.13 ± 1.53| 47.59 ± 7.63|
| 6           | >400     | >400   | >400   | >400   |
| 7           | >400     | >400   | >400   | >400   |
| 8           | 40.84 ± 1.43| 31.59 ± 2.87| 25.91 ± 3.69| 61.65 ± 4.85|
| mixture of 1,3 and 6 | 17.54 ± 0.54| 18.28 ± 1.00| 18.96 ± 2.33| 43.39 ± 2.48|
| 5-fluorouracil | 1.33 ± 0.01| 4.25 ± 0.42| 0.62 ± 0.05| 6.82 ± 1.25|

Next, we examined whether the antitumor activity of compound 5 increased with time. As shown in Fig.2, compound 5 had the weakest antitumor activity than that of 1,4 and 8 in 24h, but strongest in 48h and 72h. Moreover, compound 5 had the strongest antitumor activity among those tested compounds, indicating that with the advance of time, compound 5 released more 1, 3 and 6 to act synergestically through hydrolysis.

Figure 2 The antitumor activity of compound 1, 4, 5, and 8 with time (A549 cells were treated with every compound at dose of 20μM, and cell viability were tested by MTT method. *p < 0.05;
2.4. Long-term effect of compound 5 in PBS

According to results of MTT assay, the mixture of 1, 3 and 6 showed significantly synergetic anti-tumor effect. Furthermore, the anti-tumor activity of compound 4, 5 were lower than that of mixture of 1, 3 and 6 may be attributed to slowness of hydrolysis due to steric hindrance of diester derivatives of magnolol. To further confirm whether compound 5 is released slowly, we used HPLC to determine the release of compound 5 at pH = 7.4 PBS. As shown in Fig. 3, we found that compound 5 could be more stable than compound 4 in PBS solution with pH = 7.4, because of more steric effect of diesterification of phenolic hydroxyl groups of magnolol. The internal micro-environment of the tumor is acidic and has a large number of esterases. Therefore, compound 5 could be released slowly after being ingested by the tumor cells and possessed a long-term effect. It was more stable at pH = 7.4, which guaranteed the high lipo-solubility of compound 5 and was beneficial to cell uptake.

![Figure 3 Study on stability of compound 5 by HPLC (mobile phase was water (contain 0.1%TFA): methanol =20:80 and detection wave was 220nm).](image)

2.5. Cellular uptake of Compound 5

The cellular uptake of compound 5 was analyzed on hepatocellular carcinoma (HepG2) cells. Due to fluorescence of compound 5, the uptake of compound 5 could be visualized by fluorescence microscopy. As shown in Fig. 3, the time-dependent measurements showed that relatively less blue fluorescence was observed in cells at 1 h and fluorescence intensity increased greatly during 3 h and 6h of Incubation, followed the descent at 9 h.
Figure 4 Fluorescence spectrum of compound 3, 5 PBS buffer (pH 7.4) at room temperature, ex 325 nm.

Figure 5 Intracellular uptake of compound 5 with time (fluorescence microscope analysis of HepG2 cells treated with compound 5 for 9 h. a: 0 h, b: 0.5 h, c: 1 h, d: 3 h, e: 6 h, f: 9 h.)

2.6. Conclusions
In the present research, based on multifunctional drug approach, we designed and synthesized a novel derivative of magnolol capable of slowly releasing magnolol, 3 and 6 like other prodrugs to attack different targets. On this foundation, compound 5 and related compounds were evaluated for their antitumor activity against several tumor cell lines. The antitumor activity of compound 5 is stronger than that of parent magnolol, but weaker than that of mixture of 1, 3 and 6 in 48h. To further prove whether the difficulty of hydrolysis hinders action of antitumor for compound 5, the increase of antitumor activity of compound 5 with time was further evaluated. As we expected, compound 5 released more 1, 3 and 6 with the time to act synergistically through hydrolysis, indicating that
compound 5 is long-acting compound. In the following hydrolysis experiment in vitro also indicated that compound 5 hydrolyzed slowly to release 1, 3 and 6 potentially, our finding may aid in design of new derivatives of magnolol.

3. Experimental section

3.1. Chemistry Materials and apparatuses.
Magnolol, 7-hydroxy-4-methylcoumarin and d all were purchased from Dalian Meilun Biotechnology Co., LTD (China). DMEM, RPMI 1640, trypsin, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sangon Biotech (Shanghai) Co. Ltd (China). All other chemicals and solvents were obtained commercially and used without further purification. 1H NMR and 13C NMR spectra were recorded on a Bruker DRX spectrometer at 300 or 400 MHz, d in parts per million and J in hertz, using TMS as an internal standard. ESI-MS spectra were recorded on an API 4000 spectrometer.

HepG2, A549, A431 and HeLa cells were purchased from the American Type Culture Collection (ATCC). The cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C.

3.2. Methods.

3.2.1. Synthesis of compound 3
4-MU (40 mmol), 3-bromopropionic acid (40mmol) and K2CO3 (160mmol) in 250 mL of distilled acetone was stirred for 24h at reflux. After that, acetone was removed from resultant solution under reduced pressure and appropriate amount of water was added to dissolve reaction products. The aqueous solution was washed three times by ethyl acetate and was acidified with HCl. White colored solid precipitated and was filtered off, washed with water and dried to obtain the desired white compounds (3), yield 73%. White solid. m.p. 139–142 °C. 1H NMR (300 MHz, DMSO-d6) δ 12.44 (s, 1H), 7.66 (d, J= 8.6 Hz, 1H), 6.97 (dd, J =8.6,2.5 Hz, 1H), 6.93 (d, J = 2.5 Hz, 1H), 6.20 (d, J = 1.1 Hz,1H), 4.27 (t, J = 5.9 Hz, 2H), 2.74 (t, J = 6.0 Hz, 2H), 2.38 (d, J = 1.1 Hz, 3H), m/z (ESI-MS) 249.5[M+H]+.

3.2.2. General procedure for synthesis of the target compounds 4, and 8
To a solution of 3 or 6 (1.2mmol, 1.0 equiv) and magnolol (1.8 mmol, 1.5equiv) in CH2Cl2 40 mL were added EDCI (1.2mmol, 1.0equiv) and DMAP (0.12 mmol, 0.1equiv) under N2 flow. The reaction mixture was stirred for 2-4h at room temperature and monitored by TLC. After the reaction was finished, appropriate water was added to quench the reaction. After that, the CH2Cl2 layer was washed with 10%HCl and brine separately and was dried over Na2SO4. After removal of the solvent, the resulting crude mixture was purified by silica gel column chromatography (Hexane/EtOAc = 60/40, 50/50) to give the desired product as a colorless oil.

**Compound 4**
Yield 83%, colorless oil. 1H NMR (300 MHz, CDC13) δ 7.46 (d, J = 8.8 Hz, 1H), 7.25 (dd, J = 8.0, 2.1 Hz, 1H), 7.20 (d, J = 1.7 Hz, 1H), 7.13 (d, J = 8.2 Hz, 1H), 7.06 (dd, J = 8.2, 2.0 Hz, 1H), 6.95 (d, J = 2.0 Hz, 1H), 6.89 (d, J = 8.3 Hz, 1H), 6.77 (dd, J = 8.8, 2.4 Hz, 1H), 6.70 (d, J = 2.3 Hz, 1H), 6.14 (s, 1H), 6.04-5.83 (m, 2H), 5.19- 4.96 (m, 4H), 4.10 (t, J = 6.5 Hz, 2H), 3.42 (d, J = 6.7 Hz, 2H), 3.30 (d, J = 6.6 Hz, 2H), 2.83 (t, J = 6.5 Hz, 2H), 2.39 (s, 3H).

m/z (ESI-MS) 497.4[M]+,498.5[M+H]+

**Compound 8**
Yield 76%, colorless oil. 1H NMR (300 MHz, CDC13) δ 7.32 – 7.24 (m, 3H), 7.23 – 7.17 (m, 2H), 7.13 – 7.02 (m, 4H), 6.92 (dd, J = 12.2, 5.2 Hz, 2H), 6.07 – 5.82 (m, 2H), 5.19 – 4.98 (m, 4H), 3.44 (d, J = 6.7 Hz, 2H), 3.29 (d, J = 6.7 Hz, 2H), 2.46 (t, J = 7.6 Hz, 2H), 2.33 (t, J = 7.4 Hz, 2H), 1.83 – 1.70 (m, 2H). 13C NMR (101 MHz, CDC13) δ 172.46 (s), 151.34 (s), 146.95 (s), 141.20 (s), 138.75 (s),
137.69 (s), 136.75 (s), 131.99 (s), 131.91 (s), 130.59 (s), 130.13 (s), 129.77 (s), 129.72 (s), 128.49 (s),
128.37 (s), 126.00 (s), 124.00 (s), 122.87 (s), 116.52 (s), 116.36 (s), 115.61 (s), 39.58 (s), 39.29 (s),
34.72 (s), 33.30 (s), 26.22 (s).

m/z (ESI-MS) 430.6[M+H2O]+, 435.7[M+Na]+

3.2.3. General procedure for synthesis of the target compounds 5, 7
To a solution of 6 (1.2 mmol, 1.0 equiv) and 4 or 3 (1.2 mmol, 1.0 equiv) in CH2Cl2 40 mL were added
EDCI (1.0 mmol, 1.0 equiv) and DMAP (0.1 mmol, 0.1 equiv) under N2 flow. The reaction mixture was
stirred for 2-4 h at room temperature and monitored by TLC. After the reaction was finished,
appropriate water was added to quench the reaction. After that, the CH2Cl2 layer was washed with
10% HCl and brine separately and was dried over Na2SO4. After removal of the solvent, the resulting
crude mixture was purified by silica gel column chromatography (Hexane/EtOAc = 70/30, 60/40) to
give the desired product as a colorless oil.

**Compound 5.**
Yield 67%. colorless oil. 1H NMR (400 MHz, CDCl3) δ 7.46 (d, J = 8.6 Hz, 1H), 7.24 (d, J = 8.4 Hz,
2H), 7.18 (t, J = 9.2 Hz, 3H), 7.13 – 7.01 (m, 6H), 6.77 (d, J = 8.8 Hz, 1H), 6.72 (s, 1H), 6.14 (s,
1H), 6.00 – 5.83 (m, 2H), 5.11 – 5.01 (m, 4H), 4.11 (t, J = 5.6 Hz, 2H), 3.37 (s, 4H), 2.81 (t, J = 5.7 Hz,
2H), 2.47 (t, J = 7.5 Hz, 2H), 2.39 (s, 3H), 2.31 (t, J = 7.1 Hz, 2H), 1.77 (p, J = 7.0 Hz, 2H). 13C NMR
(101 MHz, CDCl3) δ 171.89 (s), 168.83 (s), 161.43 (s), 161.23 (s), 155.20 (s), 152.44 (s), 146.40 (s),
146.13 (s), 141.34 (s), 138.00 (s), 137.76 (s), 136.92 (s), 136.84 (s), 131.30 (s), 131.22 (s), 130.30 (s),
129.14 (s), 129.05 (s), 128.44 (s), 128.34 (s), 125.96 (s), 125.49 (s), 122.42 (s), 122.22 (s), 116.32 (s),
116.30 (s), 113.83 (s), 112.51 (s), 112.20 (s), 101.61 (s), 63.61 (s), 39.51 (s), 34.82 (s), 34.25 (s), 33.37 (s),
26.28 (s), 18.69 (s). m/z (ESI-MS) 643.4[M+H]+, 660.2[M+H2O]+

**Compound 7.**
Yield 53%. colorless oil. 1H NMR (300 MHz, CDCl3) δ 7.61 (d, J = 8.6 Hz, 1H), 7.34 (dd, J = 10.3,
4.3 Hz, 2H), 7.29 – 7.21 (m, 3H), 7.10 (d, J = 2.2 Hz, 1H), 7.06 (dd, J = 8.6, 2.2 Hz, 1H), 6.28 (d, J =
0.9 Hz, 1H), 2.77 (t, J = 7.5 Hz, 2H), 2.63 (t, J = 7.5 Hz, 2H), 2.20 – 2.03 (m, 2H). m/z (ESI-MS)
323.5[M]+, 324.5[M+H]+

In Vitro Cellular Cytotoxicity Assays.
Cells were seeded in 96-well plates at 5000 cells per well in 100 μL of complete medium, and
incubated in a 5% CO2 atmosphere at 37 °C for 24 h. The culture medium was then replaced with 100 μL of freshly prepared culture medium containing drugs at different concentrations. The cells were
further incubated for 48 h, and then the medium in each well was replaced with fresh culture medium and the MTT solution was added. The cells were incubated for another 4 h to allow viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. Finally, culture medium containing MTT was sucked and 150 µL of DMSO was added to the wells and the cell were incubated for another 10 min at 37 °C with shake. The absorbance was measured at 570 nm using a Bio-Rad 680 microplate reader. The IC50 values were calculated using GraphPad Prism software (version 5.01),
which were based on three parallel experiments.

HPLC Studies.
Compound 4 or 5 (250 μM) was dissolved in PBS(50 mM, pH 7.4)/CH3CN (1:1) or PBS(50 mM, pH 4.4)/CH3CN (1:1); the mixture was incubated at 37 °C for 72 h. Aliquots (taken at 1, 4, 8, 12, 18,
24, 36, 48, 60 and 72 h) were analyzed by HPLC. HPLC analyses were performed as on an Agilent
1200 system equipped with a Kromasil C18 column (250×4.6 mm, 5 µm). HPLC profiles were
recorded by UV detector at 220 nm and mobile phase was water (contain 0.1%TFA): methanol
=20:80.

Uptake of compound 5.
HepG2 cells were seeded in 24-well plates at a density of 10^5 cells per well. After 24 h of incubation
at 37°C in 5% CO2, the cells were treated with compound 5 (100 μM). After further incubation for
different times (0.5, 1, 3, 6 and 9 h), the medium was removed and cells were washed three times with PBS. The fluorescence imaging of cells was analyzed by fluorescence microscopy.

Supporting Information
The $^1$H NMR, $^{13}$C NMR, ESI-MS, and HPLC spectra for the synthesized compounds. The Supporting Information is available free of charge via the Internet at http://sioc-journal.cn.

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