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Synthesis and evaluation of antitumour activity *in vitro* and *in vivo* of chrysin salicylate derivatives

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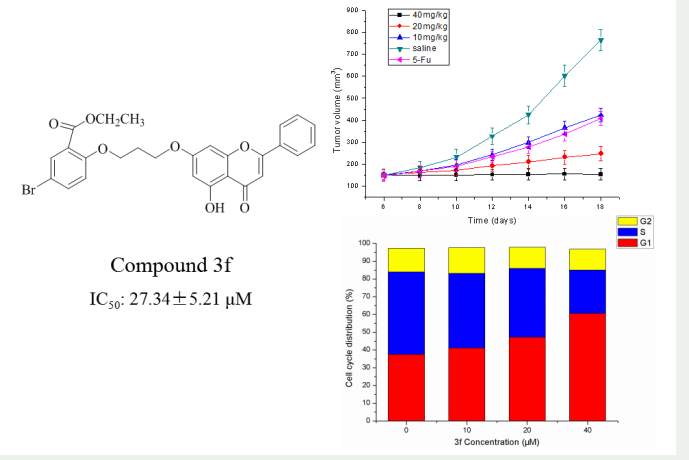
ABSTRACT

A series of chrysin salicylate derivatives as potential antitumour agents were synthesised and evaluated their antitumour activities *in vitro* and *in vivo*. Most of the compounds exhibited moderate to good activities against MCF-7 cells, HepG2 cells, MGC-803cells and MFC cells. Among them, compound **3f** showed the most potent activity against MGC-803 cells and MFC cells with IC_{50} values of 23.83 ± 3.68 and 27.34 ± 5.21 μM, respectively. The flow cytometry assay reconfirmed that compound **3f** promoted the occurrence of tumour cells' G1/S block under the inhibiting effect of compound **3f**. Compound **3f** possessed higher antitumour efficacy in tumour-bearing mice, compared with the positive control 5-Fu and the blank control saline.



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1. Introduction

Chrysin (5,7-dihydroxyflavone) is a natural flavonoid from many plants and has all kinds of biological activities such as antitumour, antiviral, antibactericidal, anti-inflammatory, antiallergic, antimutagenic, antianxiolytic and antioxidant effects (Hamilton-Miller 1995; Han 1997; Bae et al. 2000; Lim et al. 2011; Du et al. 2012; Wang et al. 2014; Aksu et al. 2016; Miura et al. 1995). A lot of studies have been done by synthesis of chrysin derivatives to improve its pharmaceutical activity. Tran Thanh Dao designed and synthesised 25 chrysin derivatives, some of which showed great inhibition activities against cyclooxygenase-2 (COX-2) catalysed prostaglandin E_2 (PGE_2) production (Dao et al. 2004), suggesting that antitumour activities of chrysin and its derivatives may be related to the COX-2/PGE_2 pathway. Zhu et al. (2014) modified chrysin by adding some hydrophobic groups in the search for potential antitumour agents.

Salicylic acid (SA) and its derivatives have been used for many years as the leading non-steroidal anti-inflammatory drugs. It was reported that SA and its derivatives prevented tumour cell growth, induced apoptosis (Grivennikov et al. 2010; Thun et al. 2012) and decreased risk of gastric carcinoma, colorectal, oesophageal, breast, lung, prostate, liver and skin tumours (Harris et al. 2005; Kaiser 2012; Sahasrabuddhe et al. 2012; Dovizio et al. 2013; Gamba et al. 2013). The antitumour effect of acetylsalicylic acid (ASA) has been owed to its effects on cyclo-oxygenase (COX) (Loll et al. 1995). Nevertheless, COX negative colon tumour cell line SW480 went through apoptosis under ASA actions, revealing that ASA also can trigger cell apoptosis by other mechanisms distinct from COX inhibition (Lai et al. 2008). Recently, it has been demonstrated that ASA inhibit 6-phosphofructo-1-kinase (PFK) that is one of the key enzymes in the glycolysis to change tumour glucose utilisation and promote cell apoptosis (Spitz et al. 2009).

As antitumour activity is especially important for clinic application, the aim of present study was to improve the antitumour efficacy of chrysin derivatives. So, in our study, the 7-OH of chrysin was modified by adding SA and its derivatives in order to find out some efficient and low toxicity antitumour drugs. A series of chrysin salicylate derivatives were firstly synthesised and investigated their antitumour activity against three human tumours and one mice tumour *in vitro*. Compound **3f** (Figure 1) showed the most potent activity against MGC-803 cells and MFC cells with IC_{50} values of 23.83 ± 3.68 and 27.34 ± 5.21 μM, respectively. Furthermore, cell cycle assay *in vitro*, and antitumour efficacy in mice after administration of compound **3f** were also investigated.

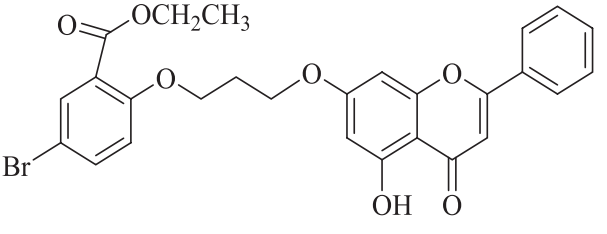


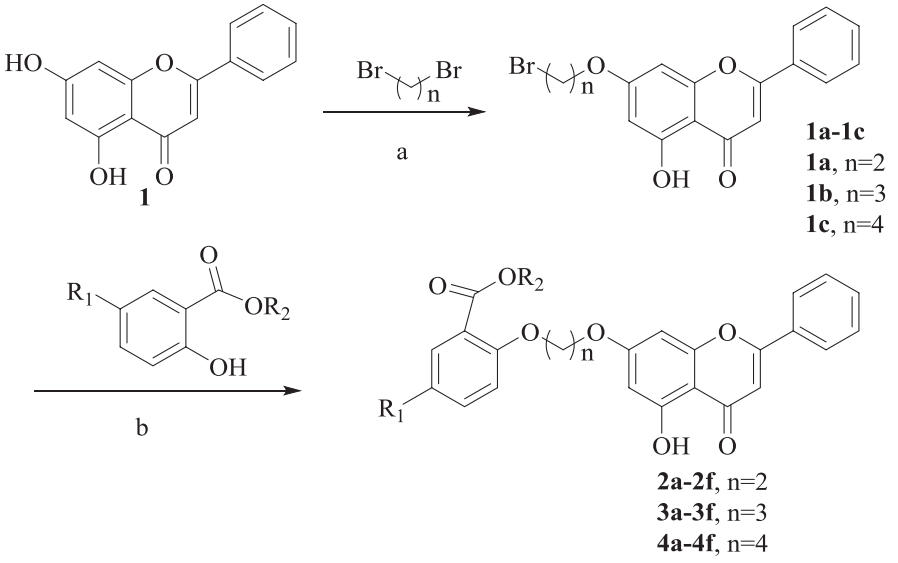
Figure 1. Structure of compound **3f**.

2. Results and discussion

The synthesis of compounds **2a–f**, **3a–f** and **4a–f** were accomplished according to the general pathway illustrated in the Scheme 1. All of the synthetic compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures.

The antitumour efficacy of new compounds was evaluated *in vitro* and *in vivo*. The antitumour activities *in vitro* of the chrysin salicylate derivatives **2a–f**, **3a–f** and **4a–f** was researched on the human tumour cells MCF-7 (michigan tumour foundation-7), HepG2 (human liver carcinoma cells), MGC-803 (gastric carcinoma cells) and the mice tumour cells MFC (mouse fore-stomach carcinoma cells) by methyl tetrazolium (MTT) assay. Compounds were tested over a range of concentrations from 2 to 256 μmol/L, and the calculated IC_{50} values were reported in Table 1. Most compounds have good bioactivity to MCF-7 cells, HepG2 cells, MGC-803cells and MFC cells. Compound **3f** was found to show the most potent activity against MGC-803 cells and MFC cells with IC_{50} values of 23.83 ± 3.68 and 27.34 ± 5.21 μM, respectively.

The antitumour growth effect *in vivo* was evaluated by measuring tumour volume following treatment with 5-Fu (1.25 mg/mL, dissolved in saline mixed with 3% Tween 80), different doses of compound **3f** (10, 20, 40 mg/kg, dissolved in saline mixed with 3% Tween 80) and saline (mixed with 3% Tween 80). As illustrated in Figures 2 and 3, compound **3f** inhibited the tumour growth remarkably by dose-dependent as well compared with the saline and the result have statistically significant, confirming that compound **3f** had antitumour activity. It was observed that the middle dose and the high dose of compound **3f** exhibited stronger



2a, 3a, 4a; R_1=H,R_2=CH_3 **2c, 3c, 4c**; R_1=Cl,R_2=CH_3 **2e, 3e, 4e**; R_1=Br,R_2=CH_3

2b, 3b, 4b; R_1=H,R_2=C_2H_5 **2d, 3d, 4d**; R_1=Cl,R_2=C_2H_5 **2f, 3f, 4f**; R_1=Br,R_2=C_2H_5

Scheme 1. Synthesis chrysin salicylate derivatives: (a) $BrCH_2CH_2Br$ or $BrCH_2CH_2CH_2Br$ or $BrCH_2CH_2CH_2CH_2Br$, K_2CO_3, acetone; (b)substituted salicylate, K_2CO_3, DMF, heating.

Table 1. Anti-proliferative activity of compounds against the tumour cell lines.

Compounds	IC_{50}(μM)			
	MGC-803	MCF-7	HepG-2	MFC
2a	> 100	N.D	N.D	> 100
2b	> 100	> 100	> 100	N.D
2c	> 100	> 100	N.D	> 100
2d	> 100	N.D	N.D	> 100
2e	46.55 ± 2.03	94.75 ± 4.62	89.18 ± 3.83	57.49 ± 3.16
2f	84.14 ± 3.72	> 100	79.30 ± 3.62	> 100
3a	> 100	> 100	89.56 ± 3.15	> 100
3b	> 100	N.D	N.D	> 100
3c	45.93 ± 3.91	> 100	79.60 ± 4.09	60.46 ± 3.83
3d	> 100	N.D	N.D	> 100
3e	70.68 ± 4.72	89.22 ± 4.37	74.02 ± 5.22	76.30 ± 4.66
3f	23.83 ± 3.68	40.47 ± 2.03	35.73 ± 4.31	27.34 ± 5.21
4a	82.04 ± 4.54	N.D	> 100	> 100
4b	N.D	N.D	N.D	N.D
4c	N.D	N.D	N.D	N.D
4d	91.16 ± 5.37	> 100	67.76 ± 5.46	85.93±5.87
4e	> 100	N.D	N.D	> 100
4f	> 100	N.D	N.D	> 100
Chrysin	> 100	> 100	78.83 ± 4.25	> 100
5-FU	78.37 ± 1.04	57.09 ± 3.17	67.32 ± 3.47	85.59 ± 4.68

Note: N.D = not detected.

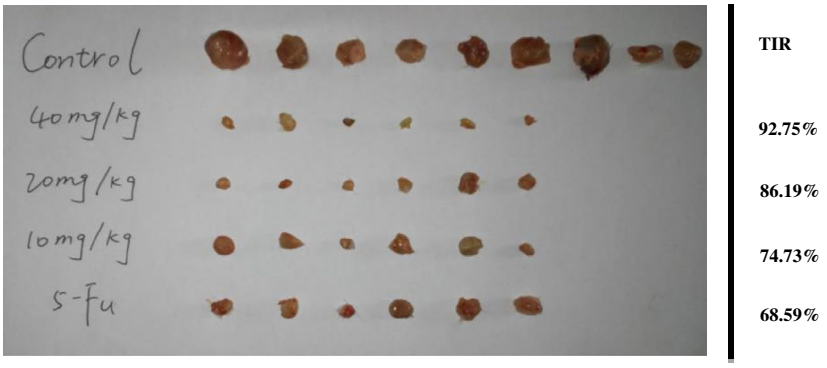


Figure 2. The TIR values of excised tumours at day 18.The differences of the average of tumour weight among the blank control group and other groups were statistically significant ($p < 0.05$). The differences of the average of tumour weight among the middle dose, the high dose of compound **3**f and 5-Fu groups were statistically significant ($p < 0.05$).

tumour regression, with TIR of 86.19 and 92.75%, compared with the 5-Fu injection (TIR of 68.59%). The differences of the average of tumour weight among the middle dose, the high dose of compound **3f** and 5-Fu groups were statistically significant ($p < 0.05$). The results confirmed that the antitumour efficacy of compound **3f** is superior to 5-Fu. Figure 4 illustrated the growth in body weight during the 18-day experimental period. The gradual increase in body weight among the low and middle dose of compound **3f** groups was consistent with that of the saline group, which represented the natural growth in body weight of MFC tumour-bearing mice. Moreover, the fur of the mice is normal by treating with different doses of compound **3f**, but the mice treated with 5-Fu have obvious change on hair removal and

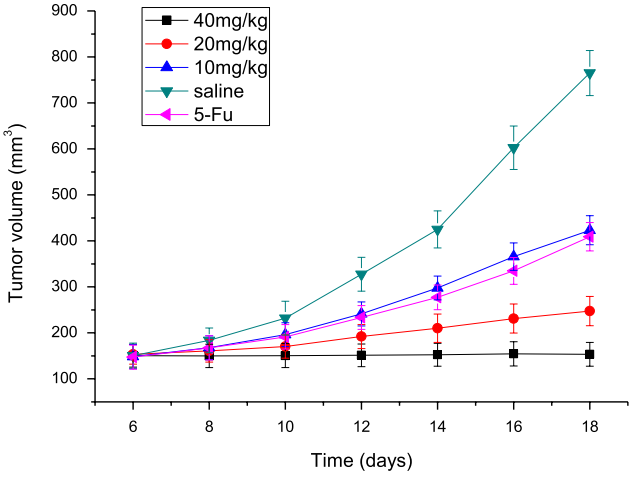


Figure 3. Tumour volume growth curves of the mice after treatment with 5-Fu (1.25 mg/mL, dissolved in saline mixed with 3% Tween 80), different doses of compound **3f** (10, 20, 40 mg/kg, dissolved in saline mixed with 3% Tween 80) and saline (mixed with 3% Tween 80). Data were showed as a mean ± SD ($n = 6$ and the mice number of blank control is 9).

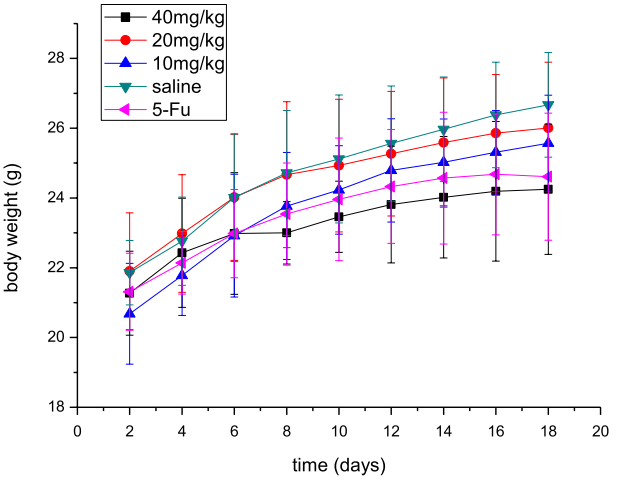


Figure 4. Body weight evolution curves of the mice after treatment with 5-Fu (1.25 mg/mL, dissolved in saline mixed with 3% Tween 80), different doses of compound **3f** (10, 20, 40 mg/kg, dissolved in saline mixed with 3% Tween 80) and saline (mixed with 3% Tween 80). Data were showed as a mean ± SD ($n = 6$ and the mice number of blank control is 9).

body weight, demonstrating that the toxicity of compound **3f** was lower than that of positive control 5-Fu.

MFC cells were treated with compound **3f** at 0, 10, 20, 40 μM for 24 h to detect cell cycle distribution. As indicated in Figure 5, with the increase of drug concentration, the percentage of cells in G1 phase increased, the percentage of cells in S phase decreased and G2/M change is not obvious. It can be shown that tumour cells occurred G1/S block under the inhibiting effect of compound **3f**.

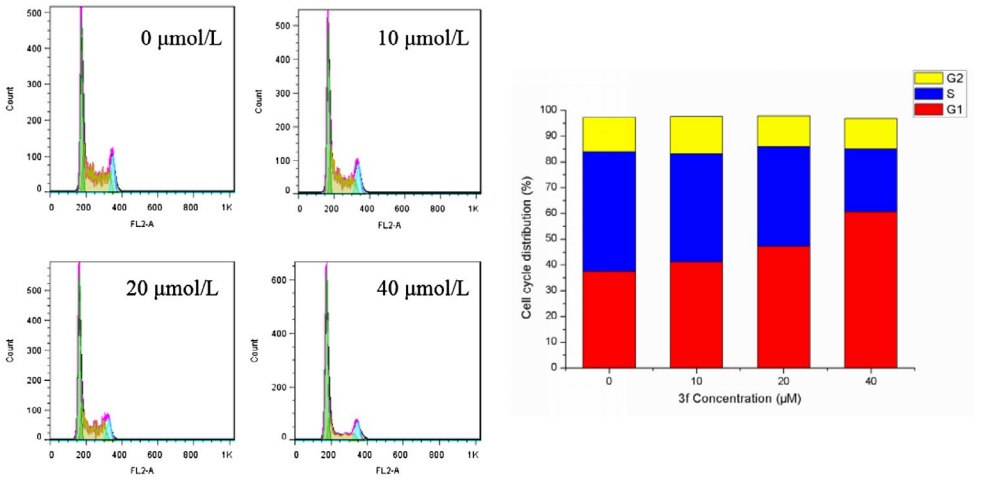


Figure 5. Flow cytometry analysis of cell cycle distribution for MFC cells treated with compound **3f** (0, 10, 20, 40 μmol/L) for 24 h.

3. Experimental

3.1. Synthesis

All chemicals (reagent grade) used were commercially available. Chrysin (>98%) was purchased from Aladdin-Reagent Co., Ltd (China). ^{1}H NMR were recorded on a Bruker AVANCE instrument (400 MHz). Melting points (uncorrected) were determined on an XT4 MP apparatus (Taike Corp., Beijing, China). ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer.

3.2. General method for synthesis of compounds 1a–1c

To a stirring solution of chrysin (compound **1**) in acetone, K_2CO_3 was added and then 1,2-dibromoethane or 1,3-dibromopropane or 1,4-dibromobutane was added dropwise to give compound **1a**–**1c**.

3.3. General method for synthesis of compounds 2a–2f

To a solution of 1a (0.36 g, 1 mmol) in 10 mL of anhydrous DMF was added Methyl salicylate (0.76 g, 5 mmol), K_2CO_3 (0.12 g, 1 mmol), followed by heating at 60 °C for 6 h. To the reaction mixture was added ice water, dropwise. The mixture was filtered, washed with water, dried over Na_2SO_4 and concentrated. The residue was purified with a silica gel column and was eluted with EA/PE 1:8 to afford 2a (0.20 g, 46%).

Compounds **2b–4f** were prepared in analogy.

The ^{1}H NMR and ESI-MS spectra data of compounds **2a–4f** were supplied as supplementary material.

3.4. In vitro *antitumour efficacy*

The human tumour cells MCF-7, HepG2, MGC-803 and the mice tumour cells MFC were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, NY, USA) containing 10% foetal bovine serum at 37 °C in a humidified incubator with 5% CO_2. MCF-7, HepG2, MGC-803 and MFC cells were chosen to evaluate the antitumour activity *in vitro*. Cells were plated in 96-well microtitre plates at a density of 8×10^3/well and incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 48 h. Test compounds were added onto triplicate wells with different concentrations and 0.1% DMSO for control. After they had been incubated for 48 h, 20μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL) was added to each well and the plate was incubated for an additional 4 h. The formazan was dissolved in 100 μL of DMSO. The absorbance (OD) was read on a WellscanMK-2 microplate reader (Labsystems) at 570 nm. The concentration causing 50% inhibition of cell growth (IC_{50}) was determined by the Logit method. All experiments were performed three times.

3.5. In vivo *antitumour efficacy*

All experiments with animals were performed in compliance with the relevant laws and institutional guidelines of China Institute of Science and Technology and institutional committees have approved the experiments. Kunming male mice (University of South China, China) were used for *in vivo* experiments. Gastric carcinoma was chosen as a model tumour to research the antitumour efficacy of the compound **3f**. Gastric tumour-bearing mice were established by subcutaneous injection of MFC cells in the ventral part that suspended in saline to 1×10^7/0.2 mL/mouse (defined as day 0). On day 1, the mice were randomly divided into five groups ($n = 6$ and the mice number of blank control is 9). The mice were treated with 5-Fu (1.25 mg/mL, dissolved in saline mixed with 3% Tween 80), different doses of compound **3f** (10, 20, 40 mg/kg, dissolved in saline mixed with 3% Tween 80) and saline (mixed with 3% Tween 80) (blank control) via intraperitoneal injection on days 2, 5, 8, 11 and 14.

On day 6, tumour volumes were monitored every other day by measuring two perpendicular diameters using Vernier caliper and calculated using the formula: Volume = $0.5 \times \text{Length} \times (\text{Width})^2$. Body weights were recorded every other day. On day 18, the animals were sacrificed and the tumour mass was dissected, weighed and photographed. The tumour inhibition ratio (TIR) was calculated using the formula: TIR (%) = (1−Wt/Ws) × 100%, where Wt and Ws represent the average tumour weight of the treatment and saline groups, respectively.

3.6. In vitro *cell cycle assay*

The MFC cells were incubated with different concentrations of compound **3f** (0, 10, 20,40 μmol/L) in a six-well plate. After 24 h coculture, the cells were washed twice with cold PBS and then resuspended cells with cold PBS. MFC cells were fixed with stored in 70% cold ethanol for 12 h at 4 °C. Then add 500 μl of Propidium staining to each tube. The cells were gently vortex and incubated for 30 min at RT (37 °C) in the dark. The cells were analysed by flow cytometry within 24 h.

4. Conclusion

In conclusion, a series of chrysin salicylate derivatives that some of them with good bioactivity have been designed and synthesised successfully. These compounds were exhibited moderate to good antitumour activity against MCF-7 cells, HepG2 cells, MGC-803cells and MFC cells. Furthermore, compound **3f** showed the most potent activity against MGC-803 cells and MFC cells with IC_{50} values of 23.83 ± 3.68 and 27.34 ± 5.21 μM, respectively. The flow cytometry assay reconfirmed that compound **3f** inhibited tumour cells by making tumour cells occurred G1/S block. Moreover, it was evident that compound **3f** had good antitumour activity and low toxicity *in vivo*. The mechanism of action of these chrysin salicylate derivatives with good bioactivity is unknown, but COX-2 and PFK have been suggested by us as a possible factor. The next work will focus on investigating the mechanism of action of these chrysin salicylate derivatives. In a word, these derivatives can be recommended as new medicine to develop new antitumour agents.

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Disclosure statement

The authors declare that there are no conflicts of interest.

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