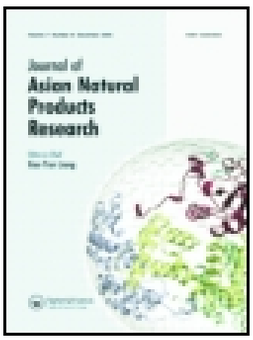

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# Synthesis, characterization and preliminary biological evaluation of chrysin amino acid derivatives that induce apoptosis and EGFR downregulation

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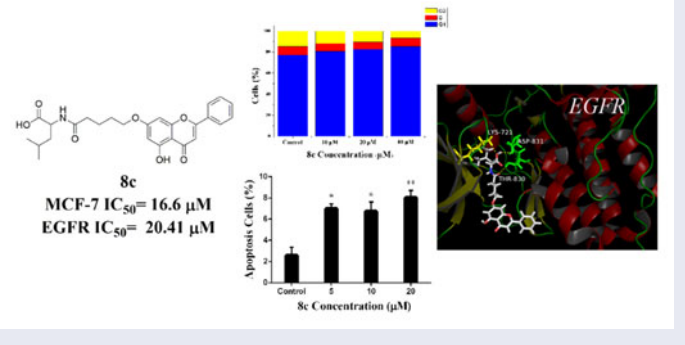
**ABSTRACT**

Chrysin amino acid derivatives were synthesized to evaluate for their antiproliferative activities. Among them, *N*-(7-((5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeryl)-L-leucine (**8c**) displayed the most remarkable inhibitory activities against MCF-7 cells with $IC_{50}$ values of 16.6 $\mu$M. Preliminary mechanistic studies showed that **8c** could inhibit the colony formation and migration of MCF-7 cells. Flow cytometry analysis demonstrated that **8c** mediated cell apoptosis and the prolongation of cell cycle progression in G1/S-phase against MCF-7 cells. Besides, **8c** displayed the moderate inhibition against EGFR. Western blot assay suggested that **8c** significantly inhibited EGFR phosphorylation. Molecular docking showed that **8c** can bind the EGFR kinase well.

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## 1. Introduction

It was reported that chryisn exhibits many biological activities and possesses a wide range of pharmacological effects, such as anti-inflammatory [1], anti-estrogenic [2] and anticancer [3]. Recently, several researchers have attempted to modify the profile of chrysin and found that chrysin derivatives have diverse activities including anti-cancer effects [4]. Noteworthy, part of chrysin derivatives showed potent epidermal growth factor receptor (EGFR) inhibitory activity [5]. EGFR, as a transmembrane, is an important class of protein tyrosine kinases [6]. Studies have shown that EGFR overexpression is associated with different types of cancers, including renal carcinoma, lung, breast, esophageal, colon, pancreatic and prostate cancer. Accordingly, the EGFR family is a major target of antitumor targets and EGFR inhibitors may be effective in the treatment of cancer [7].

Considering that amino acids are the basic substance in biological system and have special physiological functions, for example good biocompatibility and cell affinity [8]. Through modification with amino acid, the parent structure can either increase the interaction and selectivity toward tumor cells or reduce the side effect and improve the bio-availability [9].

Overexpression of EGFR contributes to cell proliferation, survival and motility as well as angiogenesis, which are responsible for all important aspects of tumorigenesis [10]. For this reason, many companies are actively developing inhibitors targeting EGFR. Several studies have been approved for the use of quercetin as inhibitors of EGFR [11, 12]. However, there are few studies about the research on the chrysin derivatives targeted EGFR. Previously, two series of amino acid derivatives containing chrysin were prepared from 7-*O*-carboxyethyl chrysin (**4a**) and 7-*O*-carboxybutyl chrysin (**4b**) and amino acid methyl easters by treatment with amidated reagent [13, 14]. Currently, we investigated whether the EGFR phosphorylation is involved in the antiproliferative activity of potent compound **8c** against MCF-7 cells. Specifically, compound **8c** was further studies to explore its mechanism on the apoptosis signal pathway by flow cytometry (FCM), clonogenicity assay, cell migration, enzyme-linked immunosorbent assay (ELISA) and western blot assay. Docking simulations were performed using the X-ray crystallographic structure of the target protein EGFR in complex with inhibitors to explore the binding modes of **8c** at the active site.

## 2. Results and discussion

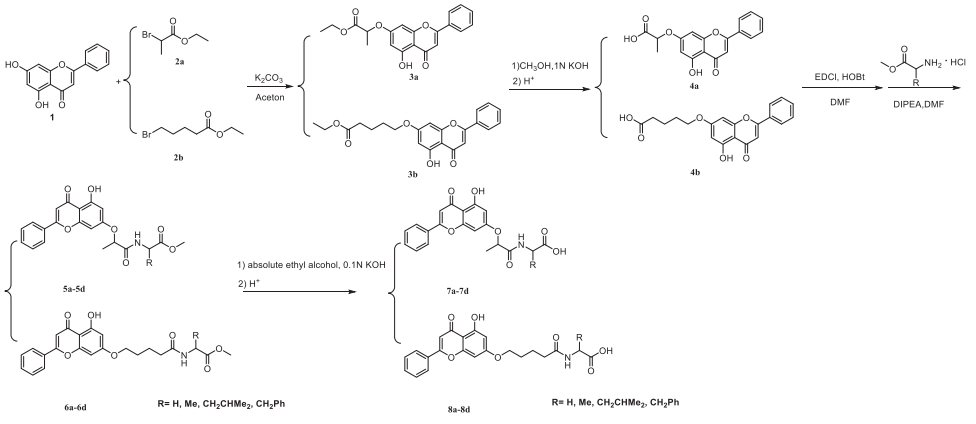
### 2.1. Chemistry

Here, we smoothly synthesized two series of chrysin amino acid analogues. All compounds were identified by spectroscopic data. The synthesis routes are outlined in Scheme 1 [13, 15].

### 2.2. Biological activity

#### 2.2.1. Antiproliferative activity

All the final products were screened for the antiproliferative activities against the human breast cancer MCF-7 cells and human liver cancer HepG-2 cells by the MTT




**Scheme 1.** Synthesis of amino acid derivatives containing chrysin.

assay. 5-Fluorouracil (5-FU) was used as reference compound. The cells were propagated for 48 hours in the test substance, and the antiproliferative results were summarized in Table 1. Against MCF-7 cells, compound **8c** showed the most potent activity with $IC_{50}$ values of 16.6 $\mu$m, which is comparable to the positive control 5-FU ($IC_{50}$=57.1 $\mu$m). Against HepG-2 cells, compounds **4b** and **6d** were the most potent agents compared with other compounds, and the $IC_{50}$ values were 39.4 and 47.1 $\mu$m, respectively. They showed inferior antiproliferative effects than 5-FU ($IC_{50}$ = 29.2 $\mu$m). Furthermore, the toxicity against normal human umbilical vein endothelial cell line HUVEC was also examined. Two series of amino acid derivatives containing chrysin manifested apparent un-toxic effect on HUVEC.

Based on our previous studies [15, 16], the introduction of the amino acid group at the C-7 position of chrysin largely improves inhibitory activities against cancer cell lines. Initially, all chrysin derivatives were evaluated in terms of their inhibitory activities in MCF-7 and HepG-2 cells. As shown in Table 1, all of compounds generally showed various degrees of inhibitory effects. In MCF-7 cells, compared with amino acid ester derivatives **5a–5d** and **6a–6d**, amino acid derivatives **7a–7d** and 8a–8d exhibited better inhibitory effects. The most obvious reason for this phenomenon is the formation of potential hydrogen bonding between the carboxyl group and target protein. Additionally, compared with compounds **7a–7d** and **8a–8d**, the results showed that chrysin coupling with long-chain can achieve good inhibitory activity. This is consistent with the conclusions reported in previous research. Nevertheless, as shown in Figure 1, L-leucine analogue 8c could maintain two hydrogen bonds with the hinge region, which should be responsible for the raised inhibitory potency of **8c**. Specifically, isobutyl group (**8c**) reaches deep into a pocket and forms hydrophobic interactions with various aromatic residues. However, the bulky phenyl group (**8a**) and the methyl group (**8d**) led to loss of inhibitory activity. Unfortunately, in HepG-2 cells, it should be noted that there are no clear structure-activity relationship for chrysin amino acid derivatives, underscoring the importance of the flavonoid scaffold and the types of amino acids for the activity.

**Table 1.** Inhibitory activity of compounds **3a–8d** against the tested cell lines.

| | $IC_{50}$ (μm) | | |
|---|---|---|---|
| Compounds | HepG-2 | MCF-7 | HUVEC |
| **3a** | 102 ± 4.9 | 49.2 ± 1.0 | >150 |
| **3b** | 110 ± 1.3 | 26.1 ± 3.2 | >150 |
| **4a** | 259 ± 4.3 | 74.4 ± 4.3 | >150 |
| **4b** | 39.4 ± 3.9 | >150 | >150 |
| **5a** | >150 | >150 | >150 |
| **5b** | >150 | 98.2 ± 5.1 | N.D. |
| **5c** | >150 | 109 ± 4.3 | >150 |
| **5d** | >150 | 91.6 ± 0.5 | N.D. |
| **6a** | >150 | 62.8 ± 4.6 | >150 |
| **6b** | >150 | 44.5 ± 0.3 | N.D. |
| **6c** | >150 | >150 | >150 |
| **6d** | 47.1 ± 1.3 | >150 | >150 |
| **7a** | >150 | 63.6 ± 3.1 | >150 |
| **7b** | 58.8 ± 0.6 | >150 | >150 |
| **7c** | >150 | 139 ± 3.9 | N.D. |
| **7d** | 72.2 ± 1.0 | >150 | N.D. |
| **8a** | 58.7 ± 1.3 | >150 | >150 |
| **8b** | >150 | 31.2 ± 1.1 | N.D. |
| **8c** | 106 ± 2.7 | 16.6 ± 0.8 | >150 |
| **8d** | 131 ± 5.2 | >150 | N.D. |
| Chrysin | >150 | 143 ± 1.9 | >150 |
| 5-FU | 29.2 ± 3.2 | 57.1 ± 3.2 | >150 |

### *2.2.2. Clonogenicity assay and cell migration*

The excellent antiproliferative inhibition of potential compound **8c** toward MCF-7 cells encouraged us to examine the effect on colony and migration of those cells. The colony formation of cancer cells indicates an indirect method of neoplastic transformation [17]. As shown in Figure 2A, treatment of MCF-7 cells with **8c** for 10 days led to obvious fewer colonies in a concentration-dependent manner compared with the control, and the colony formation was almost inhibited completely at 20 μmol/L. This study offered new evidence for the anticancer activity of **8c**. On the side, we assessed the role of **8c** on cell migration, a key determinant of malignant progression and metastases [18]. As show in Figure 2B, treatment of MCF-7 cells with **8c** for 48 h at referential concentrations obviously inhibited cell migrations in a concentration-dependent manner. This demonstrated that **8c** could block the MCF-7 cell migration significantly.

### *2.2.3. Cell apoptosis and G1/S- phase cell cycle arrest induced by compound 8c*

In order to identify the effect of **8c** on tumor cell apoptosis and cycle progression, FCM were performed on MCF-7 cells treated with different concentrations of **8c** for 48 h. As shown in Figure 3A and B, **8c** increased the proportion of cells in G1/S phase and decreased the fraction in G2-phase in a dose-dependent manner, indicating that **8c** arrested MCF-7 cells at G1/S phase. Moreover, **8c** induced cellular apoptosis in a concentration-dependent manner (Figure 3C), and the percentage of apoptotic cells induced by **8c** at 5, 10 and 20 μmol/L were 6.9%, 7.1% and 8.2%, respectively. As shown in Figure 3D, the apoptotic cells were also remarkably ($P < 0.05$ or $P < 0.01$) increased in a dose-dependent manner. These results indicated that **8c** can mediate cell apoptosis and a prolongation of cell cycle progression in G1/S phase against MCF-7.

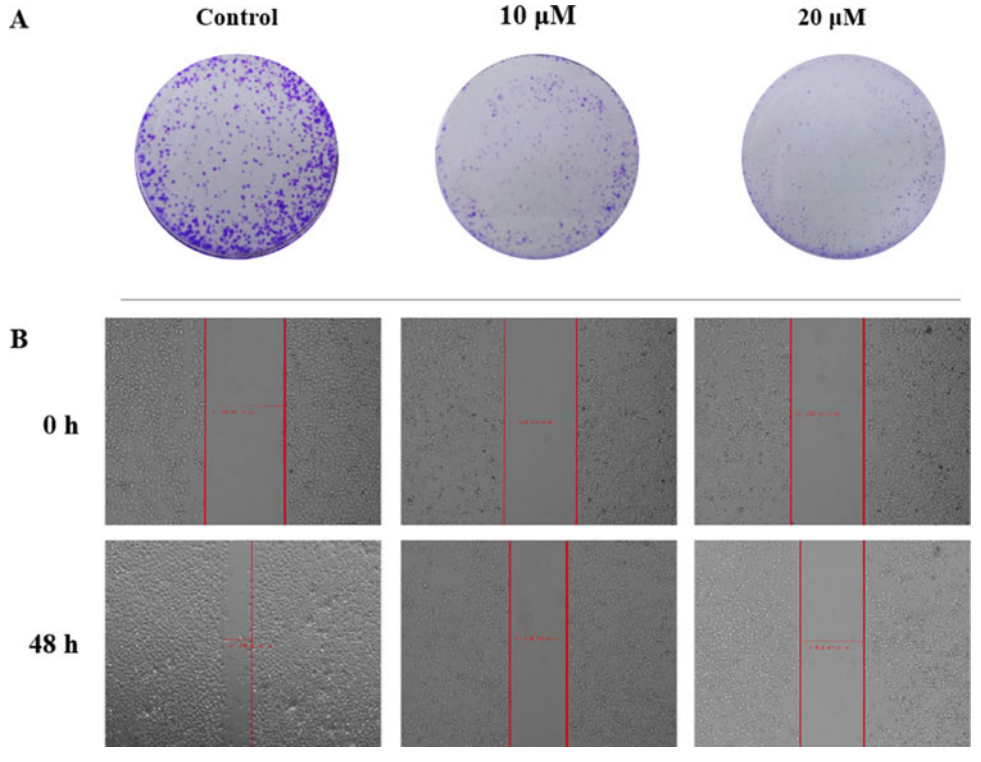



**Figure 1.** (A) Representative images of MCF-7 cell colonies after treatment with compound **8c** at different concentrations for 10 days. (B) Wound healing assay. Effect of compound **8c** on migration of MCF-7 cells. The images were captured using phase contrast microscopy before (0 h), and after 48 h of treatment with compound **8c**.

### *2.2.4. Inhibitory activity against EGFR tyrosine kinase*

Over-expression of certain growth factor receptor kinases such as epidermal growth factor receptor (EGFR) has been implicated as being important in cancer [19]. EGFR is over-expressed in plenty tumors including those derived from the brain, lung, bladder, head and neck [20]. In order to test whether compound **8c** modulates the expression of EGFR, we performed ELISA assay and western blot.

As shown in Figure 4A, the assay was used to manufacture standard curve and rapidly examine the EGFR concentration of sample. The protein levels of EGFR were measured and the results were analyzed (Figure 4B). The protein levels of EGFR in MCF-7 cells were significantly decreased in a dose-dependent manner, after treated with **8c** for 48 h. It is demonstrated that **8c** was moderately active inhibitors of EGFR kinase. As shown in Figure 5, western blot assay was employed to evaluate the inhibition of EGFR autophosphorylation of compound **8c** in MCF-7 cell lines. Importantly, **8c** did not affect the overall EGFR content in MCF-7 cells at any concentration tested, indicating that p-EGFR suppression was not an artifact of compound cytotoxicity. The results suggested that compound **8c** can block EGFR phosphorylation in a dose-dependent manner in MCF-7 cell line.

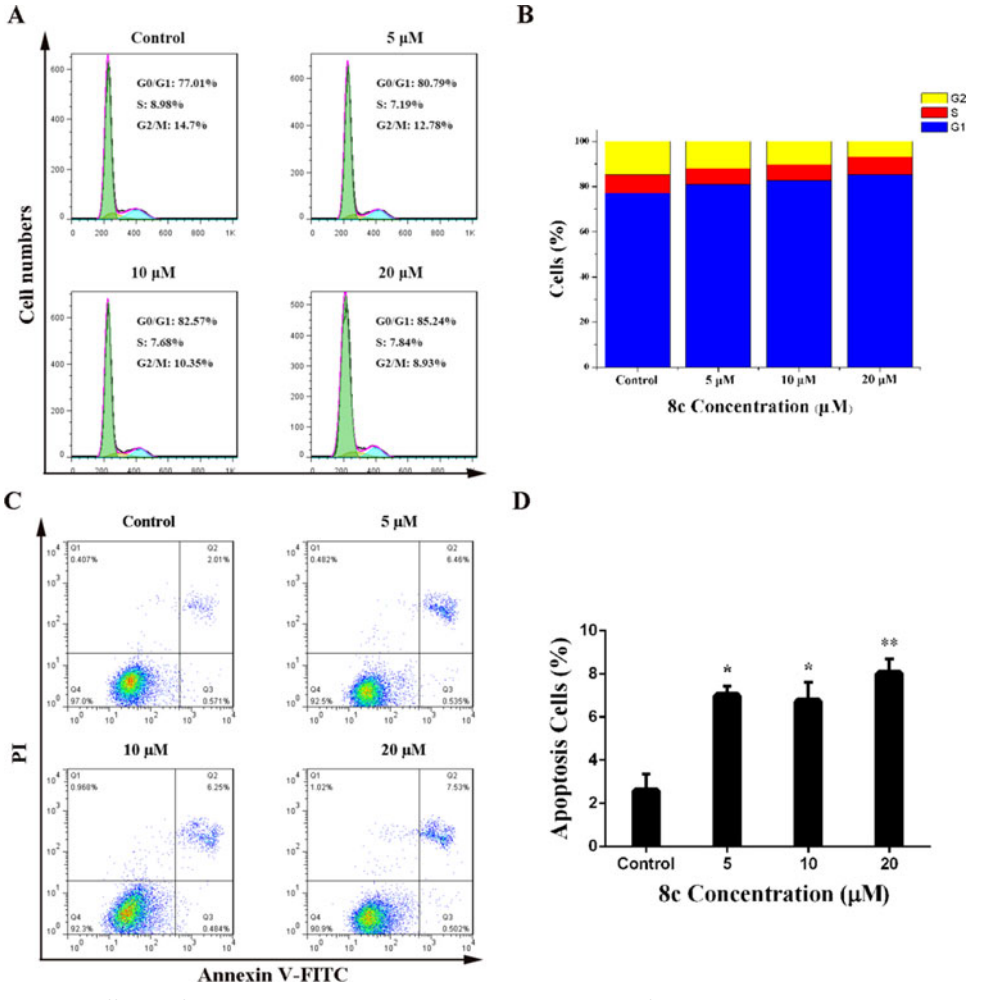



**Figure 2.** Effects of compound **8c** on cell cycle and apoptosis of MCF-7 cells. (A) Flow cytometry analysis of cell cycle distribution for MCF-7 cells treated with compound **8c** (0, 5, 10, 20 μm) for 48 h. (B) Compound **8c** blocked cell cycle G1/S arrest of MCF-7 cells and also had effects in the G2 phase. (C) Apoptotic effect of compound **8c** on MCF-7 cell line after treatment for 48 h. Q1, Q2, Q3 and Q4 respectively represent cells damaged during the procedure, the earlier apoptotic cells, the late apoptotic cells and live cells. (D) Compound **8c** increased cell apoptotic numbers. Error bar show the SD, "*" $p < 0.05$, "**" $p < 0.01$, compared with the control cells at 48 h.

### *2.2.5. Binding mode of 8c into EGFR*

To get deeper insights into the possible binding mode of the designed chrysin derivatives in target kinase, the most potent inhibitor **8c** was docked into the ATP binding site of this model of EGFR complex structure (PDB ID: 1M17). This model was able to give an explanation and understanding of good activity observed. As shown in Figure 1, in the binding model of compound **8c** and EGFR, there are three hydrogen bonds. The carboxyl group on the L-leucine formed a hydrogen bond with ASP-831, and carbonyl group of L-leucine also formed H-bond with LYS-721. Moreover, the oxygen on the 7-position formed a H-bond with the backbone of THR-830. The

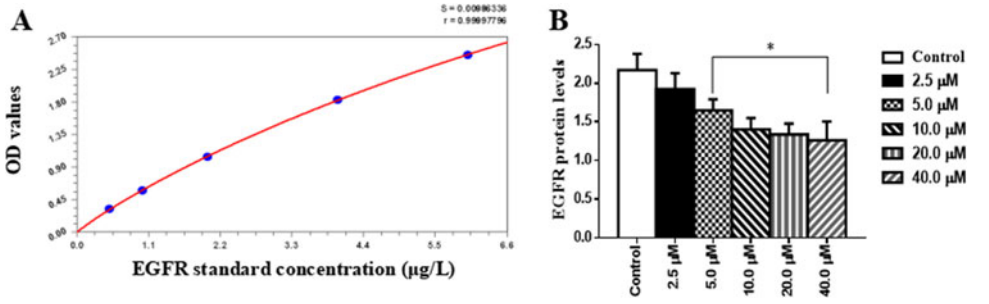



**Figure 3.** (A) The double antibody sandwich-ELISA was used to manufacture standard curve and rapidly examine the EGFR concentration of sample. (B) EGFR kinase inhibitory activity of **8c** on MCF-7 cells after treatment for 48 h. Error bar show the SD, "*" $p < 0.05$, compared with the control cells at 48 h.

molecular docking results, along with the kinase assay data, illustrated that compound **8c** may have a potential inhibitory effect on EGFR.

## 3. Experimental

### 3.1. Chemistry

The reagents (chemical) and solvents were bought from *Aladdin Chemical* (Shanghai, China), and used without further purification. The tetramethylsilane (TMS) and solvent signals were used as internal standards at 25 °C, and $^1$H-NMR was recorded by using a Bruker AVANCE-500 spectrometer (Sweden, Germany). Melting points were determined on a *Thermo Scientific* electrothermal digital melting point apparatus (California, USA). Mass spectra were acquired on APCI-MS spectrometer (Thermo, California, USA) and elemental analysis was measured on *Perkin Elmer* 2400 CHN (Waltham, USA).

The $^1$H NMR and $^{13}$C NMR spectra data of compounds **3a–8d** were supplied as supplementary material.

### 3.2. Ethyl 2-((5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy) propanoate (3a)

The mixture of chrysin (2.54 g, 0.01 mol) and $K_2CO_3$ (1.59 g, 0.0115 mol) was added in acetone (150 ml), keeping the reaction solution below 60 °C. The resulting mixture was stirred at 60 °C for 1 h, then ethyl 2-bromopropionate (1.81 g, 0.011 mol) was added dropwise into the solution. The mixture was kept stirring at 60 °C for 12 h. After TLC detection to show no starting materials, the mixture was cooled to room temperature and then the precipitate was removed by filtration. The filtrate was concentrated and the residue was purified by column chromatography on silica gel, eluting with dichloromethane/acetone (50:1) to give a light yellow solid **3a** (2.55 g, yield 60%) [14]. m.p.100–103 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.81 (s, 1 H), 8.09 - 7.95 (m, 2 H), 7.69 – 7.65 (m, 3 H), 6.77 (d, $J = 2.0$ Hz, 2 H), 6.38 (d, $J = 2.0$ Hz, 1 H), 5.21 - 5.18 (m, 1 H), 4.19 - 4.12 (m, 2 H), 1.56 (d, $J = 6.7$ Hz, 3 H), 1.21 (s, 3 H).; $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 171.1 (s), 164.1 (s), 163.7 (s), 161.7 (s), 157.7

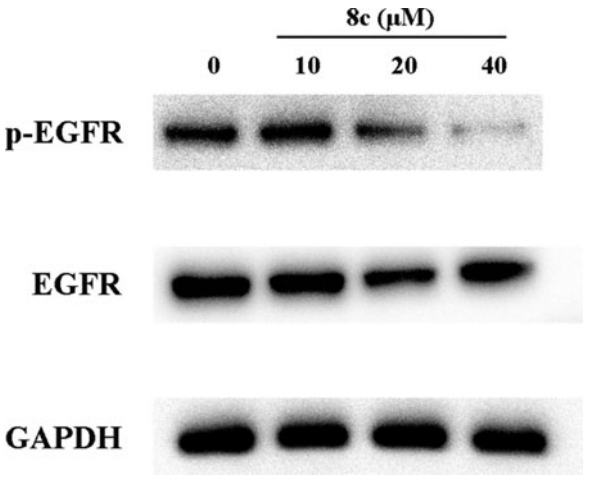



**Figure 4.** Inhibition of EGFR autophosphorylation of compound **8c** in MCF-7 cell line.

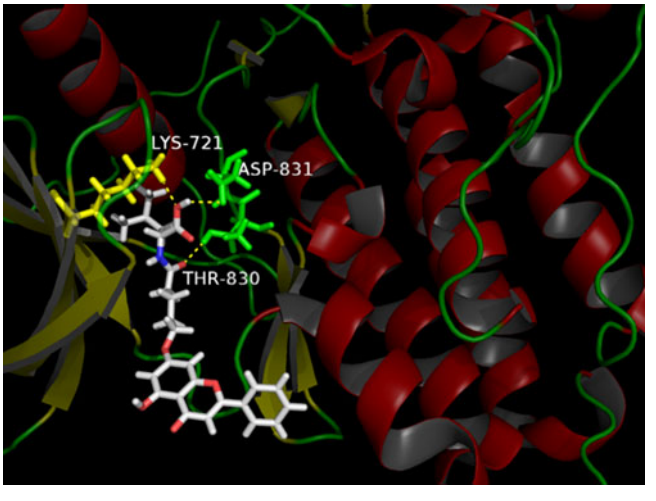



**Figure 5.** The crystal structure of EGFR (PDB ID:1M17, download from the PDB) in complex with potent compound **8c**. The protein and ligand are colored in red and green, respectively. Hydrogen bonds are represented with dotted yellow lines. The image was generated with Pymol.

(s), 132.7 (s), 131.0 (s), 129.6 (s), 129.6 (s), 127.0 (s), 127.0 (s), 105.9 (s), 105.7 (s), 99.2 (s), 94.3(s), 72.6 (s), 61.6 (s), 18.4 (s), 14.5 (s). ESI-MS: *m/z* 354.11 $[M+H]^+$. Elemental analysis: Found: C, 67.82%; H, 5.09%; calcd for $C_{20}H_{18}O_6$: C, 67.79%; H, 5.12%.

### *3.3. Ethyl 5-((5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)pentanoate (3b)*

A mixture of chrysin (2.54 g, 0.01 mol) and anhydrous potassium carbonate (1.59 g, 0.0115 mol) was dissolved in acetone (150 ml) and stirred at 60 °C for 1 h. Ethyl 5-bromovalerate (2.09 g, 0.011 mol) was added to the reaction mixture and then stirred for 12 h at 60 °C. When TLC showed the reaction was completed, evaporation of the solvent under reduced pressure gave a residue, which was purified by column

chromatography on silica gel, eluting with dichloromethane/acetone (50:1) to give a light yellow solid **3b** [14]. m.p.101.0–102.1 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.77 (s, 1 H), 8.07 - 8.00 (m, 2 H), 7.68 - 7.65 (m, 3 H), 6.99 (s, 1 H), 6.76 (s, 1 H), 6.34 (s, 1 H), 4.14 - 4.06 (m, 4 H), 2.38 (t, $J$ = 6.9 Hz, 2 H), 1.74- 1.63 (m, 4 H), 1.19 (s, 3 H).$^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 173.2 (s), 165.1 (s), 161.6 (s), 157.8 (s), 132.6 (s), 131.1 (s), 129.6 (s), 129.6 (s), 126.9 (s), 126.9 (s), 105.8 (s), 105.3 (s), 98.9 (s), 93.6 (s), 68.6 (s), 60.2 (s), 33.5 (s), 28.2 (s), 21.5 (s), 14.6 (s). ESI-MS: *m/z* 382.14 $[M+H]^+$. Elemental analysis: Found: C, 69.05%; H, 5.82%; calcd for $C_{22}H_{22}O_6$: C, 69.10%; H, 5.80%.

### 3.4. General synthetic procedure of the compounds 4a–4b

The mixture of compound **3a** or **3b** (0.005 mol) in $CH_3OH$ (50 ml) was treated with 1 N NaOH (pH = 10–11). After 6 h at 60 °C, the solvent was treated with 1 N HCl (pH= 2–3). After standing 6 h, the residue was filtered and washed with saturated salt water, 3.8% HCl and water, and dried *in vacuo* to give light yellow solid **4a** or **4b** [14].

#### 3.4.1. 2-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)propanoic acid (4a)

Yield: 90%, m.p.241.6–243.5 °C.$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.80 (s, 1 H), 8.08 - 8.00 (m, 2 H), 7.65 – 7.63 (m, 3 H), 7.02 (s, 1 H), 6.73 (d, $J$ = 1.9 Hz, 1 H), 6.35 (d, $J$ = 1.9 Hz, 1 H), 5.13 - 5.07 (m, 1 H), 1.56 (d, $J$ = 6.7 Hz, 3 H); $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.50 (s), 172.70 (s), 164.0 (d, $J$ = 7.0 Hz), 161.7 (s), 157.7 (s), 132.6 (s), 131.0 (s), 129.6 (s), 129.6 (s), 126.9 (s), 126.9 (s), 105.8 (s), 105.6 (s), 99.2 (s), 94.1 (s), 72.5 (s), 18.5 (s). ESI-MS: *m/z* 326.08 $[M+H]^+$. Elemental analysis: Found: C, 66.26%; H, 4.32%; calcd for $C_{18}H_{14}O_6$: C, 66.30%; H, 4.29%.

#### 3.4.2. 7-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeric acid (4b)

Yield: 90%, m.p. 174.2–175.5 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1 H), 12.07 (s, 1 H), 8.10 - 8.09 (m, 2 H), 7.72 – 7.46 (m, 3 H), 7.02 (s, 1 H), 6.79 (s, 1 H), 6.37 (s, 1 H), 4.12 - 4.09 (m, 2 H), 2.30 - 2.33 (m, 2 H), 1.81 – 1.72 (m, 2 H), 1.72 – 1.59 (m, 2 H).$^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.51 (s), 174.8 (d, $J$ = 1.0 Hz), 165.2 (s), 163.9 (s), 161.6 (s), 157.8 (s), 132.6 (s), 131.1 (s), 129.6 (s), 129.6 (s), 126.9 (s), 126.9 (s), 105.8 (s), 105.3 (s), 98.9 (s), 93.7 (s), 68.7 (s), 33.7 (s), 28.3 (s), 21.5 (s). ESI-MS: *m/z* 354.11 $[M+H]^+$. Elemental analysis: Found: C, 67.81%; H, 5.09%; calcd for $C_{20}H_{18}O_6$: C, 67.79%; H, 5.12%.

### 3.5. General synthetic procedure of the compounds 5a–6d

Compound **4a** or **4b** (0.5 mmol) was dissolved in DMF (10 ml) and treated with HOBt (270.2 mg, 2 mmol) and EDCl (383.4 mg, 2 mmol). The mixture was stirred for 1 h at 0–4 °C. Then, DIPEA (349.3 $\mu$l, 2 mmol) and amino acid methyl ester hydrochloride (2 mmol) were added drop by drop. After reaction at room temperature for 24 h, the cold water (50 ml) was added to the mixture. The residue was filtered and

purified with a silia gel column and was eluted with dichloromethane/acetone (12:1) to afford a yellow solids **5a–6d**.

### 3.5.1. N-(2-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)isopropionyl)-L-glycine methyl ester (5a)

Yield: 60%, m.p.176.0–177.8 °C.$^{1}$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1 H), 8.71 (t, $J$ = 5.9 Hz, 1 H), 8.11 - 8.13 (m, 2 H), 7.69 – 7.55 (m, 3 H), 7.06 (s, 1 H), 6.80 (d, $J$ = 2.0 Hz, 1 H), 6.42 (d, $J$ = 2.0 Hz, 1 H), 5.00 - 4.98 (m, 1 H), 3.91 - 3.87 (m, 2 H), 3.63 (s, 3 H), 1.51 (d, $J$ = 6.6 Hz, 3 H).$^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.1 (s), 171.0 (s), 164.5 (s), 163.1 (s), 161.1 (s), 157.2 (s), 132.2 (s), 130.6 (s), 129.1 (d), 126.5 (d), 105.4 (s), 105.2 (s), 99.2 (s), 93.2 (s), 73.8 (s), 51.7 (s), 40.4 (s), 18.5 (s). ESI-MS: $m/z$ 397.12 $[M+H]^+$. Elemental analysis: Found: C, 63.48%; H, 4.81%; N, 3.50%; calcd for $C_{21}H_{19}O_7$: C, 63.47%; H, 4.82%; N, 3.52%.

### 3.5.2. N-(2-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)isopropionyl)-L-alanine methyl ester (5b)

Yield: 55%, m.p.178.2–180.0 °C.$^{1}$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1 H), 8.75 - 8.69 (m, 1 H), 8.11 - 8.09 (m, 2 H), 7.67 – 7.54 (m, 3 H), 7.07 (s, 1 H), 6.75 (s, 1 H), 6.40 (d, $J$ = 6.3 Hz, 1 H), 4.96 - 4.89 (m, 1 H), 4.37 - 4.36 (m, 1 H), 3.67 – 3.56 (m, 3 H), 1.51 (d, $J$ = 6.2 Hz, 3 H), 1.34 (d, $J$ = 7.2 Hz, 3 H). $^{13}$C NMR (126 MHz, $CDCl_3$) $\delta$ 182.4 (s), 172.9 (s), 170.7 (s), 164.2 (s), 162.6 (s), 162.4 (s), 157.8 (s), 132.0 (s), 131.1 (s), 129.1 (s), 126.3 (s), 106.5 (s), 105.9 (s), 99.6 (s), 99.4 (s), 93.9 (s), 93.6 (s), 75.2 (s), 52.6 (s), 47.8 (s), 18.6 (s), 18.1 (s). ESI-MS: $m/z$ 411.13 $[M+H]^+$. Elemental analysis: Found: C, 64.25%; H, 5.14%; N, 3.39%; calcd for $C_{22}H_{21}O_7$: C, 64.23%; H, 5.15%; N, 3.40%.

### 3.5.3. N-(2-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)isopropionyl)-L-leucine methyl ester (5c)

Yield: 58%, m.p. .84.9–86.4 °C.$^{1}$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.78 (s, 1 H), 8.68 - 8.67 (m, 1 H), 8.14 - 8.08 (m, 2 H), 7.67 – 7.57 (m, 3 H), 7.07 (s, 1 H), 6.72 - 6.71 (m, 1 H), 6.39 (s, 1 H), 5.01 – 4.88 (m, 1 H), 4.42 – 4.29 (m, 1 H), 3.61 - 3.59 (m, 3 H), 1.51 (t, $J$ = 6.2 Hz, 2 H), 0.91 (s, 3 H), 0.87 (s, 3 H).$^{13}$C NMR (126 MHz, $CDCl_3$) $\delta$ 182.3 (s), 172.9 (s), 172.7 (s), 171.0 (s), 162.7 (s), 132.0 (s), 131.0 (s), 129.1 (s), 126.2 (s), 105.8 (s), 99.7 (s), 99.5 (s), 93.9 (s), 93.5 (s), 75.3 (s), 52.3 (s), 50.3 (s), 41.1 (s), 25.0 (s), 24.8 (s), 22.7 (s), 21.9 (s), 21.5 (s), 18.50 (s), 1.0 (s). ESI-MS: $m/z$ 453.18 $[M+H]^+$. Elemental analysis: Found: C, 66.18%; H, 6.02%; N, 3.10%; calcd for $C_{25}H_{27}O_7$: C, 66.21%; H, 6.00%; N, 3.09%.

### 3.5.4. N-(2-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)isopropionyl)-L-phenyl-alanine methyl ester (5d)

Yield: 59%, m.p 162.3–164.0 °C.$^{1}$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1 H), 8.84 - 8.83 (m, 1 H), 8.10 - 8.09 (m, 2 H), 7.67 – 7.55 (m, 3 H), 7.20 - 7.12 (m, 5 H), 7.05 (d, $J$ = 3.7 Hz, 1 H), 6.65 - 6.63 (m, 1 H), 6.33 - 6.31 (m, 1 H), 4.94 - 4.87 (m, 1 H), 4.55 - 4.54 (m, 1 H), 3.66 (s, 3 H), 3.15 – 2.90 (m, 2 H), 1.36 (d, 6.6 Hz, 3 H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 172.2 (s), 172.0 (s), 171.1 (s), 170.8 (s), 163.6

(s), 161.7 (s), 161.5 (s), 157.6 (s), 137.8 (d), 132.7 (d), 131.1 (s), 129.7 (s), 129.5 (s), 128.6 (s), 128.4 (s), 126.9 (s), 105.3 (d), 99.9(s), 94.6(s), 74.3(s), 53.9 (s), 52.4 (s), 18.9 (s). ESI-MS: *m/z* 487.16 $[M+H]^+$. Elemental analysis: Found: C, 69.03%; H, 5.17%; N, 2.89%; calcd for $C_{28}H_{25}O_7$: C, 68.99%; H, 5.17%; N, 2.87%.

### 3.5.5. N-(7-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeryl)-L-glycine methyl ester (6a)

Yield: 55%, m.p. 164.3–166.0 °C.$^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 12.67 (s, 1 H), 7.86 - 7.84 (m, 2 H), 7.57 – 7.45 (m, 3 H), 7.29 (s, 1 H), 6.64 (s, 1 H), 6.47 – 6.25 (m, 3 H), 4.10 - 4.07 (m, 3 H), 3.76 - 3.74 (m, 3 H), 2.40 - 2.37 (m, 2 H), 1.87 (s, 3 H).$^{13}$C NMR (126 MHz, $CDCl_3$) $\delta$ 182.4 (s), 172.8 (s), 170.6 (s), 165.0 (s), 163.9 (s), 162.0 (s), 157.7 (s), 131.8 (s), 131.2 (s), 129.1 (s), 129.1 (s), 126.3 (s), 126.3 (s), 105.7 (d), 98.6 (s), 93.0 (s), 68.2 (s), 52.4 (s), 41.2 (s), 35.7 (s), 28.4 (s), 22.1 (s). ESI-MS: *m/z* 425.15 $[M+H]^+$. Elemental analysis: Found: 64.91%; H, 5.47%; N, 3.29%; calcd for $C_{23}H_{23}O_7$: C, 64.93%; H, 5.45%; N, 3.29%.

### 3.5.6. N-(7-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeryl)-L-alanine methyl ester (6b)

Yield: 60%, m.p. 151.8–153.2 °C.$^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 12.67 (s, 1 H), 7.85 - 7.83 (m, 2 H), 7.40 - 7.35 (m, 3 H), 6.65 (s, 1 H), 6.50 – 5.89 (m, 4 H), 4.62 (d, $J = 5.8$ Hz, 1 H), 3.76 (s, 3 H), 2.08 - 2.05 (m, 2 H), 1.86 - 1.65 (m, 4 H), 1.42 (s, 3 H).$^{13}$C NMR (126 MHz, $CDCl_3$) $\delta$ 182.4 (s), 173.7 (s), 172.2 (s), 165.0 (s), 163.9 (s), 162.0 (s), 157.7 (s), 131.8 (s), 131.2 (s), 129.1 (s), 126.3 (s), 105.7 (s), 98.6 (s), 93.0 (s), 68.2 (s), 52.5 (s), 48.0 (s), 35.8 (s), 28.4 (s), 22.1 (s), 18.4 (s). ESI-MS: *m/z* 439.16 $[M+H]^+$. Elemental analysis: Found: C, 65.60%; H, 5.71%; N, 3.20%; calcd for $C_{24}H_{25}O_7$: C, 65.59%; H, 5.73%; N, 3.19%.

### 3.5.7. N-(7-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeryl)-L-leucine methyl ester (6c)

Yield: 49%, m.p. 139.1–140.5 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1 H), 8.28 - 8.27 (m, 1 H), 8.10 - 8.09 (m, 2 H), 7.60 - 7.57 (m, 3 H), 7.02 (s, 1 H), 6.79 (s, 1 H), 6.37 (s, 1 H), 4.38 – 4.21 (m, 1 H), 4.09 - 4.07 (m, 2 H), 2.51 (s, 1 H), 2.22 - 2.20 (m, 2 H), 1.77 – 1.70 (m, 2 H), 1.69 – 1.63 (m, 2 H), 1.59 – 1.49 (m, 2 H), 0.88 (d, $J = 6.5$ Hz, 3 H), 0.83 (d, $J = 6.5$ Hz, 3 H).$^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 173.7 (s), 172.7 (s), 165.2 (s), 163.9 (s), 161.6 (s), 157.8 (s), 132.6 (s), 131.1 (s), 129.6 (s), 126.9 (s), 105.8 (s), 105.3 (s), 99.0 (s), 93.6 (s), 68.6 (s), 52.2 (s), 50.6 (s), 34.9 (s), 28.2 (s), 24.7 (s), 23.2 (s), 22.2 (s), 21.6 (s). ESI-MS: *m/z* 481.21 $[M+H]^+$. Elemental analysis: Found: C, 67.31%; H, 6.51%; N, 2.93%; calcd for $C_{27}H_{31}O_7$: C, 67.35%; H, 6.49%; N, 2.91%.

### 3.5.8. N-(7-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeryl)-L-phenylalanine methyl ester (6d)

Yield: 52%, m.p. 151.2–153.0 °C.$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.78 (s, 1 H), 8.46 - 8.45 (m, 1 H), 8.07 - 8.05 (m, 2 H), 7.59 - 7.56 (m, 3 H), 7.32 – 7.15 (m, 5 H), 6.99 (s, 1 H), 6.74 (s, 1 H), 6.33 (s, 1 H), 4.50 (d, $J = 4.2$ Hz, 1 H), 4.01 (s, 2 H), 3.60 (s,

3 H), 3.04 - 2.97 (m, 2 H), 2.16 - 1.58 (m, 6 H).$^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 172.7 (s), 172.6 (s), 165.2 (s), 163.8 (s), 161.6 (s), 157.8 (s), 137.8 (s), 132.6 (s), 131.0 (s), 129.6 (s), 129.5, 128.6 (s), 128.6 (s), 127.0 (s), 126.9, 126.9 (s), 105.7 (s), 105.3 (s), 98.9 (s), 93.6 (s), 68.6 (s), 53.9 (s), 52.3 (s), 37.1 (s), 34.9 (s), 28.1 (s), 22.1 (s). ESI-MS: *m/z* 515.19 $[M+H]^+$. Elemental analysis: Found: C, 67.86%; H, 5.69%; N, 2.73%; calcd for $C_{30}H_{29}O_7$: C, 69.89%; H, 5.67%; N, 2.72%.

### 3.6. General method of synthesis of compounds 7a–8d

0.1 N KOH (10 ml) was added to a stirred mixture of **5a–6d** (0.2 mmol) in absolute ethyl alcohol (10 ml), followed by stirring for 24 h at room temperature.The pH of mixture was adjusted to 2–3 with 1 N $H_2SO_4$ solution.The residue was filtered off, which was subsequently washed with saturated NaCl solution and water, and dried under vacuum to afford **7a–8d**.

#### 3.6.1. N-(2-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)isopropionyl)-L-glycine (7a)

Yield: 60%, m.p. 250.0–251.5 °C.$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.77 (s, 1 H), 8.57 (s, 1 H), 8.05 - 8.04 (m, 2 H), 7.59 - 7.56 (m, 3 H), 7.00 (s, 1 H), 6.75 (s, 1 H), 6.39 (s, 1 H), 4.96 (d, $J$ = 6.1 Hz, 1 H), 3.88 - 3.79 (m, 2 H), 1.51 (d, $J$ = 5.7 Hz, 3 H).$^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 171.4 (d), 164.0 (s), 163.6 (s), 161.6 (s), 157.6 (s), 132.6 (s), 131.0 (s), 129.6 (s), 129.6 (s), 126.9 (s), 126.9 (s), 105.8 (d), 99.7 (s), 94.3 (s), 74.3 (s), 41.0 (s), 19.0 (s). ESI-MS: *m/z* 383.10 $[M+H]^+$. Elemental analysis: Found: C, 62.67%; H, 4.48%; N, 3.63%; calcd for $C_{20}H_{17}O_7$: C, 62.66%; H, 4.47%; N, 3.65%.

#### 3.6.2. N-(2-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)isopropionyl)-L-alanine (7b)

Yield: 55%, m.p.163.1–164.8 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1 H), 12.77 (s, 1 H), 8.57 - 8.56 (m, 1 H), 8.07 - 8.06 (m, 2 H), 7.60 - 7.54 (m, 3 H), 7.02 (s, 1 H), 6.73 (s, 1 H), 5.02 – 4.89 (m, 1 H), 4.31 - 4.27 (m, 1 H), 1.50 (d, $J$ = 6.0 Hz, 3 H), 1.39 – 1.31 (m, 2 H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 174.1 (s), 170.7 (s), 170.5 (s), 164.1 (s), 163.7 (s), 161.6 (s), 157.6 (s), 132.6 (s), 131.1 (s), 129.6 (s), 126.9 (s), 105.9 (s), 105.6 (s), 99.5 (s), 94.2 (s), 74.1 (s), 47.9 (s), 19.1 (s), 18.8 (s), 17.5 (s). ESI-MS: *m/z* 397.12 $[M+H]^+$. Elemental analysis: Found: C, 63.49%; H, 4.78%; N, 3.54%; calcd for $C_{21}H_{19}O_7$: C, 63.47%; H, 4.82%; N, 3.52%.

#### 3.6.3. N-(2-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)isopropionyl)-L-leucine (7c)

Yield: 58%, m.p. 164.5–166.4 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.71 (s, 1 H), 8.54 - 8.53 (m, 1 H), 8.22 – 7.95 (m, 2 H), 7.60 - 7.57 (m, 3 H), 7.02 (s, 1 H), 6.68 - 6.67 (m, 1 H), 6.35 - 6.34 (m, 1 H), 5.08 – 4.78 (m, 1 H), 4.32 - 4.31 (m, 1 H), 3.39 (s, 1 H), 1.50 - 1.49 (m, 5 H), 0.81 (s, 3 H), 0.76 (s, 3 H). $^{13}$C NMR (126 MHz, $CDCl_3$) $\delta$ 182.4 (s), 171.6 (s), 164.3 (s), 162.7 (s), 157.7 (s), 157.6 (s), 132.1 (s), 130.8 (s), 130.7 (s), 129.1 (s), 129.0 (s), 126.3 (s), 126.2 (s), 106.2 (s), 105.6 (s), 105.5 (s), 99.6 (s),

93.7 (s), 75.1 (s), 40.9 (s), 24.9 (s), 22.8 (s), 21.5 (s), 18.5 (s). ESI-MS: *m/z* 437.18 $[M+H]^+$. Elemental analysis: Found: C, 65.60%; H, 5.72%; N, 3.17%; calcd for $C_{24}H_{25}O_7$: C, 65.59%; H, 5.73%; N, 3.19%.

### 3.6.4. N-(2-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)isopropionyl)-L-phenylalanine (7d)

Yield: 59%, m.p. 197.6–199.5 °C.$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.78 (s, 1 H), 8.47 - 8.46 (m, 1 H), 8.05 - 8.03 (m, 2 H), 7.58 - 7.55 (m, 3 H), 7.18 - 7.13 (m, 5 H), 7.06 – 6.94 (m, 1 H), 6.59 - 6.58 (m, 1 H), 6.32 - 6.31 (m, 1 H), 4.87 - 4.86 (m, 1 H), 4.53 (s, 1 H), 3.56 – 2.78 (m, 2 H), 1.33 (d, 6.6 Hz, 3 H), 0.82 (s, 1 H) . $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 173.2 (s), 173.0 (s), 171.0 (s), 170.6 (s), 164.0 (s), 163.6 (s), 161.6 (s), 161.5 (s), 157.6 (s), 138.0 (s), 132.6 (s), 131.1 (s), 129.6 (s), 129.4 (s), 128.5 (s), 128.3 (s), 126.9 (s), 105.9 (s), 99.6 (s), 94.3 (s), 93.9 (s), 74.3 (s), 53.5 (s), 19.0 (s), 18.9 (s). ESI-MS: *m/z* 473.15 $[M+H]^+$. Elemental analysis: Found: C, 68.53%; H, 4.92%; N, 2.98%; calcd for $C_{27}H_{23}O_7$: C, 68.49%; H, 4.90%; N, 2.96%.

### 3.6.5. N-(7-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeryl)-L-glycine (8a)

Yield: 55%, m.p. 201.8–202.5 °C.$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1 H), 8.16 - 8.15 (m, 1 H), 8.09 - 8.07 (m, 2 H), 7.60 - 7.57 (m, 3 H), 7.02 (s, 1 H), 6.79 (s, 1 H), 6.37 (s, 1 H), 4.10 (s, 2 H), 3.74 (d, $J$ = 5.4 Hz, 2 H), 2.22 (t, $J$ = 6.8 Hz, 2 H), 1.75 (s, 2 H), 1.68 (d, $J$ = 6.8 Hz, 2 H).$^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 172.8 (s), 171.9 (s), 165.2 (s), 161.6 (s), 157.8 (s), 132.6 (s), 131.1 (s), 129.6 (s), 129.6 (s), 126.9 (s), 126.9 (s), 105.8 (s), 105.3 (s), 99.0 (s), 93.6 (s), 68.7 (s), 41.2 (s), 35.0 (s), 28.3 (s), 22.2 (s). ESI-MS: *m/z* 411.13 $[M+H]^+$. Elemental analysis: Found: C, 64.25%; H, 5.14%; N, 3.39%; calcd for $C_{22}H_{21}O_7$: C, 64.23%; H, 5.15%; N, 3.40%.

### 3.6.6. N-(7-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeryl)-L-alanine (8b)

Yield: 60%, m.p. 202.4–203.5 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.72 (s, 1 H), 8.16 - 8.15 (m, 1 H), 7.99 - 7.97 (m, 2 H), 7.55 - 7.52 (m, 3 H), 6.89 (s, 1 H), 6.63 (s, 1 H), 6.25 (s, 1 H), 4.28 – 4.18 (m, 1 H), 4.01 (s, 2 H), 3.18 (s, 1 H), 2.21 (s, 2 H), 1.68 - 1.64 (m, 4 H), 1.25 (d, 7.0 Hz, 3 H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.3 (s), 174.8 (s), 172.3 (s), 165.1 (s), 163.7 (s), 161.6 (s), 157.7 (s), 132.4 (s), 131.0 (s), 129.5 (s), 129.5 (s), 126.7 (s), 126.7 (s), 105.6 (s), 105.2 (s), 98.8 (s), 93.4 (s), 68.6 (s), 47.9 (s), 35.0 (s), 28.3 (s), 22.2 (s), 17.7 (s). ESI-MS: *m/z* 425.15 $[M+H]^+$. Elemental analysis: Found: C, 64.93%; H, 5.44%; N, 3.30%; calcd for $C_{23}H_{23}O_7$: C, 64.93%; H, 5.45%; N, 3.29%.

### 3.6.7. N-(7-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeryl)-L-leucine (8c)

Yield: 49%, m.p. 152.5–154.0 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1 H), 12.47 (s, 1 H), 8.09 - 8.07 (m, 2 H), 7.74 – 7.49 (m, 3 H), 7.02 (s, 1 H), 6.79 (s, 1 H), 6.37 (s, 1 H), 4.31 – 4.21 (m, 1 H), 4.10 (t, $J$ = 6.1 Hz, 2 H), 2.21 (d, $J$ = 2.3 Hz, 2 H), 1.73 - 1.43 (m, 8 H), 0.85 (s, 3 H), 0.86 (s, 3 H).$^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 174.8 (s), 172.5 (s), 165.2 (s), 163.9 (s), 161.6 (s), 157.8 (s), 132.6 (s), 131.1 (s), 129.6 (s), 129.6 (s), 126.9 (s), 126.9 (s), 105.8 (s), 105.3 (s), 99.0 (s), 93.6 (s), 68.7 (s), 50.6 (s), 35.0 (s), 28.3 (s), 24.8 (s), 23.3 (s), 22.2 (s), 21.7 (s). ESI-MS: *m/z* 467.19

$[M+H]^+$. Elemental analysis: Found: C, 66.81%; H, 6.25%; N, 2.99%; calcd for $C_{26}H_{29}O_7$: C, 66.80%; H, 6.25%; N, 3.00%.

#### 3.6.8. N-(7-((5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yl)oxy)valeryl)-L-phenylalanine (8d)

Yield: 52%, m.p. 204.5–206.8 °C. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 12.79 (s, 1 H), 12.72 (s, 1 H), 8.21 - 8.20 (m, 1 H), 8.07 - 8.05 (m, 2 H), 7.58 - 7.55 (m, 3 H), 7.22 - 7.17 (m, 5 H), 7.00 (s, 1 H), 6.73 (s, 1 H), 6.33 (s, 1 H), 4.48 - 4.47 (m, 1 H), 4.00 (s, 2 H), 3.08 - 2.76 (m, 2 H), 2.15 - 1.58 (m, 6 H). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 182.5 (s), 173.0 (s), 170.0 (s), 164.1 (s), 164.0 (s), 161.6 (s), 157.6 (s), 138.0 (s), 132.7 (s), 131.1 (s), 129.6 (s), 129.4 (s), 129.4 (s), 128.4 (s), 128.3 (s), 127.0 (s), 126.7 (s), 106.0 (s), 105.7 (s), 99.6 (s), 94.4 (s), 94.0 (s), 78.0 (s), 53.3 (s), 36.7 (s), 28.7 (d, $J = 7.0$ Hz), 24.6 (s), 22.4 (s). ESI-MS: *m/z* 501.18 $[M+H]^+$. Elemental analysis: Found: C, 69.44%; H, 5.44%; N, 2.79%; calcd for $C_{29}H_{27}NO_7$: C, 69.45%; H, 5.43%; N, 2.79%.

### 3.7. Biology

#### 3.7.1. MTT cytotoxicity assay

The antiproliferative activities of those novel compounds were determined by the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method. Two cancer cells were seeded in a density in 96-well plates ($5 \times 10^3$ cells per well). After 24 h of incubation, cells were treated with different concentrations of compounds (16, 32, 64, 128 $\mu$mol/L) for 48 h. Then, 0.5% MTT solution was added to each well. After 4 h of incubation, formazan formed from MTT was extracted by adding 200 $\mu$l DMSO. Cell viability was assessed at 570 nm by a microplate reader (*Biotech*, Shanghai, China). The concentration causing 50% inhibition of cells growth ($IC_{50}$) was determined by the Logit method.

#### 3.7.2. Clonogenicity assay

To determine the survival of the MCF-7 cells treated with compound **8c**, 1000 tumor cells were planted in six-well plate and incubated for 24 h. Then, fresh medium containing different concentrations (0, 5, 10, 20 $\mu$mol/L) of test compound was added to the plate. After incubation for 10 day at 37 °C, the cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The cells were imaged.

#### 3.7.3. Wound healing assay

Cells were seeded in 6-well plate and allowed to grow to nearly 100% confluence in culture medium. Subsequently, a cell-free line was manually formed by scratching the confluent cell monolayers with a 200 $\mu$l pipette tip. Then the cells were treated with different concentrations of potent compound and photographed on an inverted microsope.

#### 3.7.4. Cell cycle assay

Cell cycle assay was determined by Cell Cycle and Apoptosis Analysis Kit (*Beyotime Biotechnology*, Jiangsu, China), according to the manufacturer's instructions and reference methods [21].

#### 3.7.5. Cell apoptosis assay

Cells ($2 \times 10^5$ cells per well) were treated with different concentrations of potent compound for 48 h. Then, cells were collected after incubation, washed twice in cold phosphate buffer saline (PBS), and treated with the Annexin-V-FITC/PI apoptosis kit (*MultiSciences Biotech*., Hangzhou, China). The stained cells were analyzed by flow cytometry [22].

#### 3.7.6. The assay for EGFR inhibition

Human EGFR ELISA kit was purchased from *Boster Biological Technology* (Wuhan, China. Catalog #EK0327). Human EGFR were expressed and purified as described [23]. The experiments were performed according to the manufacturer's instructions. The inhibition rate (%) was calculated using the following equation: [1-(A490/A490control)] × 100%.

#### 3.7.7. Western blotting

Western blot analysis was carried out as described previously [13]. Cells were treated with compound **8c** or DMSO for a specified period time. Then, the cells were washed twice with cold PBS and lysed in cold radioimmunoprecipitation assay (RIPA) buffer. Lysates were cleared by centrifugation. Antibodies against the following were used: phospho-EGFR and EGFR, GAPDH (All from *Cell Signaling Technology,* Beverly, MA, USA).

### 3.8. Molecule docking simulations

To explore the interaction mechanism between the newly synthesized amino acid derivatives containing chrysin and target protein, molecular docking for compound **8c** into three-dimensional EGFR complex structure (PDB ID:1M17, download from the PDB) was carried out using the SYBYL-XL 2.0 [24]. The image was generated with Pymol [25].

## Disclosure statement

The authors declare that there are no conflicts of interest.

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