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Design, synthesis and biological evaluation of chrysin long-chain derivatives as potential anticancer agents

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ABSTRACT

A series of long-chain derivatives of chrysin (compounds **3**–**22**) were synthesized to evaluate for their antiproliferative activities against the human liver cancer cell line HT-29 and EGFR inhibitory activity. Among the compounds tested, compounds *hexadecyl 2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetate* (**10**) and *N-hexadecyl 2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetamide* (**20**) displayed potent EGFR inhibitory activity with IC_{50} values of 0.048 μM and 0.035 μM), comparable to the positive control erlotinib. Docking simulation of compounds **10** and **20** was carried out to illustrate the binding mode of the molecular into the EGFR active site, and the result suggested that compound **10** and **20** can bind the EGFR kinase well. Thus, compounds **10** and **20** with potent EGFR inhibitory activity would be potential anticancer agents.

1. Introduction

Cancer chemotherapy has entered a new era of molecularly targeted therapeutics, which is highly selective and not associated with the serious toxicities of conventional cytotoxic drugs.[1] Receptor protein tyrosine kinases play a key role in signal transduction pathways that regulate cell division and differentiation. Among the growth factor receptor kinases that have been identified as being important in cancer is epidermal growth factor receptor (EGFR) kinase. Activation of EGFR may be because of overexpression, mutations resulting in constitutive activation, or autocrine expression of ligand.[2,3] The role of EGFR has been most thoroughly studied in breast cancer, where it is overexpressed in 25–30% of cases and is correlated with a poor prognosis. EGFR overexpression is also seen in ovarian cancer,[4] lung cancer (especially lung adenocarcinomas)[5–7] and in hormone-refractory prostate cancer.[8]

Compounds that inhibit the kinase activity of EGFR and/or HER-2 after binding of its cognate ligand are of potential interest as new therapeutic antitumor agents.[9,10]

Flavonoids are natural polyphenolic phytochemicals that are ubiquitous in plants and present in the average human diet. Chrysin is a natural flavonoid found in many plant extracts, honey, and propolis, which has been reported to have many different biological activities such as anti-viral, anti-cancer, anti-bactericidal, anti-inflammatory, anti-allergic, DNA cleavage, vasodilator, anti-mutagenic, anti-anxiolytic and anti-oxidant effects.[11–18] However, to our knowledge, few reports have been dedicated to the synthesis and EGFR inhibitory activity of chrysin long-chain derivatives.[19] Herein, in continuation to extend our research on anticancer compounds with EGFR inhibitory activity,[20,21] in this study, we describe the synthesis and the SAR of a series of chrysin long-chain derivatives. Biological evaluation indicated that compounds **10** and **20** showed the most potent EGFR inhibitory activities with IC_{50} values of 0.048 μM and 0.035 μM. Docking simulations were performed using the X-ray crystallographic structure of the EGFR in complex with inhibitors to explore the binding modes of these compounds at the active site.

2. Chemistry

The routes adapted for the synthesis of chrysin long-chain derivatives are outlined in Scheme 1. To a stirring solution of chrysin in acetone, K_2CO_3 was added and then a mixture of ethyl bromoacetate and acetone was added dropwise to give compound **2**. Compound **2** was treated in Na_2CO_3 solution (DMSO) and after that most of the volatiles were evaporated. The residue was dissolved in water and the solution was adjusted to pH 2 by using HCl solution to give compound **3**. Compound **3** was suspended in DMF with stirring at room temperature, then long-chain alcohol or long-chain amine, 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC·HCl) were added. The reaction solution was stirred for 12 h at room temperature. Then, compounds **3–22** were obtained by subsequent purification with flash chromatography. All of the synthetic compounds gave satisfactory analytical and spectroscopic data, which were in full accordance with their depicted structures.

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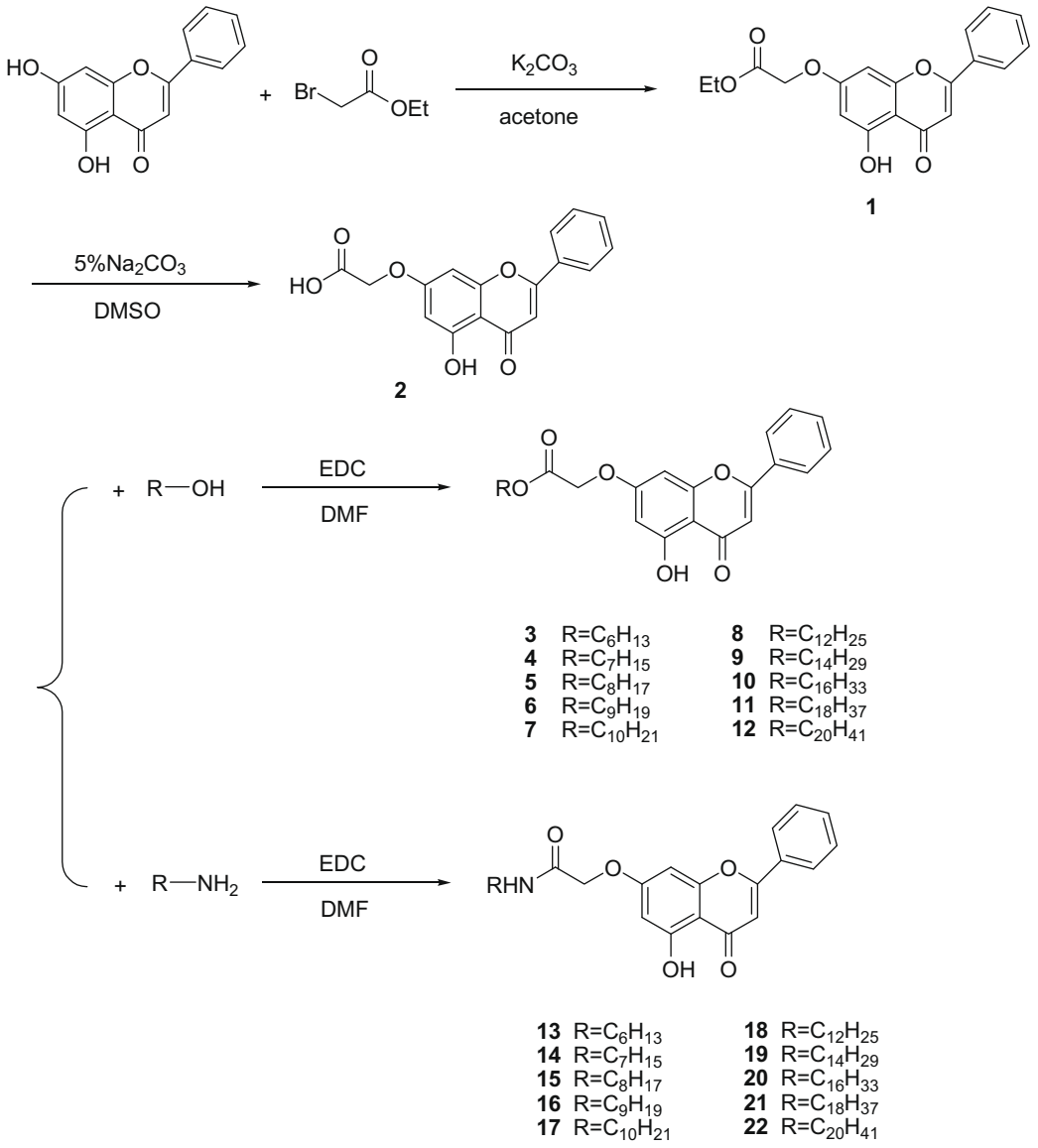

Scheme 1. Synthesis routes of compounds **1**–**22**.

3. Result and discussion

3.1. Biological activity

The in vitro antiproliferative activities of the chrysin long-chain derivatives **3**–**22** was studied on the human liver cancer cell line HT-29 by applying the MTT colorimetric assay. Compounds were tested over a range of concentrations from 0.1 to 100 μg/mL, and the calculated IC_{50} values, that is, the concentration (μg/mL) of a compound able to cause 50% of cell death with respect to the control culture, are reported in Table 1. As shown in Table 1, among the ester and amide derivatives, compounds **10** and **20** were found to show the most potent activity with IC_{50} values of 8.7 and 4.2 μg/mL, which is comparable to the positive control Iressa (IC_{50} = 9.8 μg/mL).

Furthermore, compounds **3–22** were also evaluated for their ability to inhibit the autophosphorylation of EGFR kinases using a solid-phase ELISA assay. A number of synthesized compounds displayed potent EGFR kinase inhibitory activity. The results have the same trends with the antiproliferative activities against HT-29 due to that EGFR is an important factor in colon cancer (Table 1). Compounds **10** and **20** displayed potent inhibitory activity (IC_{50} = 0.048 μM and 0.035 μM, respectively).

EGFR kinase inhibition IC_{50} values of long-chain ester and amide derivatives are similarly parabolic curves with the increase in the number of carbon atoms (Fig. 1). As the chain length increases, the inhibitory activities of those derivatives are increased. When the number of carbon atoms of long-chain part is more than 16, the activity is no further increase. The results indicated that, chrysin coupled with long-chain could get good antiproliferative activity. However, when the carbon number of long-chain part is more than 16 it gives a negative impact on the antiproliferative activity.

3.2. Binding mode of 20 into EGFR kinase

The ICM Suite of Software provides a way to dock flexible ligand and receptor, and to give intuitionistic structure insights of ligand/enzyme interactions. Molecular docking of the most potent activity compound **20** into the ATP binding site of EGFR kinase was performed on the binding model based on the EGFR complex structure (PDB code: 1M17,[9]) giving an explanation and understanding of good activity observed. The binding models of compounds **10**

Table 1
Antiproliferative activities data, inhibition of EGFR kinase and yields of compounds **3**–**22**

Compound	HT-29 IC_{50} (μg/mL)	EGFR Inhibition IC_{50} (μM)	Yield (%)
3	77 ± 14	9.12 ± 0.18	40
4	58 ± 13	7.23 ± 0.14	51
5	51 ± 6	5.84 ± 0.12	44
6	43 ± 16	4.96 ± 0.10	38
7	31 ± 8	3.11 ± 0.08	50
8	24 ± 12	1.72 ± 0.05	43
9	16 ± 6	1.01 ± 0.07	43
10	8.7 ± 0.4	0.048 ± 0.003	49
11	15 ± 3	0.692 ± 0.017	45
12	20 ± 5	1.589 ± 0.024	33
13	67 ± 17	8.71 ± 0.15	38
14	65 ± 14	6.28 ± 0.12	47
15	51 ± 12	5.33 ± 0.10	30
16	39 ± 21	3.58 ± 0.08	37
17	25 ± 11	2.14 ± 0.09	42
18	16 ± 7	1.27 ± 0.06	35
19	8 ± 4	0.316 ± 0.012	46
20	4.2 ± 0.5	0.035 ± 0.002	44
21	11 ± 4	0.464 ± 0.014	45
22	19 ± 8	1.35 ± 0.09	40
Iressa	9.8 ± 0.3	0.033 ± 0.001	—

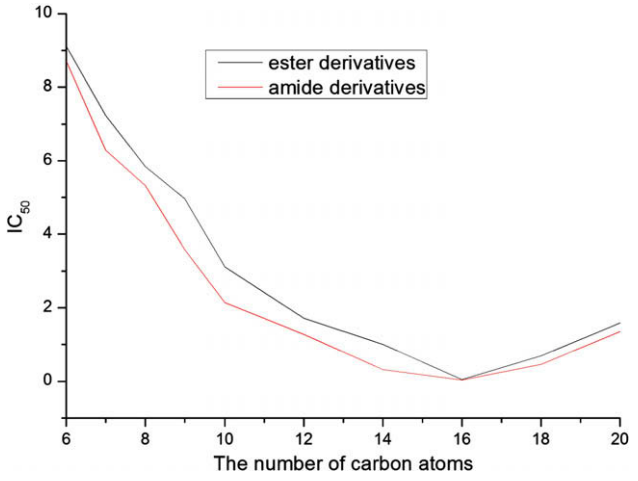

Figure 1. The curves of EGFR inhibition IC_{50} value and the number of carbon atoms.

and **20** into EGFR are depicted in Figures 2 and 3. Docking studies of compound **10** and **20** into the active site of EGFR provided well-clustered solutions. In the binding model of compound **10** and EGFR, the hydroxyl group and the carbonyl group of compound **10** form three hydrogen bonds with Met 769. Moreover, in the binding model of compound **20** and EGFR, there are two hydrogen bonds. Compound **20** is nicely bound to the region with the hydroxyl group project toward Thr766, with the hydroxyl group forming a more optimal H-bond interaction, and carbonyl group of compound **20** also forms hydrogen bond with Met 769. The molecular docking results, along with the enzyme assay data, suggesting that compound **10** and **20** are potential inhibitors of EGFR.

4. Conclusions

A series of long-chain derivatives of chrysin were synthesized to evaluate for antiproliferative activities against the human cancer cell line HT-29 and EGFR inhibitory activity. The results showed that chrysin coupling with long-chain can get good antiproliferative activity and the compounds with 16 carbon atoms chain were found to display potent antiproliferative activity. EGFR inhibitory ability of these synthesized compounds was also evaluated using

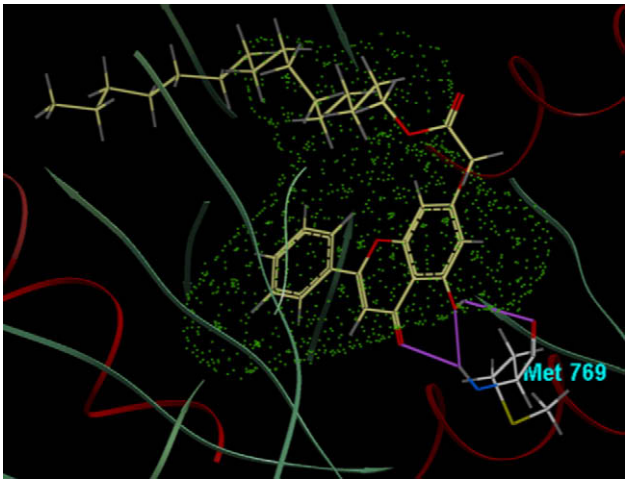

Figure 2. Binding mode of compound **10** with EGFR kinase. For clarity, only interacting residues are displayed. Ligand and interacting key residues are represented as stick models, while the proteins are represented as ribbons. The H-bond (purple) is displayed as line, and the green dots show the binding sites of EGFR.

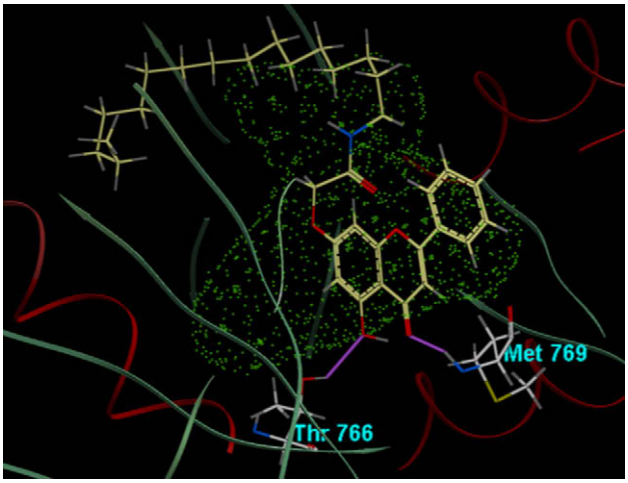

Figure 3. Binding mode of compound **20** with EGFR kinase. For clarity, only interacting residues are displayed. Ligand and interacting key residues are represented as stick models, while the proteins are represented as ribbons. The H-bond (purple) is displayed as line, and the green dots show the binding sites of EGFR.

a solid-phase ELISA assay, the results have the same trend as the antiproliferative activities assay. Moreover, docking simulations were performed to give the probable binding modes of compound **10** and **20** into the ATP binding site of EGFR kinase.

5. Experimental section

5.1. Chemistry

All chemicals (reagent grade) used were purchased from Aldrich (U.S.A) and Sinopharm Chemical Reagent Co., Ltd (China). Melting points (uncorrected) were determined on a XT4 MP apparatus (Taike Corp., Beijing, China). TLC was run on the silica gel coated aluminum sheets (Silica Gel 60 GF_{254}, E. Merk, Germany) and visualized in UV light (254 nm). EI mass spectra were obtained on a Waters GCT mass spectrometer, and ^1H NMR spectra were recorded on a Bruker DPX-300, AV-300 or AV-500 spectrometer at 25 °C with TMS and solvent signals allotted as internal standards. Chemical shifts were reported in ppm (δ). Elemental analyses were

performed on a CHN–O-rapid instrument and were within ±0.4% of the theoretical values.

5.1.1. Ethyl 2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetate (1)

Chrysin (2.54 g 0.01 mol) and anhydrous potassium carbonate (1.66 g 0.011 mol) were added in one portion to a stirred solution of 100 mL acetone, then drop ethyl bromoacetate (1.84 g 0.011 mol) into the solution. The reaction mixture was stirred at 50–60 °C for 6 h. On cooling, the precipitate was removed by filtration, the filtrate was concentrated and the residue was purified by column chromatography on silica gel, eluting with petroleum ether/EtOAc (3:1) to give a light yellow solid 2.95 g (yield: 87%). Mp: 145–148 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 1.21 (t, J = 7.0 Hz, 3H); 4.20 (p, J = 2.2 Hz, 2H); 4.96 (s, 2H); 6.44 (d, J = 2.2 Hz, 1H); 6.86 (d, J = 2.2 Hz, 1H); 7.05(s, 1H); 7.62 (m, 3H); 8.10 (m, 2H); 12.81 (s, 1H). MS (ESI):340.1 ([M+H]$^+$). Anal. Calcd for $C_{19}H_{16}O_6$: C, 67.05; H, 4.74. Found: C, 66.77; H, 4.91.

5.1.2. 2-(5-Hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetic acid (2)

A mixture of compound **1 (0.680 g 0.002 mol)** and 10 mL 5% sodium carbonate solution in 40 mL DMSO was stirred at 80–90 °C for 10 h. Then the mixture was pour into 10% 300 mL dilute hydrochloric acid solution. After standing overnight, The yellow solid was filtered off, washed with water, and dried in vacuo to give a yellow solid 0.441 g (yield: 71%). Mp: 279–282 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 4.96 (s, 2H); 6.41 (d, J = 2.2 Hz, 1H); 6.83 (d, J = 2.1 Hz, 1H); 7.05(s, 1H); 7.62 (t, J = 7.2 Hz, 3H); 8.10 (m, 2H); 12.81 (s, 1H). MS (ESI):312.1 ([M+H]$^+$). Anal. Calcd for $C_{17}H_{12}O_6$: C, 65.39; H, 3.87%. Found: C, 65.14; H, 4.02.

5.2. General method of synthesis 3–22

Equimolar quantities (1.0 mmol) of (**2**) and long-chain alcohol or long-chain amine were dissolved in DMF (10 mL), and 1.2 mmol EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide) were also added to the solution as catalyst. The solution was then stirred at 80–90 °C for approximately 12 h. The product was extracted by EtOAc. Then evaporated of the solvent, the residue was purified by column chromatography on silica gel, eluting with petroleum ether/EtOAc (6:1) to give a yellow solid.

5.2.1. Hexyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (3)

Light yellow powder, yield: 40%. Mp: 56–57 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.79 (t, J = 6.9 Hz, 3H); 1.23 (m, 6H); 1.56 (m, 2H); 4.13 (t, J = 6.4 Hz, 2H); 4.98 (s, 2H); 6.43 (d, J = 2.2 Hz, 1H); 6.85 (d, J = 2.2 Hz, 1H); 7.06 (s, 1H); 7.61 (m, 3H); 8.10 (m, 2H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.3, 168.1, 164.2, 163.7, 161.2, 157.1, 132.0, 131.1, 129.7, 126.4, 105.8, 104.9, 98.7, 94.2, 65.1, 64.8, 31.5, 28.8, 25.5, 22.3, 14.0. MS (ESI):396.2 ([M+H]$^+$). Anal. Calcd for $C_{23}H_{24}O_6$: C, 69.68; H, 6.10. Found: C, 69.45; H, 6.17.

5.2.2. Heptyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (4)

Light yellow powder, yield: 51%. Mp: 56–58 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.80 (t, J = 6.6 Hz, 3H); 1.17 (m, 8H); 1.57 (m, 2H); 4.14 (t, J = 6.4 Hz, 2H); 4.98 (s, 2H); 6.44 (d, J = 2.4 Hz, 1H); 6.85 (d, J = 2.4 Hz, 1H); 7.07 (s, 1H); 7.62 (m, 3H); 8.10 (m, 2H); 12.82 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.5, 168.7, 164.1, 163.7, 160.8, 156.6, 133.2, 131.5, 129.8, 127.1, 125.8, 105.9, 98.5, 94.3, 65.1, 64.7, 31.6, 28.8, 28.3, 25.6, 22.5, 14.1. MS (ESI): 410.2 ([M+H]$^+$). Anal. Calcd for $C_{24}H_{26}O_6$: C, 70.23; H, 6.38. Found: C, 70.53; H, 6.12.

5.2.3. Octyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (5)

Light yellow powder, yield: 44%. Mp: 58–60 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.81 (t, J = 6.7 Hz, 3H); 1.22 (m, 10H); 1.56 (m, 2H); 4.13 (t, J = 6.2 Hz, 2H); 4.98 (s, 2H); 6.43 (d, J = 2.2 Hz, 1H); 6.85 (d, J = 2.2 Hz, 1H); 7.07 (s, 1H); 7.62 (m, 3H); 8.10 (m, 2H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.4, 168.5, 164.0, 163.9, 163.6, 157.6, 132.5, 130.9, 129.5, 126.8, 105.7, 105.6, 98.9, 93.6, 65.4, 65.0, 31.5, 28.9, 28.8, 28.4, 25.6, 22.4, 14.2. MS (ESI): 424.2 ([M+H]$^+$). Anal. Calcd for $C_{25}H_{28}O_6$: C, 70.74; H, 6.65. Found: C, 70.95; H, 6.52.

5.2.4. Nonyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (6)

Light yellow powder, yield: 38%. Mp: 61–63 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.82 (t, J = 6.7 Hz, 3H); 1.23 (m, 12H); 1.56 (m, 2H); 4.13 (t, J = 6.4 Hz, 2H); 4.98 (s, 2H); 6.44 (d, J = 2.2 Hz, 1H); 6.85 (d, J = 2.2 Hz, 1H); 7.06 (s, 1H); 7.61 (m, 3H); 8.10 (m, 2H); 12.82 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.3, 167.8, 164.1, 163.5, 162.3, 157.5, 131.8, 131.4, 131.1, 129.0, 128.9, 126.2, 105.8, 98.4, 93.3, 65.7, 65.2, 31.5, 30.7, 29.5, 28.7, 28.4, 25.6, 22.4, 13.9. MS (ESI): 438.2 ([M+H]$^+$). Anal. Calcd for $C_{26}H_{30}O_6$: C, 71.21; H, 6.90. Found: C, 71.49; H, 6.76.

5.2.5. Decyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (7)

Light yellow powder, yield: 50%. Mp: 64–66 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.82 (t, J = 6.8 Hz, 3H); 1.23 (m, 14H); 1.56 (m, 2H); 4.13 (t, J = 6.4 Hz, 2H); 4.98 (s, 2H); 6.43 (d, J = 2.4 Hz, 1H); 6.85 (d, J = 2.4 Hz, 1H); 7.07 (s, 1H); 7.61 (m, 3H); 8.10 (m, 2H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.5, 168.1, 165.3, 164.1, 162.8, 157.4, 132.2, 131.7, 131.2, 130.8, 129.2, 127.6, 106.7, 99.1, 94.5, 66.7, 65.3, 31.6, 30.7, 29.8, 29.4, 28.6, 28.2, 25.5, 22.3, 13.9. MS (ESI): 452.2 ([M+H]$^+$). Anal. Calcd for $C_{27}H_{32}O_6$: C, 71.66; H, 7.13. Found: C, 71.91; H, 7.07.

5.2.6. Dodecyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (8)

Light yellow powder, yield: 43%. Mp: 67–69 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.84 (t, J = 7.2 Hz, 3H); 1.24 (m, 18H); 1.56 (m, 2H); 4.13 (t, J = 6.3 Hz, 2H); 4.98 (s, 2H); 6.44 (d, J = 2.2 Hz, 1H); 6.85 (d, J = 2.2 Hz, 1H); 7.07 (s, 1H); 7.61 (t, 3H); 8.10 (m, 2H); 12.82 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.5, 167.3, 164.8, 163.4, 162.1, 158.6, 132.5, 131.9, 129.8, 129.5, 128.7, 126.4, 106.2, 99.1, 92.8, 65.7, 65.1, 31.8, 30.6, 29.9, 29.5, 29.2, 28.3, 28.1, 25.6, 22.5, 13.9. MS (ESI): 480.2 ([M+H]$^+$). Anal. Calcd for $C_{29}H_{36}O_6$: C, 72.48; H, 7.55. Found: C, 72.66; H, 7.39%.

5.2.7. Tetradecyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (9)

Light yellow powder, yield: 43%. Mp: 70–72 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.85 (t, J = 6.8 Hz, 3H); 1.24 (m, 22H); 1.56 (m, 2H); 4.14 (t, J = 6.4 Hz, 2H); 4.98 (s, 2H); 6.44 (d, J = 2.2 Hz, 1H); 6.85 (d, J = 2.4 Hz, 1H); 7.06(s, 1H); 7.62 (m, 3H); 8.10 (m, 2H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.4, 168.2, 165.3, 163.4, 162.8, 158.4, 132.4, 131.4, 131.1, 129.8, 128.4, 126.1, 105.4, 98.5, 93.1, 66.1, 65.4, 31.6, 31.2, 30.8, 30.1, 29.7, 29.4, 29.0, 28.2, 28.0, 25.4, 22.4, 14.1. MS (ESI): 508.3 ([M+H]$^+$). Anal. Calcd for $C_{31}H_{40}O_6$: C, 73.20; H, 7.93. Found: C, 73.51; H, 7.82.

5.2.8. Hexadecyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (10)

Light yellow powder, yield: 49%. Mp: 71–72 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.85 (t, J = 6.4 Hz, 3H); 1.25 (m, 26H); 1.57 (m, 2H); 4.14 (t, J = 6.3 Hz, 2H); 4.99 (s, 2H); 6.45 (d,

J = 2.2 Hz, 1H); 6.86 (d, J = 2.2 Hz, 1H); 7.09 (s, 1H); 7.63 (m, 3H); 8.11 (m, 2H); 12.82 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.7, 168.1, 164.3, 163.4, 162.1, 157.4, 132.4, 131.8, 131.7, 129.5, 128.7, 126.8, 126.2, 98.7, 93.2, 65.8, 65.4, 32.1, 31.5, 31.1, 30.8, 30.2, 29.7, 29.5, 29.1, 28.9, 28.4, 28.2, 25.4, 22.3, 14.0. MS (ESI): 536.3 ([M+H]$^+$). Anal. Calcd for $C_{33}H_{44}O_6$: C, 73.85; H, 8.26. Found: C, 74.09; H, 8.19.

5.2.9. Octadecyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (11)

Light yellow powder, yield: 45%. Mp: 73–75 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.85 (t, J = 7.0 Hz, 3H); 1.22 (m, 30H); 1.57 (m, 2H); 4.13 (t, J = 6.5 Hz, 2H); 4.97 (s, 2H); 6.43 (d, J = 2.2 Hz, 1H); 6.84 (d, J = 2.2 Hz, 1H); 7.05 (s, 1H); 7.60 (m, 3H); 8.10 (m, 2H); 12.80 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.3, 168.2, 164.5, 163.8, 162.4, 157.4, 132.5, 131.8, 131.2, 129.4, 128.6, 126.4, 105.9, 98.8, 93.6, 65.8, 65.1, 32.4, 32.2, 31.7, 31.4, 30.6, 30.1, 29.6, 29.3, 29.0, 28.7, 28.4, 28.1, 25.7, 22.3, 14.1. MS (ESI): 564.4 ([M+H]$^+$). Anal. Calcd for $C_{35}H_{48}O_6$: C, 74.44; H, 8.57. Found: C, 74.70; H, 8.38.

5.2.10. Icosyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetate (12)

Light yellow powder, yield: 33%. Mp: 74–75 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.85 (t, J = 6.8 Hz, 3H); 1.25 (m, 34H); 1.57 (m, 2H); 4.13 (t, J = 6.5 Hz, 2H); 4.98 (s, 2H); 6.45 (d, J = 2.2 Hz, 1H); 6.85 (d, J = 2.2 Hz, 1H); 7.07 (s, 1H); 7.60 (m, 3H); 8.11 (m, 2H); 12.80 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.4, 168.4, 165.1, 163.8, 162.4, 157.4, 131.8, 131.1, 129.4, 128.6, 126.4, 105.9, 98.6, 93.6, 65.9, 65.1, 32.3, 31.9, 31.6, 31.2, 30.9, 30.5, 30.2, 29.8, 29.4, 29.0, 28.8, 28.6, 28.3, 25.7, 22.3, 14.1. MS (ESI): 592.4 ([M+H]$^+$). Anal. Calcd for $C_{37}H_{52}O_6$: C, 74.96; H, 8.84. Found: C, 75.21; H, 8.45.

5.2.11. *N*-Hexyl-2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetamide (13)

Yellow powder, yield: 38%. Mp: 120–122 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.80 (t, J = 6.8 Hz, 3H); 1.11 (m, 6H); 1.41 (m, 2H); 3.12 (d, J = 6.4 Hz, 2H); 4.63 (s, 2H); 6.45 (d, J = 2.2 Hz, 1H); 6.81 (d, J = 2.2 Hz, 1H); 7.06 (s, 1H); 7.62 (m, 3H); 8.11 (m, 3H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.6, 168.3, 164.9, 162.5, 160.8, 158.3, 133.4, 132.4, 128.7, 125.2, 106.5, 105.2, 98.5, 94.8, 65.6, 64.1, 32.2, 29.1, 25.7, 22.6, 14.1. MS (ESI): 395.2 ([M+H]$^+$). Anal. Calcd for $C_{23}H_{25}NO_5$: C, 69.86; H, 6.37; N, 3.54. Found: C, 70.12; H, 6.48; N, 3.37.

5.2.12. *N*-Heptyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetamide (14)

Yellow powder, yield: 47%. Mp: 121–123 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.80 (t, J = 6.6 Hz, 3H); 1.08 (m, 8H); 1.42 (m, 2H); 3.12 (d, J = 6.2 Hz, 2H); 4.63 (s, 2H); 6.45 (d, J = 2.2 Hz, 1H); 6.81 (d, J = 2.2 Hz, 1H); 7.05 (s, 1H); 7.62 (m, 3H); 8.11 (m, 3H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.3, 167.4, 165.8, 164.2, 161.3, 155.3, 134.1, 131.8, 129.4, 127.1, 126.8, 106.3, 98.7, 95.4, 65.1, 64.5, 31.8, 29.1, 28.5, 25.3, 22.4, 14.0. MS (ESI): 409.2 ([M+H]$^+$). Anal. Calcd for $C_{24}H_{27}NO_5$: C, 70.40; H, 6.65; N, 3.42. Found: C, 70.73; H, 6.82; N, 3.24.

5.2.13. *N*-Octyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetamide (15)

Yellow powder, yield: 30%. Mp: 124–126 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.82 (t, J = 6.8 Hz, 3H); 1.17 (m, 10H); 1.41 (m, 2H); 3.14 (d, J = 6.6 Hz, 2H); 4.64 (s, 2H); 6.45 (d, J = 2.2 Hz, 1H); 6.81 (d, J = 2.2 Hz, 1H); 7.07 (s, 1H); 7.62 (m, 3H); 8.11 (m, 3H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.7, 167.5, 165.2, 164.2, 163.6, 158.1, 133.2, 131.3, 128.7, 125.6, 106.1, 106.0, 98.4, 93.1, 65.1, 65.5, 31.7, 28.3, 28.0, 27.3, 26.4, 22.2, 14.3. MS (ESI): 423.2 ([M+H]$^+$). Anal. Calcd for $C_{25}H_{29}NO_5$: C, 70.90; H, 6.90; N, 3.31. Found: C, 71.11; H, 6.99; N, 3.22.

5.2.14. *N*-Nonyl 2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy)acetamide (16)

Yellow powder, yield: 37%. Mp: 128–130 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.83 (t, J = 6.6 Hz, 3H); 1.17 (m, 12H); 1.42 (m, 2H); 3.12 (d, J = 6.0 Hz, 2H); 4.63 (s, 2H); 6.44 (d, J = 2.2 Hz, 1H); 6.81 (d, J = 2.2 Hz, 1H); 7.06 (s, 1H); 7.62 (m, 3H); 8.11 (m, 3H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.4, 166.8, 165.1, 164.3, 163.8, 157.6, 132.1, 131.8, 130.6, 129.5, 128.1, 126.0, 106.3, 97.6, 94.5, 65.1, 66.3, 31.7, 30.1, 29.8, 28.8, 28.2, 25.7, 22.3, 13.8. MS (ESI): 437.2 ([M+H]$^+$). Anal. Calcd for $C_{26}H_{31}NO_5$: C, 71.37; H, 7.14; N, 3.20. Found: C, 71.74; H, 7.03; N, 3.02.

5.2.15. *N*-Decyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetamide (17)

Yellow powder, yield: 42%. Mp: 129–131 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.85 (t, J = 6.2 Hz, 3H); 1.19 (m, 14H); 1.41 (m, 2H); 3.13 (d, J = 6.4 Hz, 2H); 4.63 (s, 2H); 6.45 (d, J = 2.2 Hz, 1H); 6.81 (d, J = 2.2 Hz, 1H); 7.07 (s, 1H); 7.62 (m, 3H); 8.11 (m, 3H); 12.80 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.4, 169.3, 166.4, 163.7, 161.5, 157.1, 133.5, 132.2, 131.7, 130.4, 128.6, 127.3, 107.6, 99.4, 94.8, 66.2, 65.1, 31.9, 31.4, 29.5, 29.0, 28.4, 28.1, 25.7, 22.4, 14.1. MS (ESI): 451.2 ([M+H]$^+$). Anal. Calcd for $C_{27}H_{33}NO_5$: C, 71.82; H, 7.37; N, 3.10. Found: C, 72.15; H, 7.02; N, 2.99.

5.2.16. *N*-Dodecyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetamide (18)

Yellow powder, yield: 35%. Mp: 135–137 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.84 (t, J = 6.2 Hz, 3H); 1.19 (m, 18H); 1.41 (m, 2H); 3.12 (d, J = 6.2 Hz, 2H); 4.63 (s, 2H); 6.45 (d, J = 1.8 Hz, 1H); 6.81 (d, J = 1.8 Hz, 1H); 7.07 (s, 1H); 7.62 (m, 3H); 8.11 (m, 3H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.3, 168.4, 165.3, 164.0, 163.2, 159.1, 133.6, 132.5, 129.7, 128.3, 127.4, 126.1, 107.8, 99.6, 92.1, 65.3, 65.0, 31.9, 30.2, 29.8, 29.5, 29.0, 28.4, 28.1, 25.3, 22.6, 14.0. MS (ESI): 479.3 ([M+H]$^+$). Anal. Calcd for $C_{29}H_{37}NO_5$: C, 72.62; H, 7.78; N, 2.92. Found: C, 72.98; H, 7.52; N, 2.74.

5.2.17. *N*-Tetradecyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetamide (19)

Yellow powder, yield: 46%. Mp: 135–137 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.83 (t, J = 6.6 Hz, 3H); 1.17 (m, 22H); 1.42 (m, 2H); 3.13 (d, J = 6.2 Hz, 2H); 4.63 (s, 2H); 6.45 (d, J = 2.2 Hz, 1H); 6.81 (d, J = 2.2 Hz, 1H); 7.07(s, 1H); 7.62 (m, 3H); 8.11 (m, 3H); 12.80 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.6, 167.5, 166.1, 162.8, 161.3, 158.7, 131.8, 131.1, 130.7, 129.4, 127.8, 126.7, 105.1, 98.8, 93.4, 66.5, 65.1, 31.9, 31.4, 30.5, 30.0, 29.5, 29.3, 29.0, 28.7, 28.1, 25.2, 22.6, 14.1. MS (ESI): 507.3 ([M+H]$^+$). Anal. Calcd for $C_{31}H_{41}NO_5$: C, 73.34; H, 8.14; N, 2.76. Found: C, 73.53; H, 8.39; N, 2.88.

5.2.18. *N*-Hexadecyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetamide (20)

Yellow powder, yield: 44%. Mp: 137–139 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.82 (t, J = 6.8 Hz, 3H); 1.22 (m, 26H); 1.41 (m, 2H); 3.12 (d, J = 6.0 Hz, 2H); 4.63 (s, 2H); 6.45 (d, J = 2.2 Hz, 1H); 6.81 (d, J = 2.2 Hz, 1H); 7.06(s, 1H); 7.62 (m, 3H); 8.11 (m, 3H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.4, 168.7, 165.6, 164.5, 163.7, 158.2, 133.5, 132.4, 131.8, 129.1, 127.3, 126.2, 125.4, 98.1, 93.7, 65.6, 65.2, 32.5, 31.7, 31.3, 30.5, 30.1, 29.7, 29.5, 29.3, 28.6, 28.4, 28.0, 25.3, 22.1, 13.9. MS (ESI): 535.3 ([M+H]$^+$). Anal. Calcd for $C_{33}H_{45}NO_5$: C, 73.99; H, 8.47; N, 2.61. Found: C, 74.42; H, 8.63; N, 2.44.

5.2.19. *N*-Octadecyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy)acetamide (21)

Yellow powder, yield: 45%. Mp: 138–140 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.85 (t, J = 6.4 Hz, 3H); 1.22 (m, 30H); 1.42 (m, 2H); 3.13 (d, J = 6.2 Hz, 2H); 4.63 (s, 2H); 6.44 (d, J = 2.0 Hz, 1H); 6.81 (d, J = 2.0 Hz, 1H); 7.05 (s, 1H); 7.61 (m, 3H); 8.10 (m, 3H); 12.80 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.6, 167.6, 163.7, 163.2, 162.1, 158.5, 131.4, 131.0, 130.5, 129.1, 127.6, 125.3, 105.4, 98.3, 93.1, 65.7, 64.6, 32.7, 32.4, 31.5, 31.2, 30.9, 30.4, 29.4, 29.2, 28.7, 28.5, 28.4, 27.6, 25.1, 22.5, 14.0. MS (ESI): 563.3 ([M+H]$^+$). Anal. Calcd for $C_{35}H_{49}NO_5$: C, 74.57; H, 8.76; N, 2.48. Found: C, 74.93; H, 9.02; N, 2.23.

5.2.20. *N*-Icosyl 2-(5-hydroxy-4-oxo-2-phenyl-4*H*-chromen-7-yloxy) acetamide (22)

Yellow powder, yield: 40%. Mp: 137–139 °C. ^{1}H NMR (300 MHz, DMSO-d_6, δ ppm): 0.85 (t, J = 6.4 Hz, 3H); 1.22 (m, 34H); 1.42 (m, 2H); 3.12 (d, J = 6.0 Hz, 2H); 4.63 (s, 2H); 6.44 (d, J = 2.0 Hz, 1H); 6.81 (d, J = 2.2 Hz, 1H); 7.06 (s, 1H); 7.61 (m, 3H); 8.11 (m, 3H); 12.81 (s, 1H). ^{13}C NMR (DMSO-d_6, δ ppm): 182.6, 169.4, 166.5, 164.3, 162.7, 156.5, 132.3, 130.8, 129.1, 127.7, 126.5, 105.3, 98.5, 94.6, 65.8, 64.3, 32.6, 31.7, 31.4, 31.2, 30.8, 30.4, 30.0, 29.6, 29.2, 29.0, 28.7, 28.5, 28.1, 25.8, 22.6, 14.1. MS (ESI): 591.4 ([M+H]$^+$). Anal. Calcd for $C_{37}H_{53}NO_5$: C, 75.09; H, 9.03; N, 2.37. Found: C, 74.76; H, 9.17; N, 2.19.

5.3. Antiproliferative activities assay

The antiproliferative activities of compounds **3–22** were determined using a standard (MTT)-based colorimetric assay (Sigma). Briefly, cell lines were seeded at a density of 7×10^3 cells/well in 96-well microtiter plates (Costar). After 24 h, exponentially growing cells were exposed to the indicated compounds at final concentrations ranging from 0.1 to 100 μg/mL. After 48 h, cell survival was determined by the addition of an MTT solution (10 μL of 5 mg/mL MTT in PBS). After 4 h, 100 μL of 10% SDS in 0.01 N HCl was added, and the plates were incubated at 37 °C for a further 18 h; optical absorbance was measured at 570 nm on an LX300 Epson Diagnostic microplate reader. Survival ratios are expressed in percentages with respect to untreated cells. IC_{50} values were determined from replicates of six wells from at least two independent experiments.

5.4. Cell culture

Colorectal cancer cell line, HT-29, was obtained from Jiangsu Institute of Hematology and Cells were cultured in Mccoy's 5A culture medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA). The cultures were incubated at 37 °C in humidified 5% CO_2 incubator. When experiments were performed in the absence of FBS, FBS was eliminated 24 h before initiating the experiments.

5.5. EGFR inhibitory assay

A 1.6 kb cDNA encoded for the EGFR cytoplasmic domain (EGFR-CD, amino acids 645–1186) were cloned into baculoviral expression vector pFASTBacHTc. A sequence that encodes $(His)_6$ was located at the 5′ upstream to the EGFR sequence. Sf-9 cells were infected for three days for protein expression. Sf-9 cell pellets were solubilized at 0 °C in a buffer at pH 7.4 containing 50 mM HEPES, 10 mM NaCl, 1% Triton, 10 μM ammonium molybdate, 100 μM sodium vanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 16 μg/mL benzamidine HCl for 20 min followed by 20 min centrifugation. Crude extract supernatant was passed through an equilibrated Ni-NTA superflow packed column and washed with 10 mM and then 100 mM imidazole to remove nonspecifically bound material. Histidine tagged proteins were eluted with 250 and 500 mM imidazole and dialyzed against 50 mM NaCl, 20 mM HEPES, 10% glycerol, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin for 2 h. The entire purification procedure was performed at 4 °C or on ice.

The EGFR kinase assay was set up to assess the level of autophosphorylation based on DELFIA/Time-Resolved Fluorometry. Compounds **3–22** were dissolved in 100% DMSO and diluted to the appropriate concentrations with 25 mM HEPES at pH 7.4. In each well, 10 μL of compound was incubated with 10 μL (12.5 ng for HER-2 or 5 ng for EGFR) of recombinant enzyme (1:80 dilution in 100 mM HEPES) for 10 min at room temperature. Then, 10 μL of 5 mM buffer (containing 20 mM HEPES, 2 mM $MnCl_2$, 100 μM Na_3VO_4, and 1 mM DTT) and 20 μL of 0.1 mM ATP-50 mM $MgCl_2$ was added for 1 h. Positive and negative controls were included in each plate by incubation of enzyme with or without ATP-$MgCl_2$. At the end of incubation, liquid was aspirated, and plates were washed three times with wash buffer. A 75 μL (400 ng) sample of europium labeled anti-phosphotyrosine antibody was added to each well for another 1 h of incubation. After washing, enhancement solution was added and the signal was detected by Victor (Wallac Inc.) with excitation at 340 nm and emission at 615 nm. The percentage of autophosphorylation inhibition by the compounds was calculated using the following equation: 100% − [(negative control)/(positive control − negative control)]. The IC_{50} was obtained from curves of percentage inhibition with eight concentrations of compound. As the contaminants in the enzyme preparation are fairly low, the majority of the signal detected by the antiphosphotyrosine antibody is from EGFR.

5.6. Docking simulations

Molecular docking of compounds **10** and **20** into the three-dimensional EGFR complex structure (PDB code: 1M17, download from the PDB) was carried out using the Molsoft ICM-Pro software package (version 3.5-0a).[22,23]

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