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# Design and synthesis of 4,6-substituted-(diaphenylamino)quinazolines as potent EGFR inhibitors with antitumor activity

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ABSTRACT

A type of novel 4,6-substituted-(diaphenylamino)quinazolines, which designed based on the 4-(phenylamino)quinazoline moiety, have been discovered as potential EGFR inhibitors. These compounds displayed good antiproliferative activity and EGFR-TK inhibitory activity. Especially, 4-((4-(3-bromophenylamino)quinazolin-6-ylamino)methyl)phenol (**5b**), showed the most potent inhibitory activity ($IC_{50}$ = 0.28 μM for Hep G2, $IC_{50}$ = 0.59 μM for A16-F10 and $IC_{50}$ = 0.87 μM for EGFR) and effectively induces apoptosis in a dose-dependent manner in the Hep G2 cell line. Molecular docking of **5b** into EGFR TK active site was also performed. This inhibitor nicely fitting the active site might well explain its excellent inhibitory activity.


## 1. Introduction

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.[1,2] Expression of a dominant negative Ras mutant in EGFR overexpressing cells also results in a significant potentiation of EGFR induced apoptosis suggesting that Ras activation is a key survival signal generated by the EGFR. The role of EGFR has been most thoroughly studied in breast cancer,[3] lung cancer (especially lung adenocarcinomas)[4–6] and in hormone-refractory prostate cancer.[7] Compounds that inhibit the kinase activity of EGFR after binding of its cognate ligand are of potential interest as new therapeutic antitumor agents.[8,9]

The 6,7-dialkoxy-4-(phenylamino)quinazolines are a class of potent, selective, ATP-competitive inhibitors of EGFR tyrosine kinase.[10,11] Tosu group and Parke-Davis group developed irreversible inhibitors the butynamide-substituted 4-anilinoquinazoline **A**,[12] as well as the acrylamide-substituted 4-anilinoquinazoline **B**,[13] based on the 4-(phenylamino)quinazoline core structure that have an attached Michael acceptor functional group at the C-6 position. Molecular modeling studies suggested, and experimental results confirmed, that the Michael acceptor side chain of these inhibitors forms a covalent linkage with the sulfhydryl group of the Cys 773 of EGFR (Scheme 1). These compounds proved to be potent inhibitors of tumor growth in a human epidermoid carcinoma xenograft (A431) model that overexpresses EGFR-TK.[13] However, these compounds exhibit poor bioavailability and the induction of apoptosis by these EGFR-TK inhibitors in solid tumors have not been researched. In continuation of our earlier studies focus on EGFR inhibitors as antitumor agents,[14,15] we now discovery a series of 4,6-substituted-(diaphenylamino)quinazolines as new EGFR-TK inhibitors. As discussed above, molecular modeling suggests that a favorable site to attach new phenylamino groups based on the 4-(phenylamino)quinazoline core structure would be at the C-6 position, since these positions point toward the outside of the protein. These compounds are potent in inhibition of cell growth in two cancer cell lines (Hep G2 and A16-F10) and with high levels of EGFR proteins and effectively induces apoptosis in a dose-dependent manner in the Hep G2 cell line.

## 2. Results and discussion

### 2.1. Chemsitry

Other workers have prepared 4-anilinoquinazolines by reacting substituted anilines with 4-chloroquinazolines.[16] We designed a more efficient synthesis that allowed ring cyclization and incorporation of the 4-anilino group in a single step, as shown in Scheme 2. Commercially available 5-nitroanthranilonitrile **1** was converted

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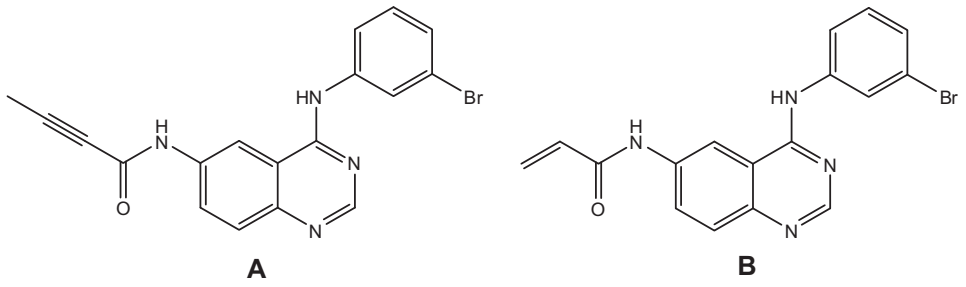
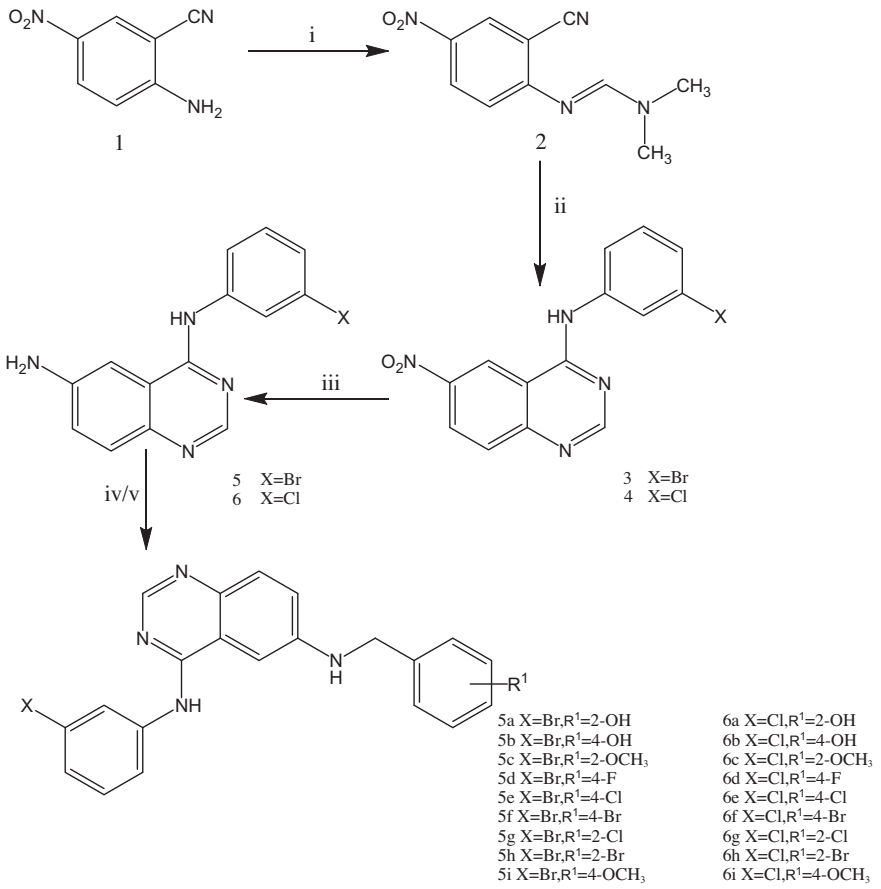



**Scheme 1.**




**Scheme 2.** Synthetic routes of 4-(phenylamino)quinazolines. Reagents and conditions: (i) dimethylformamide dimethyl acetal, 70–75 °C; (ii) $ArNH_2$/AcOH, 70–75 °C; (iii) Fe/AcOH/EtOH/$H_2O$, 70–80 °C; (iv) benzaldehyde/ethanol, 40 °C; (v) sodium borohydride/ethanol, 40–50 °C.

into the corresponding formamidine **2** using DMF acetal. Heating a solution of formamidine **2** and 3-X-aniline in HOAc gave 6-nitro-4-(3-X-phenylamino)quinazolines **3** and **4**. Reduction of the nitro group of **3** and **4** with iron in HOAc yielded the intermediate 6-aminoquinazolines **5** and **6**.

Schiff bases of 6-aminoquinazolines were obtained in high yields using various substituted benzaldehyde and EtOH as the carbonyl activator. Then schiff bases of 6-aminoquinazolines were submitted to the Zn/HCOOH (aq) reductive conditions, leading to the corresponding, 6-substituted-(diaphenylamino)quinazolines **5a–5i** and **6a–6i** with moderate to high overall yields (Scheme 2). All new 4,6-substituted-(diaphenylamino)quinazolines were fully characterized by spectroscopic methods and the elemental analysis together with a crystal structure (**5d** Fig. 1).

## 2.2. Antiproliferative activity

In former reports, the N1, N3 of the 4-(phenylamino)quinazoline core structure would interact with active centre probably by hydrogen bond, water bridge or else. So we saved the quinazoline skeleton in the new designed compunds.[17] The *meta* substituent of the aniline group is oriented toward the kinase pocket. This mode of binding is in line with the structure–activity relationship observed in the quinazoline inhibition series. This mode of binding

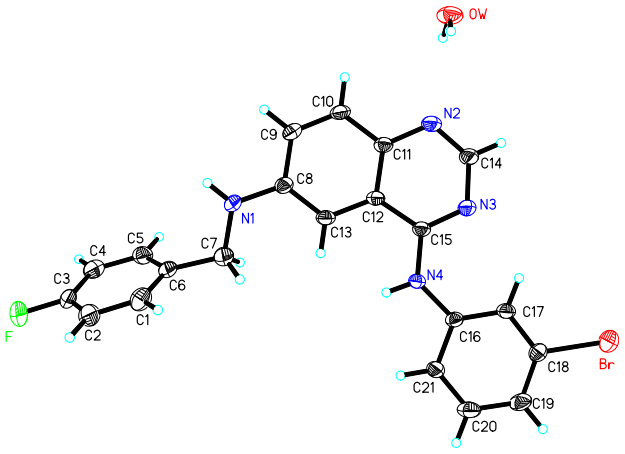



**Figure 1.** Crystal structure of compounds **5d**.

is in line with the structure–activity relationship observed in the quinazoline inhibition series. Small substituents are tolerated at the 3′-aniline moiety, preferentially halogens and electron-donating group at the 6- and/or 7-positions that enhance the binding of N1 and N3. Thus, in this study, we choose halogen atom (Br and Cl) at the 3′-X-aniline moiety to develop the novel 4,6-substituted-(diaphenylamino)quinazolines.

The synthesized 4,6-substituted-(diaphenylamino)quinazolines were evaluated for their antiproliferative activities against Hep G2 and A16-F10 cells by applying the MTT colorimetric assay. The results were summarized in Table 1. Compounds were tested over a range of concentrations from 0.01 to 100 μg/ml, and the calculated $IC_{50}$ values, that is, the concentration (μg/mL) of a compound that was able to cause 50% cell death with respect to the control culture, were reported differently according to different cancer cells. As expected, these compounds containing 3′-X = Br or Cl all exhibited remarked effects on antiproliferative activities, and generally the results showed which those applied to Hep G2 cells would performed better than that in A16-F10 cells, was normal and acceptable. Especially, 4-((4-(3-bromophenylamino)quinazolin-6-ylamino)methyl)phenol (**5b**), showed the most potent inhibitory activity ($IC_{50}$ = 0.28 μM for Hep G2 and $IC_{50}$ = 0.59 μM for A16-F10), and comparable to the positive control erlotinib ($IC_{50}$ = 0.12 μM for Hep G2 and $IC_{50}$ = 0.02 μM for A16-F10).

**Table 1**
Structure of 4,6-substituted-(diaphenylamino)quinazolines and the antiproliferative effects

| Compd | X | $R^1$ | $IC_{50}$ (μM) | |
|---|---|---|---|---|
| | | | Hep G2 | A16-F10 |
| **5a** | Br | 2-OH | 1.26 ± 0.04 | 1.87 ± 0.36 |
| **5b** | Br | 4-OH | 0.28 ± 0.15 | 0.59 ± 0.15 |
| **5c** | Br | 2-OMe | 8.82 ± 1.14 | 12.5 ± 2.2 |
| **5d** | Br | 4-F | 1.77 ± 0.16 | 2.09 ± 0.43 |
| **5e** | Br | 4-Cl | 0.92 ± 0.16 | 1.82 ± 0.36 |
| **5f** | Br | 4-Br | 1.41 ± 0.06 | 2.21 ± 1.05 |
| **5g** | Br | 2-Cl | 1.82 ± 0.13 | 3.74 ± 0.83 |
| **5h** | Br | 2-Br | 2.76 ± 0.07 | 4.38 ± 0.61 |
| **5j** | Br | 4-OMe | 5.43 ± 0.76 | 10.4 ± 2.7 |
| **6a** | Cl | 2-OH | 2.08 ± 0.16 | 3.18 ± 0.54 |
| **6b** | Cl | 4-OH | 1.52 ± 0.07 | 1.58 ± 0.28 |
| **6c** | Cl | 2-OMe | 9.85 ± 2.12 | 14.8 ± 3.1 |
| **6d** | Cl | 4-F | 2.69 ± 0.21 | 2.95 ± 0.28 |
| **6e** | Cl | 4-Cl | 1.78 ± 0.21 | 2.67 ± 0.27 |
| **6f** | Cl | 4-Br | 3.81 ± 0.32 | 4.01 ± 0.14 |
| **6g** | Cl | 2-Cl | 5.33 ± 0.67 | 7.12 ± 0.79 |
| **6h** | Cl | 2-Br | 4.17 ± 0.48 | 5.83 ± 0.51 |
| **6j** | Cl | 4-OMe | 5.26 ± 0.18 | 8.43 ± 1.26 |
| Erlotinib | | | 0.12 | 0.02 |

Structure–activity relationships in these new compounds demonstrated that compounds containing 3′-X = Br showed better activity than 3′-X = Cl, indicating little tolerance of bulkiness in the hydrophobic pocket of the ATP site of EGFR of the latter compounds. Compounds with $R^1$ substitution at the *para* position (**5b**, **5d**, **5e** and **5f**) showed better activities than those with substitution at the *meta* position (**5a**, **5c**, **5g** and **5h**) based both on cytotoxicity and enzyme inhibitory activity, respectively. Hydroxyl derivatives at $R^1$ position are more potent then corresponding methoxy derivatives, so the increased lipophilicity of methoxy derivatives at $R^1$ position may be detrimental to the inhibitory activity.

### 2.3. EGFR inhibitory activity and molecular docking

To evaluate the EGFR inhibitory potency of new compounds, their ability to block EGFR TK was tested in an EGFR TK assay. We chose ten compounds behaved well for antiproliferation and their EGFR inhibitory activities were displayed at Table 2. This could indicate that 4,6-substituted-(diaphenylamino)quinazolines basically follow the EGFR inhibition path against cancer cell. As shown in Table 2, compound **5b** and **5e** displayed the most potent inhibitory activity ($IC_{50}$ = 0.87 μM and 1.64 μM for EGFR), less comparable to the positive control erlotinib ($IC_{50}$ = 0.03 μM for EGFR).

To help understand the SARs observed at the EGFR and guide further SAR studies, molecular docking of the most potent inhibitor **5b** into ATP binding site of EGFR kinase was performed on the binding model based on the EGFR complex structure (1M17.pdb). The binding model of compound **5b** and EGFR was depicted in Figure 2A and Figure 2B. In the binding model, compound **5b** is nicely bound to the region of EGFR, the quinazoline ring be inserted nicely inside the pocket. The $R^1$ substituted phenyl group in **5b** projects into a hydrophobic region, which is comprised of the side chains of Pro 717, Leu 768 and Ile 716, that was important for the potent inhibitory activity of **5b.** The modeling also suggested that there is a π-cation interaction between quinazoline ring of compound **5b** and Lys828, π-cation interaction energies are of the same order of magnitude as hydrogen bonds or salt bridges and play an important role in stabilizing the three dimensional structure of a protein.[18]

### 2.4. Analysis of apoptosis induced by compounds 5b

We evaluated compound **5b** for their ability to induce apoptosis in the Hep G2 cell line using Annexin-V and propium iodide (PI) double staining by flow cytometry. The results are shown in Figure 3. As can be seen, compound **5b** is very effective in induction of apoptosis in a dose-dependent manner. Treatment of the Hep G2 cells by 1 and 2 μM of **5b** for 3 days results in 20.8% and 54.2% of apoptotic cells (early + late), as compared to 0.98% of apoptotic cells in an untreated

**Table 2**
Inhibition ($IC_{50}$) of EGFR kinase

| Compd | EGFR inhibition $IC_{50}$ (μM) |
|---|---|
| **5a** | 3.22 |
| **5b** | 0.87 |
| **5c** | 11.22 |
| **5e** | 1.64 |
| **5f** | 3.13 |
| **6a** | 3.54 |
| **6b** | 1.87 |
| **6c** | 13.88 |
| **6e** | 4.41 |
| **6f** | 5.67 |
| Erlotinib | 0.03 |

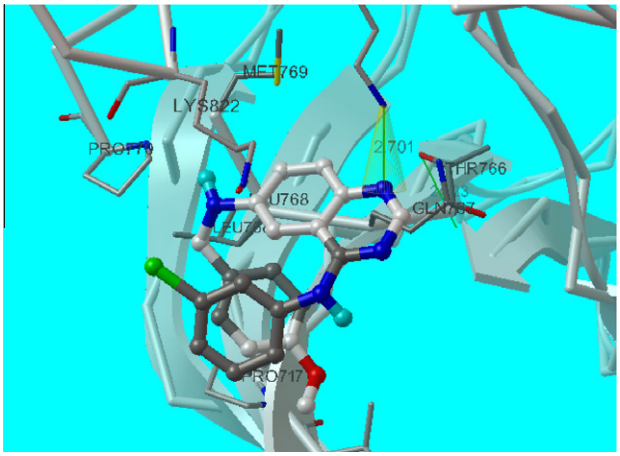



**Figure 2A.** Molecular docking modeling of compounds **5b** with EGFR kinase: The $R^1$ substituted phenyl group in **5b** projects into a hydrophobic region, which is comprised of the side chains of Pro 717, Leu 768 and Ile 716, that was important for the potent inhibitory activity of **5b.** The modeling also suggested that there is a π-cation interaction between quinazoline ring of compound **5b** and Lys828.

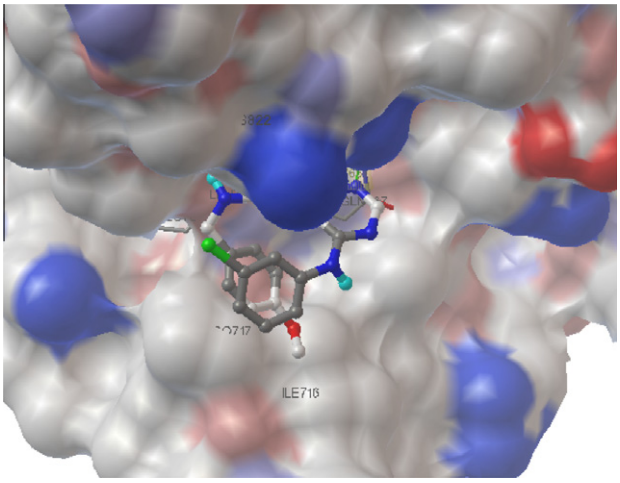



**Figure 2B.** 3D model of the interaction between compound **5b** and the ATP binding site, which binding energy is equal to −11.0.

control. This is consistent with its nice binding affinity to EGFR TK and its potent activity in inhibition of cell growth.

## 3. Conclusion

In summary, eighteen novel 4,6-substituted-(diaphenylamino)quinazolines that may function as inhibitors of EGFR kinases have been designed and prepared. As expected, these compounds containing 3′-X = Br or Cl all exhibited remarked effects on antiproliferative activity and EGFR TK inhibitory activity. Especially, 4-((4-(3-bromophenylamino)quinazolin-6-ylamino)methyl)phenol (**5b**)**,** showed the most potent inhibitory activity ($IC_{50}$ = 0.28 μM for Hep G2, $IC_{50}$ = 0.59 μM for A16-F10 and $IC_{50}$ = 0.87 μM for EGFR). The EGFR molecular docking model suggested that compound **5b** is nicely bound to the region of EGFR, the quinazoline ring be inserted nicely inside the pocket, π-cation interaction between N1, N3-quinazoline ring and Lys828 may an important role in stabilizing the three dimensional structure of a protein. Thus, compound **5b** is potent EGFR TK inhibitor and effectively induces apoptosis in a dose-dependent manner in the Hep G2 cell line as a potential anticancer agent.

## 4. Experimental

### 4.1. Chemistry general

All chemicals (reagent grade) used were purchased from Sigma–Aldrich (USA) and Sinopharm Chemical Reagent Co. , Ltd.(China).$^1$H NMR spectra were measured on a Bruker AV-300 or AV-500 spectrometer at 25 °C and referenced to $Me_4Si$. Chemical shifts are reported in ppm ($\delta$) using the residual solvent line as internal standard. Splitting patterns are designed as s, singlet; d, doublet; t, triplet; m, multiplet. ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. Elemental analyses were performed on a CHN–O-Rapid instrument and were within ± 0.4% of the theoretical values. Melting points were determined on a XT4 MP apparatus (Taike Corp., Beijing, China) and are as read. Analytical thin-layer chromatography (TLC) was performed on the glass-backed silica gel sheets (silica gel 60 Å GF254). All compounds were detected using UV light (254 or 365 nm).

### 4.2. General procedure for the preparation of compounds

#### 4.2.1. General procedure for the preparation of compounds 5 and 6

5-Nitroanthranilonitrile **1** (4.89 g, 30 mmol, 1.0 equiv) was suspended in dimethylformamide dimethyl acetal (10 mL) and the mixture was refluxed for 2 h. The resulting mixture was cooled to room temperature for 2–3 h. The yellow precipitate that formed was filtered, washed with ethyl ether, and dried to give 2 (Yield: 80–90%). A mixture of 2 (2.18 g, 10 mmol, 1.0 equiv) and 3-chloroaniline (1.1 equiv) or 3-bromoaniline (1.1 equiv) was heated and stirred at reflux in acetic acid (20 mL) for 2 h. Aniline and acetic acid would be added into the reaction flask first and then slowly added 2 using the medicine spoon within one minute. This treatment is to prevent the formation of lumps between them. The yellow precipitate that formed was filtered hot, washed with hot acetic acid, diethyl ether, and dried to give the desired nitroquinazoline 3 or 4 (Yield: 80–90%).

6-Nitroquinazoline **4** (2.0 g, 6.65 mmol, 1.0 equiv), iron (2.5 g, 45 mmol, 6.7 equiv) and acetic acid (6 mL, 90 mmol, 13.5 equiv) were suspended in aqueous ethanol (180 mL, 77.8% v/v) and heated at reflux about 70–80 °C for 5–6 h. Meantime this mixture would be stirred for one minute every half hour with a glass rod and the yellow solution would become reddish-brown slowly. The reaction mixture was cooled to room temperature and alkalinized by addition of concentrated ammonia (40 mL). Insoluble material was removed by filtration through celite, and the filtrate was evaporated under reduced pressure. The resulting solid was extracted with ethyl acetate for column chromatography. Column chromatography was performed using silica gel (200–300 mesh) eluting with ethyl acetate and petroleum ether (3:1, v/v) to give amine **6**.

#### 4.2.2. General Procedure for synthesis of compounds 5a–5i and 6a–6i

Equimolar amount of amine **5** or **6** (0.5 mmol) and various substituted benzaldehyde (1.0 mmol) were dissolved in ethanol (20 mL), and stirred 30–40 °C for 5–6 h. The next step was to add sodium borohydride (5 mmol) into the reaction mixture and this solution continued to stirred 40–50 °C for 3–4 h. The reaction mixture was concentrated. The product (**5a–5i**, **6a–6i**) was obtained by column chromatography.

**4.2.2.1. 2-((4-(3-Bromophenylamino)quinazolin-6-ylamino)methyl)phenol (5a).** Mp 235–236 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.38 (d, $J$ = 5.49 Hz, 2H, $CH_2$), 6.40 (t,

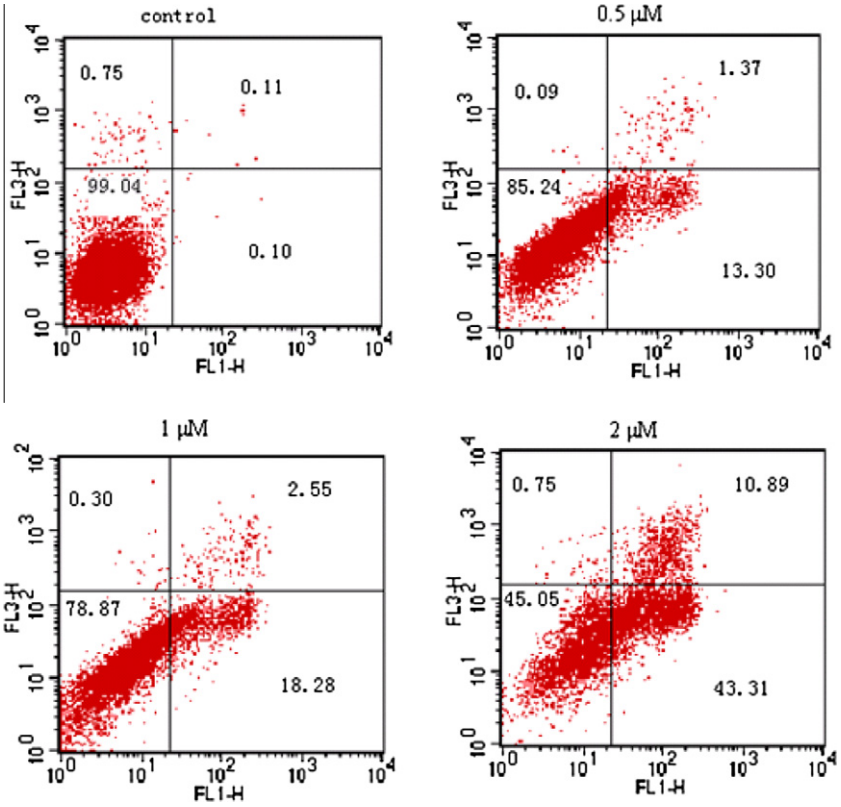



**Figure 3.** Analysis of apoptosis induced by compounds **5b** in the Hep G2 liver cancer cell line. Data represent the percentage of apoptotic cells. (a) control, (b) 0. 5 μM , (c) 1.0 μM, (d) 2.0 μM.

*J* = 5.58 Hz, 1H), 6.76 (t, *J* = 6.96 Hz, 1H), 6.86 (d, *J* = 8.07 Hz, 1H), 7.04–7.11 (m, 1H), 7.24–7.40 (m, 5H), 7.55 (d, *J* = 8.94 Hz, 1H), 7.89 (d, *J* = 8.04 Hz, 1H), 8.16 (s, 1H, NH), 8.39 (s, 1H), 9.44 (s, 1H, $NHCH_2$), 9.60 (s, 1H, OH). ESI-MS: 421.1 $C_{21}H_{18}BrN_4O$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{17}BrN_4O$: C, 59.87%; H, 4.07%; N, 13.30%. Found: C, 59.53%; H, 4.36%; N, 13.57%.

**4.2.2.2. 4-((4-(3-Bromophenylamino)quinazolin-6-ylamino) methyl)phenol (5b).** Mp 239–240 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.30 (d, *J* = 5.31 Hz, 2H, $CH_2$), 6.56 (s, 1H), 6.74 (t, *J* = 8.4 Hz, 1H), 7.24–7.37 (m, 6H), 7.54 (d, *J* = 8.97 Hz, 1H), 7.91 (d, *J* = 8.22 Hz, 1H), 8.18 (s, 1H, NH), 8.38 (s, 1H), 9.30 (s, 1H, OH), 9.43 (s, 1H, $NHCH_2$). ESI-MS: 421.1 ($C_{21}H_{18}BrN_4O$, $[M+H]^+$). Anal Calcd for $C_{21}H_{17}BrN_4O$: C, 59.87%; H, 4.07%; N, 13.30%. Found: C, 59.72%; H, 4.45%; N, 13.11%.

**4.2.2.3. N6-(2-Methoxybenzyl)-N4-(3-bromophenyl)quinazoline-4,6-diamine(5c).** Mp 105–107 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 3.85 (s, 3H, $OCH_3$), 4.41 (d, *J* = 5.67 Hz, 2H, $CH_2$), 6.47 (s, 1H), 6.92 (t, *J* = 7.40 Hz, 1H), 7.04 (d, *J* = 8.04 Hz, 1H), 7.24–7.39 (m, 6H), 7.56 (d, *J* = 8.94 Hz, 1H), 7.84 (d, *J* = 8.04 Hz, 1H), 8.15 (s, 1H, NH), 8.38 (s, 1H), 9.42 (s, 1H, $NHCH_2$). ESI-MS: 435.1 ($C_{22}H_{20}BrN_4O$, $[M+H]^+$). Anal. Calcd for $C_{22}H_{19}BrN_4O$: C, 60.70%; H, 4.40%; N, 12.87%. Found: C, 60.21%; H, 4.36%; N, 13.07%.

**4.2.2.4. N6-(4-Fluorobenzyl)-N4-(3-bromophenyl)quinazoline-4,6-diamine(5d).** Mp 122–124 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.44 (d, *J* = 5.85 Hz, 2H, $CH_2$), 6.73 (t, *J* = 5.58 Hz, 1H), 7.18 (t, *J* = 8.88 Hz, 2H), 7.26 (d, *J* = 8.58 Hz, 1H), 7.34(t, *J* = 7.95 Hz ,3H), 7.47–7.52 (m, 2H), 7.56(d, *J* = 8.94 Hz, 1H), 7.89 (d, *J* = 8.07 Hz, 1H), 8.17 (s, 1H, NH), 8.39 (s, 1H), 9.37 (s, 1H, $NHCH_2$). ESI-MS: 423.1 ($C_{21}H_{17}BrN_4O$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}BrN_4O$: C, 59.59%; H, 3.81%; N, 13.24%. Found: C, 59.83%; H, 4.26%; N, 12.87%.

**4.2.2.5. N6-(4-Chlorobenzyl)-N4-(3-bromophenyl)quinazoline-4,6-diamine (5e).** Mp 119–121 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.46 (d, *J* = 5.85 Hz, 2H, $CH_2$), 6.78 (t, *J* = 5.85 Hz, 1H), 7.25–7.37 (m, 4H), 7.41 (d, *J* = 8.58 Hz, 2H), 7.48 (d, *J* = 8.61 Hz, 2H), 7.57 (d, *J* = 8.79 Hz, 1H), 7.88 (d, *J* = 8.04 Hz, 1H), 8.16 (s, 1H, NH), 8.39 (s, 1H), 9.36 (s, 1H, $NHCH_2$). ESI-MS: 439.0 ($C_{21}H_{17}BrClN_4$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}BrClN_4$: C, 57.36%; H, 3.67%; N, 12.74%. Found: C, 59.83%; H, 4.06%; N, 12.57%.

**4.2.2.6. N6-(4-Bromobenzyl)-N4-(3-bromophenyl)quinazoline-4,6-diamine(5f).** Mp 125–127 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.44 (d, *J* = 6.03 Hz, 2H, $CH_2$), 6.78 (t, *J* = 5.94 Hz, 1H), 7.24–7.36 (m, 4H), 7.41 (d, *J* = 8.4 Hz, 2H), 7.53–7.58 (m, 3H), 7.88 (d, *J* = 8.07 Hz, 1H), 8.16 (s, 1H, NH), 8.39 (s, 1H), 9.35 (s, 1H, $NHCH_2$). ESI-MS: 482.9 ($C_{21}H_{17}Br_2N_4$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}Br_2N_4$: C, 52.09; H, 3.33%; N, 11.57%. Found: C, 52.34%; H, 3.36%; N, 11.27%.

**4.2.2.7. N6-(2-Chlorobenzyl)-N4-(3-bromophenyl)quinazoline-4,6-diamine(5g).** Mp 199–200 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.53 (d, *J* = 5.67 Hz, 2H, $CH_2$), 6.69 (t, *J* = 5.76 Hz, 1H), 6.76 (t, *J* = 6.96 Hz, 1H), 7.24–7.40 (m, 6H), 7.48–7.53 (m, 2H), 7.59 (d, *J* = 7.71 Hz, 1H), 7.87 (d, *J* = 8.22 Hz, 1H), 8.16 (s, 1H, NH), 8.40 (s, 1H), 9.41 (s, 1H, $NHCH_2$). ESI-MS: 439.0 ($C_{21}H_{17}BrClN_4$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}BrClN_4$: C, 57.36%; H, 3.67%; N, 12.74%. Found: C, 56.97%; H, 3.36%; N, 13.09%.

**4.2.2.8. N6-(2-Bromobenzyl)-N4-(3-bromophenyl)quinazoline-4,6-diamine (5h).** Mp 213–214 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.49 (d, $J$ = 5.49 Hz, 2H, $CH_2$), 6.68 (t, $J$ = 5.76 Hz, 1H), 7.23–7.40 (m, 6H), 7.50 (d, $J$ = 6.95 Hz, 1H), 7.59 (d, $J$ = 8.97 Hz, 1H), 7.67 (dd, $J_1$ = 7.86 Hz, $J_2$ = 8.04 Hz, 1H), 7.87 (d, $J$ = 8.04 Hz, 1H), 8.16 (s, 1H, NH), 8.39 (s, 1H), 9.44 (s, 1H, $NHCH_2$). ESI-MS: 482.9 ($C_{21}H_{17}Br_2N_4$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}Br_2N_4$: C, 52.09%; H, 3.33%; N, 11.57%. Found: C, 51.89%; H, 3.46%; N, 11.76%.

**4.2.2.9. N6-(4-Methoxybenzyl)-N4-(3-bromophenyl)quinazoline-4,6-diamine (5i).** Mp 115–117 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 3.37 (s, 3H, $OCH_3$), 4.37 (d, $J$ = 5.49 Hz, 2H, $CH_2$), 6.64 (t, $J$ = 5.58 Hz, 1H), 6.93 (d, $J$ = 8.61 Hz, 2H), 7.25–7.40 (m, 6H), 7.56 (d, $J$ = 8.97 Hz, 1H), 7.91 (d, $J$ = 8.04 Hz, 1H), 8.18 (s, 1H, NH), 8.39 (s, 1H), 9.39 (s, 1H, $NHCH_2$). ESI-MS: 435.1 ($C_{22}H_{20}BrN_4O$, $[M+H]^+$). Anal. Calcd for $C_{22}H_{19}BrN_4O$: C, 60.70%; H, 4.40%; N, 12.87%. Found: C, 60.43%; H, 4.34%; N, 13.07%.

**4.2.2.10. 2-((4-(3-Chlorophenylamino)quinazolin-6-ylamino) methyl)phenol (6a).** Mp 232–234 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.38 (d, $J$ = 5.49 Hz, 2H, $CH_2$), 6.41 (t, $J$ = 5.4 Hz, 1H), 6.77 (t, $J$ = 7.41 Hz, 1H), 6.86 (d, $J$ = 8.04 Hz, 1H), 7.07–7.14 (m, 2H), 7.30 (t, $J$ = 6.95 Hz, 2H), 7.40 (t, $J$ = 8.15 Hz, 2H), 7.55 (d, $J$ = 8.97 Hz, 1H), 7.83 (d, $J$ = 8.22 Hz, 1H), 8.06 (s, 1H, NH), 8.40 (s, 1H), 9.45 (s, 1H, $NHCH_2$), 9.61 (s, 1H, OH). ESI-MS: 377.1 ($C_{21}H_{18}ClN_4O$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{17}ClN_4O$: C, 66.93%; H, 4.55%; N, 14.87%. Found: C, 67.03%; H, 4.36%; N, 14.59%.

**4.2.2.11. 4-((4-(3-Chlorophenylamino)quinazolin-6-ylamino) methyl)phenol (6b).** Mp 228–230 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.31 (d, $J$ = 5.49 Hz, 2H, $CH_2$), 6.56 (s, 1H), 6.75 (d, $J$ = 8.61 Hz, 2H), 7.13 (dd, $J_1$ = 7.86 Hz, $J_2$ = 7.86 Hz, 1H), 7.25–7.43 (m, 5H), 7.30 (t, $J$ = 6.95 Hz, 2H), 7.40 (t, $J$ = 8.15 Hz, 2H), 7.55 (d, $J$ = 8.97 Hz, 1H), 7.84 (d, $J$ = 8.22 Hz, 1H), 8.07 (s, 1H, NH), 8.39 (s, 1H), 9.32 (s, 1H, OH), 9.40 (s, 1H, $NHCH_2$). ESI-MS: 377.1 ($C_{21}H_{18}ClN_4O$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{17}ClN_4O$: C, 66.93%; H, 4.55%; N, 14.87%. Found: C, 67.14%; H, 4.63%; N, 14.49%.

**4.2.2.12. N7-(2-Methoxybenzyl)-N2-(3-chlorophenyl)quinazoline-2,7-diamine (6c).** Mp 99–101 °C; $^1$H NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 3.86 (s, 3H, $OCH_3$), 4.43 (d, $J$ = 6 Hz, 2H, $CH_2$), 6.50 (t, $J$ = 5.75 Hz, 1H), 6.93 (t, $J$ = 7.5 Hz, 1H), 7.04 (d, $J$ = 8.5 Hz, 1H), 7.13 (dd, $J_1$ = 8 Hz, $J_2$ = 8 Hz, 1H), 7.26–7.31 (m, 2H), 7.35–7.42 (m, 3H), 7.57 (d, $J$ = 8.5 Hz, 1H), 7.83 (d, $J$ = 8.5 Hz, 1H), 8.05 (s, 1H, NH), 8.40 (s, 1H), 9.44 (s, 1H, $NHCH_2$). ESI-MS: 391.1 ($C_{22}H_{20}ClN_4O$, $[M+H]^+$). Anal. Calcd for $C_{22}H_{19}ClN_4O$: C, 67.60%; H, 4.90%; N, 14.33%. Found: C, 67.26%; H, 5.24%; N, 14.65%.

**4.2.2.13. N7-(4-Fluorobenzyl)-N2-(3-chlorophenyl)quinazoline-2,7-diamine (6d).** Mp 115–117 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.44 (d, $J$ = 5.67 Hz, 2H, $CH_2$), 6.74 (t, $J$ = 5.85 Hz, 1H), 7.12–7.21 (m, 3H), 7.33–7.43 (m, 3H), 7.47–7.58 (m, 3H), 7.82 (d, $J$ = 8.22 Hz, 1H), 8.06 (s, 1H, NH), 8.40 (s, 1H), 9.39 (s, 1H, $NHCH_2$). ESI-MS: 379.1 ($C_{21}H_{17}ClFN_4$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}ClFN_4$: C, 66.58%; H, 4.26%; N, 14.79%. Found: C, 66.83%; H, 4.62%; N, 14.41%.

**4.2.2.14. N6-(4-Chlorobenzyl)-N4-(3-chlorophenyl)quinazoline-4,6-diamine (6e).** Mp 121–123 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.46 (d, $J$ = 5.85 Hz, 2H, $CH_2$), 6.79 (s, 1H), 7.13 (d, $J$ = 9.15 Hz, 1H), 7.31–7.49 (m, 6H), 7.57 (d, $J$ = 8.94 Hz, 1H), 7.81 (d, $J$ = 9.15 Hz, 1H), 8.05 (s, 1H, NH), 8.39 (s, 1H), 9.38 (s, 1H, $NHCH_2$). ESI-MS: 395.0 ($C_{21}H_{17}Cl_2N_4$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}Cl_2N_4$: C, 63.81%; H, 4.08%; N, 14.17%. Found: C, 64.03%; H, 4.21%; N, 13.84%.

**4.2.2.15. N6-(4-Bromobenzyl)-N4-(3-chlorophenyl)quinazoline-4,6-diamine (6f).** Mp 126–128 °C; $^1$H NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 4.41 (d, $J$ = 6 Hz, 2H, $CH_2$), 6.76 (t, $J$ = 5.75 Hz, 1H), 7.07 (dd, $J_1$ = 7.5 Hz, $J_2$ = 8 Hz, 1H), 7.29–7.38 (m, 6H), 7.49 (d, $J$ = 8.2 Hz, 2H), 7.56 (d, $J$ = 9 Hz, 1H), 7.79 (d, $J$ = 8 Hz, 1H), 8.04 (s, 1H, NH), 8.39 (s, 1H), 9.38 (s, 1H, $NHCH_2$). ESI-MS: 439.0 ($C_{21}H_{17}BrClN_4$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}BrClN_4$: C, 57.36; H, 3.67%; N, 12.74%. Found: C, 57.24%; H, 3.39%; N, 13.05%.

**4.2.2.16. N6-(2-Chlorobenzyl)-N4-(3-chlorophenyl)quinazoline-4,6-diamine (6g).** Mp 195–197 °C; $^1$H NMR (500 MHz, DMSO-$d_6$, $\delta$ ppm): 4.54 (d, $J$ = 5.5 Hz, 2H), 6.70 (t, $J$ = 5.5 Hz, 2H), 7.12 (dd, $J_1$ = 6.5 Hz, $J_2$ = 6.5 Hz, 1H), 7.31–7.33 (m, 3H), 7.37–7.40 (m, 2H), 7.48–7.52 (m, 2H), 7.60 (d, $J$ = 9 Hz, 1H), 7.81 (d, $J$ = 8 Hz, 1H), 8.05 (s, 1H, NH), 8.41 (s, 1H), 9.43 (s, 1H, $NHCH_2$). ESI-MS: 394.1 ($C_{21}H_{17}Cl_2N_4$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}Cl_2N_4$: C, 63.81%; H, 4.08%; N, 14.17%. Found: C, 64.07%; H, 3.95%; N, 14.09%.

**4.2.2.17. N6-(2-Bromobenzyl)-N4-(3-chlorophenyl)quinazoline-4,6-diamine (6h).** Mp 203–205 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 4.50 (d, $J$ = 5.67 Hz, 2H, $CH_2$), 6.70 (s, 1H), 7.14 (d, $J$ = 7.68 Hz, 1H), 7.25 (t, $J$ = 6.95 Hz, 1H), 7.33–7.43 (m, 4H), 7.59 (d, $J$ = 8.97 Hz, 1H), 7.50 (dd, $J_1$ = 7.86 Hz, $J_2$ = 7.5 Hz, 1H), 7.59 (d, $J$ = 9.15 Hz, 1H), 7.67 (d, $J$ = 7.86 Hz, 1H), 7.80 (d, $J$ = 8.97 Hz, 1H), 8.04 (s, 1H, NH), 8.41 (s, 1H), 9.46 (s, 1H, $NHCH_2$). ESI-MS: 439.0 ($C_{21}H_{17}BrClN_4$, $[M+H]^+$). Anal. Calcd for $C_{21}H_{16}BrClN_4$: C, 57.36%; H, 3.67%; N, 12.74%. Found: C, 56.99%; H, 3.42%; N, 13.06%.

**4.2.2.18. N6-(4-Methoxybenzyl)-N4-(3-chlorophenyl)quinazoline-4,6-diamine (6i).** Mp 103–104 °C; $^1$H NMR (300 MHz, DMSO-$d_6$, $\delta$ ppm): 3.73 (s, 3H, $OCH_3$), 4.38 (d, $J$ = 5.49 Hz, 2H, $CH_2$), 6.64 (t, $J$ = 5.48 Hz, 1H), 6.93 (d, $J$ = 8.61 Hz, 2H), 7.13 (dd, $J_1$ = 8.07 Hz, $J_2$ = 7.86 Hz, 1H), 7.33–7.43 (m, 5H), 7.56 (d, $J$ = 8.97 Hz, 1H), 7.85 (d, $J$ = 8.25 Hz, 1H), 8.07 (s, 1H, NH), 8.40 (s, 1H), 9.40 (s, 1H, $NHCH_2$). ESI-MS: 391.1 ($C_{22}H_{20}IN_4O$, $[M+H]^+$). Anal. Calcd for $C_{22}H_{19}ClN_4O$: C, 67.60%; H, 4.90%; N, 14.33%. Found: C, 67.13%; H, 5.12%; N, 14.36%.

### 4.3. Cell proliferation assay

The antiproliferative activities of chalcone thiosemicarbazide derivatives were determined using a standard (MTT)-based colorimetric assay (Sigma). Briefly, cell lines were seeded at a density of $7 \times 10^3$ 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 40 mg/mL. After 48 h, cell survival was determined by the addition of an MTT solution (20 μL of 5 mg/mL MTT in PBS). After 6 h, 100 mL of 10% SDS in 0.01 N HCl was added, and the plates were incubated at 37 °C for a further 4 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 6 wells from at least two independent experiments.

### 4.4. General procedure for preparation, purification of 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 vectors pBlueBacHis2B and pFASTBacHTc (Huakang Company China), separately. A sequence that encodes $(His)_6$ was located at the 5′ upstream to the EGFR sequences. Sf-9 cells were infected for 3 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 non-specifically bound material. Histidinetagged 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.[19]

The EGFR kinase assay was set up to assess the level of autophosphorylation based on DELFIA/Time-Resolved Fluorometry. Compounds **20–42** were dissolved in 100% DMSO and diluted to the appropriate concentrations with 25 mM HEPES at pH 7.4. In each well, 10 μL compound was incubated with 10 μL (5 ng for EGFR) recombinant enzyme (1:80 dilution in 100 mM HEPES) for 10 min at room temperature. Then, 10 μL of 5 × 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 $MgCl_2$ were 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 europiumlabeled 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 anti-phosphotyrosine antibody is from EGFR.

### 4.5. Molecular docking modeling

Molecular docking of compound **5b** into the three-dimensional EGFR complex structure (1M17.pdb, downloaded from the PDB) was carried out using the AutoDock software package (version 4.0) as implemented through the graphical user interface AutoDock Tool Kit (ADT 1.4.6).

### 4.6. Analysis of apoptosis induced in cells

The Hep G2 cells were seeded in 6-well plates at a seeding density of 105 cells/ml. When all the cells were adhered, various concentrations of compound **5b** added. Cells were treated with compounds **5b** for 3 days and apoptosis was analyzed using Annexin-V and propium iodide (PI) double staining by flow cytometry. Early apoptotic cells were defined as Annexin-V positive/PI-negative, late apoptotic cells as Annexin-V/PI-double positive and necrotic cells as Annexin-V positive/PI positive.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.085.

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