ORIGINAL RESEARCH



Synthesis, structure, and biological assay of cinnamic amides as potential EGFR kinase inhibitors

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Abstract A series of derivatives of cinnamic amide (compounds 2a-2v) were synthesized and evaluated for antiproliferative activities against the human breast cancer cell line MCF-7- and EGFR-inhibitory activities. The structures of compounds 2b and 2i were determined by single-crystal X-ray diffraction analysis. Compounds 2fand 2j showed moderate EGFR inhibitory activity with IC₅₀ values of 5.16 and 7.37 μ M, respectively. Docking simulation of compound 2f was carried out to illustrate the binding mode of the molecule into the EGFR active site. Structure–activity relationship analysis found that the *N*-phenyl rings are required for enhancing the activities.

Keywords Cinnamic amides · MCF-7 · EGFR · Docking simulation · Structure–activity relationship

Introduction

Conventional cytotoxic drugs for cancer chemotherapy are associated with serious toxic side effects, while drugs

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designed to modulate or inhibit molecular targets are found to show high selectivities and low toxicities (Seymour, 1999). One family of the targets is receptor tyrosine kinases (RTKs), which play a crucial role in signal transduction pathways that regulate various cellular mechanisms (Herbst, 2004). Among the RTKs, epidermal growth factor receptor tyrosine kinase (known as EGFR, HER-1, or ErbB-1) is considered to be of great value in cancer therapy (Traxler, 2003; Zhang et al., 2007). Possibly owing to overexpressions, mutations, or autocrine expressions of ligands (EGR or TGF α) (Slamon *et al.*, 1987), hyperactivation of EGFR induces aggressive tumor progression and reduces sensitivity to antitumor drugs (Yarden and Sliwkowski, 2001; Oda et al., 2005). EGFR overexpression is widely found in breast cancer (Lv et al., 2010a, b), ovarian cancer (Bull-Phelps et al., 2008), lung cancer (Yamamoto et al., 2009), and hormone-refractory prostate cancer (Wikstrand et al., 1997). Thus, searching for small molecules to inhibit the EGFR activities shows great significance in developing new anticancer agents (Bridges, 1999).

Cinnamic acid, which is ubiquitous in cinnamon oil and many other balsams, is a naturally occurring aromatic fatty acid of low toxicity with a long history of human exposure (Liu *et al.*, 1995). Commercially, it is widely applied in the perfume industry because of its floral odor. Certain substituted cinnamic amides containing cyano or fluoro moieties are of particular value due to their inhibitory effect in mitochondrial pyruvate transport (Hoskins, 1984). Cinnamic amides were reported to have many different biological activities such as anticancer, antimitotic, antioxidant, and seed-germination inhibitory effects (Leslie *et al.*, 2010; Natella *et al.*, 1999; Cutillo *et al.*, 2003). However, to our knowledge, few reports have been dedicated to the EGFR inhibitory activity and the structure– activity relationship (SAR) of cinnamic amide derivatives.

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Herein, our interest is to seek how simple substituents on the *N*-phenyl ring of cinnamic amides affect the EGFR inhibitory activities. Therefore, we select a series of substituents for their electronic properties to modify the *N*-phenyl ring leaving the other phenyl ring as a constant. The synthesis route, biological assay and the SARs are described here. Docking simulations were performed by the X-ray crystallographic structure of the EGFR in complex with inhibitors to explore the binding modes of these compounds at the active site.

Results and discussion

Chemistry

In order to investigate how substituents on the *N*-phenyl ring influence the activities, 20 substituted anilines and two alkylamines were treated to react with cinnamoyl chloride. The substituents introduced here include –Me, –iPr, –OMe, –OEt, –F, –Cl, and –Br. The synthesis route outlined in Scheme 1 gave satisfactory yields.

The crystal structures of compounds 2b and 2i

Among these compounds, crystal structures of compounds **2b** and **2i** were determined by X-ray diffraction analysis.

The crystal data are presented in Table 1, and Fig. 1a, b give perspective views of **2b** and **2i** with the atomic labeling system. Selected bond lengths, bond angles, and torsion angles are listed in Table 2. All the bond lengths and angles are in the normal ranges.

Biological assay

The in vitro antiproliferative activities of the cinnamic amides **2a–2v** are exhibited in Table 3. The research was carried out by the MTT colorimetric assay on the human tumor cell line MCF-7 which overexpresses EGFR. The IC₅₀ values, the concentration (μ M) at which only 50 % survival of cells was allowed, were calculated. As shown in Table 3, compound **2f** was found to show the most potent activity with IC₅₀ value of 2.01 μ M.

Furthermore, compounds 2a-2v were also evaluated for their ability to inhibit the autophosphorylation of EGFR kinase by a solid-phase ELISA assay. A number of synthesized compounds displayed moderate EGFR kinase inhibitory activities, which can be seen in Table 3. Owing to that EGFR is an important factor to breast carcinoma cancer, the results show the same trend with the antiproliferative activities against MCF-7. Compounds **2f** and **2j** displayed the most potent inhibitory activities (IC₅₀ = 5.16 and 7.37 µM, respectively).



Reagents and conditions: (i) SOCl₂, 80-90°C, reflux for 2h; (ii) CH_2Cl_2 , DMAP, NaHCO₃, 2-3 h.

Scheme 1 Synthesis of cinnamic amides 2a-2v. Reagents and conditions: i SOCl₂, 80-90 °C, reflux for 2 h; ii CH₂Cl₂, DMAP, NaHCO₃, 2-3 h

Table 1 Crystallographic data for compounds 2b and 2i

Compounds	2b	2i
Empirical formula	C ₁₆ H ₁₅ NO	C ₁₇ H ₁₇ NO ₂
Formula weight	237.29	267.32
Crystal system	Orthorhombic	Monoclinic
Space group	Pbca	$P2_{1}/c$
Crystal color	White	Pale gray
<i>a</i> (Å)	14.227(17)	18.012(7)
<i>b</i> (Å)	9.504(11)	10.205(4)
<i>c</i> (Å)	19.78(2)	7.753(3)
α (°)	90.00	90.00
β (°)	90.00	93.418(4)
γ (°)	90.00	90.00
$V(\text{\AA})$	2674(6)	1422(2)
Ζ	8	4
$D_{\rm calc}~({\rm g~cm^{-3}})$	1.179	1.248
θ range (°)	2.51-24.87	2.27-27.63
hkl range	$-9 \le h \le 17;$ $-11 \le k \le 11;$ $-23 \le l \le 24$	$-22 \le h \le 22;$ $-11 \le k \le 12;$ $-9 \le l \le 9$
<i>F</i> (0 0 0)	1,008	568.0
No. collected refl.	12,708	10,425
No. ind refl. (R_{int})	2,734 (0.061)	2,887 (0.062)
Data/restraints/parameters	2,734/0/164	2,887/0/182
Absorption coefficient (mm^{-1})	0.073	0.082
$R_I; wR_2 [I > 2\sigma(I)]$	0.0580; 0.1417	0.0494; 0.1173
R_1 ; wR_2 (all data)	0.0943; 0.1737	0.0777; 0.1377
GOOF	1.038	1.034

Fig. 1 a ORTEP view showing the atom-labeling scheme with thermal ellipsoids drawn at 30 % probability for compound 2b. b ORTEP view of compound 2i drawn in the same way as 2b



01

SAR analysis

It could be observed in Table 3 that there exists a gap of activities between 2a-2t and 2u-2v, which demonstrates that *N*-phenyl rings are required for enhancing the activities. For 2a-2t, there is a weak fluctuation in the inhibitory activities showing that o - > p - > m- substituents and replacements of Me groups by alkoxyl groups (RO) or halogens groups (X) result in the increase of activities in general. But, the IC₅₀ values of 2a-2t cover such narrow ranges (threefold for MCF-7 and 4.5-fold for EGFR), revealing that the addition of simple groups to the *N*-phenyl rings does not seem to be an essential point for enhancing the inhibitory activities. Thus, we do not declare it to be a general rule. We hope this finding could be of help for future exploring.

Binding model of compound 2f into EGFR

In order to help to explain the SARs observed at the EGFR inhibition, molecular docking of the most potent inhibitor **2f** into the ATP binding site of EGFR kinase was performed on the binding model based on the EGFR complex structure (1M17.pdb), which is depicted in Fig. 2. In the binding model, compound **2f** is nicely bound to the region with the MeO group projecting toward the HO of THR 830 forming a more optimal hydrogen bond interaction. Moreover, carbonyl group of compound **2f** also forms a H-bond with the HO of THR 766. Together with the SAR study, this molecular docking result suggests the MeO to be favorable for enhancing the activity.

Table 2 Selected bond lengths, bond angles, and torsion angles of compounds 2b and 2i

Table 3	The antip	roliferative	activities	and	EGFR	inhibitory	activi-
ties of co	ompounds	2a–2v					

Compounds	2b	2i
Bond lengths (Å)		
C ₁ -C ₂	1.369(4)	1.375(3)
C ₄ –C ₇	1.463(3)	1.465(3)
C ₇ –C ₈	1.316(3)	1.321(3)
C ₈ –C ₉	1.477(3)	1.477(3)
O ₁ -C ₉	1.231(3)	1.230(2)
N ₁ -C ₉	1.339(3)	1.352(2)
N ₁ -C ₁₀	1.426(3)	1.419(2)
$C_{15}-C_{16}$ (2b)	1.502(4)	
O ₂ –C ₁₃ (2i)		1.373(2)
O ₂ –C ₁₆ (2i)		1.422(3)
C ₁₆ –C ₁₇ (2i)		1.495(3)
Bond angles (°)		
$H_1A-N_1-C_9$	117.0	117.5
C ₉ -N ₁ -C ₁₀	125.93(18)	124.94(16)
$C_1 - C_2 - C_3$	120.1(3)	120.3(2)
$N_1 - C_9 - O_1$	122.7(2)	122.61(18)
$N_1 - C_9 - C_8$	114.44(19)	114.84(17)
$C_4 - C_7 - C_8$	127.5(2)	128.12(19)
C_{10} – C_{15} – C_{16} (2b)	121.6(3)	
C_{15} - C_{16} - $H_{16}A$ (2b)	109.5	
C_{13} - O_2 - C_{16} (2i)		118.26(15)
$O_2 - C_{16} - C_{17}$ (2i)		107.6(2)
$H_{16}A-C_{16}-C_{17}$ (2i)		110.2
Torsion angles (°)		
$C_3 - C_4 - C_7 - C_8$	-17.7(4)	-171.2(2)
$C_4 - C_7 - C_8 - C_9$	-178.8(2)	-178.69(17)
$C_7 - C_8 - C_9 - N_1$	174.1(2)	-166.19(18)
$N_1 - C_{10} - C_{11} - C_{12}$	-178.9(2)	177.74(17)
C_{11} - C_{10} - N_1 - C_9	-47.8(3)	45.2(3)
$O_1 - C_9 - N_1 - C_{10}$	-3.1(4)	0.8(3)
$N_1 - C_{10} - C_{15} - C_{16}$ (2b)	-2.9(4)	
C_{11} - C_{10} - C_{15} - C_{16} (2b)	178.5(3)	
C_{11} - C_{12} - C_{13} - O_2 (2i)		-179.08(16)
C_{17} – C_{16} – O_2 – C_{13} (2i)		177.67(19)

Compounds MCF-7 (IC50/µM) EGFR (IC50/µM) 5.24 ± 0.94 23.16 ± 0.62 2.9 2b 3.17 ± 0.54 12.29 ± 0.37 2c 5.83 ± 0.97 19.69 ± 0.61 2d 3.26 ± 0.59 12.69 ± 0.32 2e 5.39 ± 1.08 14.58 ± 0.41 2f 2.01 ± 0.28 5.16 ± 0.14 2.34 ± 0.35 9.05 ± 0.32 2g 2h 2.43 ± 0.46 9.35 ± 0.27 2i 3.30 ± 0.50 10.23 ± 0.27 2j 2.38 ± 0.33 7.37 ± 0.20 2.56 ± 0.41 10.02 ± 0.25 2k 21 4.14 ± 0.83 11.69 ± 0.28 $4.65\,\pm\,0.90$ 13.23 ± 0.44 2m 2.89 ± 0.52 12.88 ± 0.39 2n 20 2.37 ± 0.38 9.86 ± 0.27 4.37 ± 0.61 14.59 ± 0.31 2p 2q 3.49 ± 0.63 13.71 ± 0.37 $4.00\,\pm\,0.77$ 16.17 ± 0.40 2r 4.47 ± 0.92 13.93 ± 0.36 2s2t 4.05 ± 0.64 15.74 ± 0.49 2u 13.40 ± 2.42 38.46 ± 0.92 2v 11.13 ± 1.78 29.85 ± 0.78 0.22 ± 0.03 0.34 ± 0.01 Erlotinib



Conclusions

A series of cinnamic amides were synthesized. The crystal structures of **2b** and **2i** were determined. All these compounds were evaluated for antiproliferative activities against the human cancer cell line MCF-7- and EGFR-inhibitory activities, and the two series of activities show the same trend. Moreover, docking simulations were performed to give the probable binding modes of compound **2f** into the ATP binding site of EGFR kinase. Biological assay shows that *N*-phenyl rings are required to enhance the activities. Although certain rules are found in the SAR

Fig. 2 Binding model of compound 2f with EGFR kinase. *Note* Only interacting residues are displayed for clarity. Ligand and interacting key residues are represented as stick models, while the rest are represented as ribbons. The H-bonds are displayed as *green dotted lines* (Color figure online)

study, no strong evidences of the data support the beneficial substituents, revealing that the simple substitution on the N-phenyl does not bring a leap in activities. The results of

this study may be helpful for researching new potential antitumor agents.

Experimental part

Chemistry general

In this study, 22 corresponding cinnamic amides were prepared. Different amines acted as nucleophilic reagents to react with cinnamoyl chloride (1). Reactions were monitored by TLC using silica gel-coated glass slides (silica gel 60 GF 254, Qingdao Haiyang Chemical, China). Detections were done in UV (254 nm). Melting points were measured on a SPSIC WRS-1B digital melting-point apparatus (Shanghai, China); uncorrected. ¹H-NMR spectra were recorded on a Bruker Avance III 400 NMR spectrometer with CDCl₃ as solvent. The chemical shifts (δ) are reported in ppm with reference to internal TMS and coupling constants (J) are given in Hz. ESI-MS spectra were recorded on a Mariner System 5304 mass spectrometer. All reagents bought from Aldrich (USA), Aladdin (China) or Sinopharm Chemical Reagent Co., Ltd (China) were of pure analytical grades and used without further treatments.

General procedure for the synthesis and purification of the cinnamic amides

The series of compounds were synthesized according to literatures with some modifications (Narasimhan *et al.*, 2004; Zeller *et al.*, 2010). A mixture of cinnamic acid (0.14 g, 1 mmol) and SOCl₂ (15 mL, acted as both reactant and solvent) was stirred and refluxed at 80–90 °C for 2 h. Remaining SOCl₂ was removed by distillation under reduced pressure. Thus, the intermediate cinnamoyl chloride (1) was achieved, which was used directly in the next step without any further purification.

Intermediate **1** was then dissolved in anhydrous CH_2Cl_2 (40–50 mL), and NaHCO₃ (excessive, about 1.0 g) was added to the solution. Stirring was continued for 5 min to destroy residual SOCl₂. Then, 4-dimethylaminopyridine (DMAP, 10 mg) was added to the mixture followed by another 1–2 min stirring. Afterward, appropriate substituted aniline or alkylamine (1 mmol) was added slowly (finished in 1 min) and the mixture was stirred at r.t. for 2–3 h.

The reaction mixture was washed with HCl solution (5 %, 50 mL) and NaOH solution (5 %, 50 mL) several times. The CH₂Cl₂ solution was collected and dried by anhydrous Na₂SO₄. Further purification was carried out by silica gel column chromatography with petroleum ether/ EtOAc (10:1) as eluent. After recrystallization from a

mixture of EtOAc and hexane (1:1), final compounds **2a**–**2v** were obtained.

(E)-N-phenylcinnamamide (2a)

White crystal. yield 82 %. m.p. 148–149 °C, $\delta_{\rm H}$ (CDCl₃): 6.55 (d, *J* 15.6, 1H, H-8), 7.14 (t, *J* 7.2, 1H, H-4'), 7.34–7.42 (m, 5H, H-3,4,5,3',5'), 7.54 (d, *J* 6.0, 2H, H-2,6), 7.62 (d, *J* 6.0, 2H, H-2',6'), 7.76 (d, *J* 15.6, 1H, H-7). *m/z* (ESI): 224.1 [(M + H)⁺]; 222.0 [(M – H)⁻]. (Anal. Calcd. for C₁₅H₁₃NO: C, 80.72; H, 5.83; N, 6.78. Found: C, 80.39; H 6.27; N, 6.41 %.)

(E)-N-(o-tolyl)cinnamamide (2b)

White crystal. yield 86 %. m.p. 175–177 °C. $\delta_{\rm H}$ (CDCl₃): 2.33 (s, 3H, –CH₃); 6.58 (d, *J* 15.6, 1H, H-8); 7.13 (m, 2H, H-4',5'); 7.22 (m, 2H, H-3',6'); 7.38 (m, 3H, H-3,4,5); 7.55 (d, *J* 7.0, 2H, H-2,6); 7.80 (d, *J* 15.6, 1H, H-7), 7.99 (br. s, 1H, NH). *m*/z (ESI): 238.1 [(M + H)⁺]; 250.1 [(M + Na)⁺]. (Anal. Calcd. for C₁₆H₁₅NO: C, 81.01; H, 6.33; N, 5.91. Found: C, 80.63; H, 6.67; N, 6.24 %.)

(E)-N-(m-tolyl)cinnamamide (2c)

White crystal. yield 81 %. m.p. 111–112 °C. $\delta_{\rm H}$ (CDCl₃): 2.35 (s, 3H, –CH₃); 6.55 (d, *J* 15.6, 1H, H-8); 6.95 (d, 1H, H-4'); 7.23 (t, *J* 7.8, 1H, H-5'); 7.40–7.43 (m, 4H, H-3,4,5,2'); 7.49–7.54 (m, 3H, H-2,6,6'); 7.75 (d, *J* 15.6, 1H, H-7). *m/z* (ESI): 238.1 [(M + H)⁺]; 250.1 [(M + Na)⁺]. (Anal. Calcd. for C₁₆H₁₅NO: C, 81.01; H, 6.33; N, 5.91. Found: C, 81.38; H, 6.07; N, 5.67 %.)

(E)-N-(p-tolyl)cinnamamide (2d)

White crystal. yield 84 %. m.p. 168–170 °C. $\delta_{\rm H}$ (CDCl₃): 2.34 (s, 3H, –CH₃); 6.54 (d, *J* 14.8, 1H, H-8); 7.16 (d, *J* 7.2, 2H, H-2',6'); 7.38 (m, 3H, H-3,4,5); 7.46–7.53 (m, 4H, H-2,6,3',5'); 7.75 (d, *J* 14.8, 1H, H-7). *m/z* (ESI): 238.1 [(M + H)⁺]; 250.1 [(M + Na)⁺]. (Anal. Calcd. for C₁₆H₁₅NO: C, 81.01; H, 6.33; N, 5.91. Found: C, 81.40; H, 6.62; N, 5.57 %.)

(E)-N-(4-isopropylphenyl)cinnamamide (2e)

Yellow crystal. yield 73 %. m.p. 159–160 °C. $\delta_{\rm H}$ (CDCl₃): 1.24 (d, *J* 7.2, 6H, 2 × -CH₃); 2.89 (m, *J* 6.8, 1H, -CH-); 6.55 (d, *J* 15.6, 1H, H-8); 7.21 (d, *J* 8.4, 2H, H-2',6'); 7.36–7.41 (m, 3H, H-3,4,5); 7.53 (m, 4H, H-2,6,3',5'); 7.75 (d, *J* 15.6, 1H, H-7). *m*/*z* (ESI): 266.2 [(M + H)⁺]; 264.1 [(M – H)⁻]. (Anal. Calcd. for C₁₈H₁₉NO: C, 81.51; H, 7.17; N, 5.28. Found: C, 81.18; H, 6.78; N, 5.62 %.)

(E)-N-(2-methoxyphenyl)cinnamamide (2f)

Yellow crystal. yield 79 %. m.p. 140–141 °C. $\delta_{\rm H}$ (CDCl₃): 3.93 (s, 3H, –OCH₃); 6.60 (d, *J* 15.6, 1H, H-8); 6.91 (d, *J* 8.0, 1H, H-3'); 6.99–7.10 (m, 2H, H-4',5'); 7.41 (m, 3H, H-3,4,5); 7.58 (d, *J* 6.8, 2H, H-2,6); 7.76 (d, *J* 15.6, 1H, H-7); 7.98 (d, *J* 7.2, 1H, H-6'); 8.54 (br. s, 1H, NH). *m/z* (ESI): 254.1 [(M + H)⁺]. (Anal. Calcd. for C₁₆H₁₅NO₂: C, 75.89; H, 5.93; N, 5.53. Found: C, 76.22; H, 5.57; N, 5.87 %.)

(E)-N-(4-methoxyphenyl)cinnamamide (2g)

White powder. yield 88 %. m.p. 163–164 °C. $\delta_{\rm H}$ (CDCl₃): 3.79 (s, 3H, –OCH₃); 6.55 (d, *J* 15.5, 1H, H-8); 6.88 (d, *J* 8.3, 2H, H-3',5'); 7.36 (m, 3H, H-3,4,5); 7.47–7.55 (m, 4H, H-2,6,2',6'); 7.74 (d, *J* 15.5, 1H, H-7). *m*/*z* (ESI): 254.1 [(M + H)⁺]; 276.1 [(M + Na)⁺]. (Anal. Calcd. for C₁₆H₁₅NO₂: C, 75.89; H, 5.93; N, 5.53. Found: C, 76.35; H, 5.63; N, 5.30 %.)

(E)-N-(2-ethoxyphenyl)cinnamamide (2h)

White powder. yield 79 %. m.p. 123–124 °C. $\delta_{\rm H}$ (CDCl₃): 1.53 (t, *J* 7.2, 3H, –CH₃); 4.18 (q, *J* 7.2, 2H, –OCH₂–); 6.62 (d, *J* 15.6, 1H, H-8); 6.92 (d, *J* 8.0, 1H, H-3'); 7.05 (m, 2H, H-4',5'); 7.42 (m, 3H, H-3,4,5); 7.61 (d, *J* 6.9, 2H, H-2,6); 7.78 (d, *J* 15.2, 1H, H-7); 8.00 (br. s, 1H, H-6'); 8.56 (br. s, 1H, NH). *m*/*z* (ESI): 268.1 [(M + H)⁺]; 290.2 [(M + Na)⁺]. (Anal. Calcd. for C₁₇H₁₇NO₂: C, 76.40; H, 6.37; N, 5.24. Found: C, 76.18; H, 6.66; N, 4.95 %.)

(E)-N-(4-ethoxyphenyl)cinnamamide (2i)

Pale gray lamellar crystal. yield 83 %. m.p. 150–152 °C. $\delta_{\rm H}$ (CDCl₃): 1.41 (t, *J* 7.2, 3H, –CH₃); 4.01 (q, *J* 6.8, 2H, – OCH₂–); 6.54 (d, *J* 15.6, 1H, H-8); 6.87 (d, *J* 8.4, 2H, H-3',5'); 7.37(m, 3H, H-3,4,5); 7.52 (m, 4H, H-2,6,2',6'); 7.74 (d, *J* 15.6, 1H, H-7). *m*/*z* (ESI): 268.1 [(M + H)⁺]; 290.2 [(M + Na)⁺]. (Anal. Calcd. for C₁₇H₁₇NO₂: C, 76.40; H, 6.37; N, 5.24. Found: C, 76.75; H, 6.59; N, 4.97 %.)

(E)-N-(2-fluorophenyl)cinnamamide (2j)

White crystal. yield 76 %. m.p. 116–117 °C. $\delta_{\rm H}$ (CDCl₃): 6.58 (d, *J* 15.2, 1H, H-8); 7.06–7.19 (m, 3H, H-3',4',5'); 7.40 (dd, 3H, H-3,4,5); 7.52 (d *J* 6.8, 1H, H-6'); 7.57 (dd, 2H, H-2,6); 7.78 (d, *J* 15.6, 1H, H-7); 8.48 (br. s, 1H, NH). *m*/*z* (ESI): 242.1 [(M + H)⁺]; 240.0 [(M – H)[–]]. (Anal. Calcd. for C₁₅H₁₂FNO: C, 74.69; H, 4.98; N, 5.81. Found: C, 74.30; H, 5.41; N, 5.50 %.)

(E)-N-(4-fluorophenyl)cinnamamide (2k)

White crystal. yield 79 %. m.p. 155–157 °C. $\delta_{\rm H}$ (CDCl₃): 6.54 (d, *J* 15.2, 1H, H-8); 7.04 (t, *J* 8.4, 2H, H-3',5'); 7.38

(m, 3H, H-3,4,5); 7.43 (m, 2H, H-2,6); 7.52 (d, *J* 6.2, 2H, H-2',6'); 7.76 (d, *J* 15.6, 1H, H-7). *m/z* (ESI): 242.1 $[(M + H)^+]$; 240.0 $[(M - H)^-]$. (Anal. Calcd. for $C_{15}H_{12}FNO$: C, 74.69; H, 4.98; N, 5.81. Found: C, 74.98; H, 5.37; N, 5.52 %.)

(E)-N-(2,4-difluorophenyl)cinnamamide (2l)

Pale purple crystal. yield 72 %. m.p. 140–141 °C. $\delta_{\rm H}$ (CDCl₃): 6.58 (d, *J* 15.2, 1H, H-8); 6.90 (m, 2H, H-3',5'); 7.40 (m, 3H, H-3,4,5); 7.45 (m, 1H, H-6'); 7.55 (d, *J* 6.0, 2H, H-2,6), 7.77 (d, *J* 15.6, 1H, H-7); 8.42 (br. s, 1H, NH). *m/z* (ESI): 260.1 [(M + H)⁺]. (Anal. Calcd. for C₁₅H₁₁F₂NO: C, 69.50; H, 4.25; N, 5.41. Found: C, 69.13; H, 4.61; N, 5.16 %.)

(E)-N-(3,5-difluorophenyl)cinnamamide (2m)

White crystal. yield 86 %. m.p. 154–155 °C. $\delta_{\rm H}$ (CDCl₃): 6.51 (d, *J* 15.6, 1H, H-8); 6.58 (tt, *J* 7.2, 2.0 1H, H-4'); 7.25 (m, 2H, H-2',6'); 7.40 (m, 3H, H-3,4,5); 7.54 (m, 2H, H-2,6); 7.78 (d, *J* 15.6, 1H, H-7). *m/z* (ESI): 260.1 [(M + H)⁺]. (Anal. Calcd. for C₁₅H₁₁F₂NO: C, 69.50; H, 4.25; N, 5.41. Found: C, 69.17; H, 4.64; N, 5.12 %.)

(E)-N-(3,4,5-trifluorophenyl)cinnamamide (2n)

White powder. yield 72 %. m.p. 170–171 °C. $\delta_{\rm H}$ (CDCl₃): 6.49 (d, *J* 15.6, 1H, H-8); 7.34–7.42 (m, 2H, H-2',6'); 7.40–7.42 (m, 3H, H-3,4,5); 7.54–7.57 (m, 2H, H-2,6); 7.78 (d, *J* 15.6, 1H, H-7). *m*/*z* (ESI): 278.1 [(M + H)⁺]; 276.1 [(M – H)⁻]. (Anal. Calcd. for C₁₅H₁₀F₃NO: C, 64.98; H, 3.61; N, 5.05. Found: C, 65.33; H, 3.27; N, 5.43 %.)

(E)-N-(2-chlorophenyl)cinnamamide (20)

White crystal. yield 81 %. m.p. 137–139 °C. $\delta_{\rm H}$ (CDCl₃): 6.61 (d, *J* 15.2, 1H, H-8); 7.07 (t, *J* 8.8, 1H, H-4'); 7,26 (m, 1H, H-3'); 7.33 (t, *J* 7.6, 1H, H-5'); 7.40–7.44 (m, 3H, H-3,4,5); 7.59 (m, 2H, H-2,6); 7.79 (d, *J* 15.6, 1H, H-7); 7.81 (br. s, 1H, H-6'); 8.55 (br. s, 1H, NH). *m/z* (ESI): 258.2 [(M + H)⁺]; 280.1 [(M + Na)⁺]. (Anal. Calcd. for C₁₅H₁₂ClNO: C, 69.90; H, 4.66; N, 5.44. Found: C, 70.26; H, 4.90; N, 5.02 %.)

(E)-N-(3-chlorophenyl)cinnamamide (2p)

White crystal. yield 84 %. m.p. 124–125 °C. $\delta_{\rm H}$ (CDCl₃): 6.54 (d, *J* 15.6, 1H, H-8); 7.12 (d, *J* 7.6, 1H, H-4'); 7.28 (t, *J* 7.8, 1H, H-5'); 7.36 (s, 1H, H-2'); 7.40–7.41 (m, 3H, H-3,4,5); 7.47 (d, *J* 8.0, 1H, H-6'); 7.54 (m, 2H, H-2,6); 7.78 (d, *J* 15.6, 1H, H-7). *m/z* (ESI): 258.1 [(M + H)⁺]; 280.1 [(M + Na)⁺]. (Anal. Calcd. for C₁₅H₁₂ClNO: C, C, 69.90; H, 4.66; N, 5.44. Found: C, 70.32; H, 4.94; N, 5.17 %.)

(E)-N-(4-chlorophenyl)cinnamamide (2q)

White crystal. yield 89 %. m.p. 188–189 °C. $\delta_{\rm H}$ (CDCl₃): 6.54 (d, *J* 15.6, 1H, H-8); 7.32 (d, *J* 8.8, 2H, H-3',5'); 7.40 (m, 2H, H-2',6'); 7.53–7.55 (m, 3H, H-3,4,5); 7.59 (d, *J* 8.0, 2H, H-2,6); 7.77 (d, *J* 15.6, 1H, H-7). *m/z* (ESI): 258.1 [(M + H)⁺]; 280.1 [(M + Na)⁺]. (Anal. Calcd. for C₁₅H₁₂ClNO: C, 69.90; H, 4.66; N, 5.44. Found: C, 69.56; H, 4.36; N, 5.75 %.)

(E)-N-(2,4-dichlorophenyl)cinnamamide (2r)

White powder. yield 81 %. m.p. 171–173 °C. $\delta_{\rm H}$ (CDCl₃): 6.58 (d, *J* 15.5, 1H, H-8), 7.29 (d, *J* 8.8, 1H, H-5'), 7.41 (m, 4H, H-3,4,5,3'), 7.58 (m, 2H, H-2,6), 7.74 (br. s, 1H, H-6'), 7.78 (d, *J* 15.5, 1H, H-7), 8.52 (d, *J* 8.8, 1H, NH). *m/z* (ESI): 293.0 [(M + H)⁺]. (Anal. Calcd. for C₁₅H₁₁Cl₂NO: C, 61.64; H, 3.77; N, 4.69. Found: C, 61.30; H, 4.03; N, 4.34 %.)

(E)-N-(3,5-dichlorophenyl)cinnamamide (2s)

Pale purple crystal. yield 78 %. m.p. 142–143 °C. $\delta_{\rm H}$ (CDCl₃): 6.51 (d, *J* 15.6, 1H, H-8); 7.13 (s, 1H, H-4'); 7.27–7.29 (m, 3H, H-3,4,5); 7.42 (m, 2H, H-2,6); 7.57 (m, 2H, H-2',6'); 7.78 (d, *J* 15.6, 1H, H-7). *m/z* (ESI): 293.0 [(M + H)⁺]. (Anal. Calcd. for C₁₅H₁₁Cl₂NO: C, 61.64; H, 3.77; N, 4.69. Found: C, 61.26; H, 4.12; N, 4.39 %.)

(E)-N-(2-bromophenyl)cinnamamide (2t)

White crystal. yield 89 %. m.p. 148–149 °C. $\delta_{\rm H}$ (CDCl₃): 6.60 (d, *J* 15.6, 1H, H-8); 7.01 (t, *J* 8.0, 1H, H-4'); 7.37 (t, *J* 8.0, 1H, H-5'); 7.41–7.45 (m, 3H, H-3,4,5); 7.56–7.61 (m, 3H, H-2,6,3'); 7.79 (d, *J* 15.6, 1H, H-7); 7.81 (br. s, 1H, H-6'); 8.53 (br. s, 1H, NH). *m/z* (ESI): 302.0 [(M + H)⁺]. (Anal. Calcd. for C₁₅H₁₂BrNO: C, 59.62; H, 3.97; N, 4.64. Found: C, 59.25; H, 4.33; N, 4.40 %.)

(E)-N-dodecylcinnamamide (2u)

White crystal. yield 91 %. m.p. 73–74 °C. $\delta_{\rm H}$ (CDCl₃): 0.88 (t, J 5.8, 3H, –CH₃); 1.25–1.32 (m, 18H, –C₉H₁₈–); 1.57 (m, 2H, –CH₂–); 3.38 (m, 2H, –NCH₂–); 6.38 (d, J 15.5, 1H, H-8); 7.35–7.37 (m, 3H, H-3,4,5); 7.50 (d, J 6.8, 2H, H-2,6); 7.62 (d, J 15.5, 1H, H-7). *m/z* (ESI): 316.3 [(M + H)⁺]; 338.3 [(M + Na)⁺]. (Anal. Calcd. for C₂₁H₃₃NO: C, 80.00; H, 10.48; N, 4.44. Found: C, 79.78; H, 10.84; N, 4.09 %.)

(E)-N-(tert-butyl)cinnamamide (2v)

White powder. yield 85 %. m.p. 139–141 °C. $\delta_{\rm H}$ (CDCl₃): 1.44 (s, 9H, 3 × –CH₃); 6.32 (d, *J* 15.6, 1H, H-8); 7.35 (m, 3H, H-3,4,5); 7.48 (d, *J* 6.8, 2H, H-2,6); 7.57 (d, *J* 15.6, 1H, H-7). *m/z* (ESI): 204.3 [(M + H)⁺]. (Anal. Calcd. for C₁₃H₁₇NO: C, 76.85; H, 8.37; N, 6.90. Found: C, 76.46; H, 8.74; N, 6.58 %.)

Crystallographic studies

X-ray single-crystal diffraction data for compounds **2b** and **2i** were collected on a Bruker SMART APEX CCD diffractometer at 296 (2) K using Mo K α radiation ($\lambda = 0.71073$ Å) by the ω scan mode. The program SAINT was used for integration of the diffraction profiles. Structure was solved by direct methods by means of the SHELXS program of the SHELXTL package and refined by full-matrix least-squares methods by means of SHELXL (Sheldrick, 1997). All non-hydrogen atoms of compounds **2b** and **2i** were refined with anisotropic thermal parameters. All hydrogen atoms were placed in geometrically idealized positions and constrained to ride on their parent atoms.

Cell proliferative activities assay

The antiproliferative activities of compounds 2a-2v were determined as described (Lv et al., 2010a, b; Li et al., 2010) by 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 μ g mL⁻¹. After 48 h, cell survival was determined by the addition of an MTT solution (10 µL of 5 mg mL⁻¹ 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. IC50 values were determined from replicates of six wells from at least two independent experiments.

EGFR inhibitory assay

The preparation, purification, and the inhibitory assay of EGFR were conducted as described (Lv *et al.*, 2010a, b; Li *et al.*, 2010). A 1.6-kb cDNA encoded for the EGFR cytoplasmic domain (amino acids 645–1,186) was 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 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 μ M ammonium molybdate, 100 μ M sodium vanadate, 10 μ g mL⁻¹ aprotinin, 10 μ g mL⁻¹ leupeptin, 10 μ g mL⁻¹ pepstatin, and 16 μ g mL⁻¹ 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 μ g mL⁻¹ 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 2a-2v 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 μ L (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 MnCl₂, 100 µM Na₃VO₄, and 1 mM DTT) and 20 µL of 0.1 mM ATP-50 mM MgCl₂ was added for 1 h. Positive and negative controls were included in each plate by incubation of enzyme with or without ATP-MgCl₂. 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 antiphosphotyrosine 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 (WallacInc.) with excitation at 340 nm and emission at 615 nm. The percentage of autophosphorylation inhibition by the compounds was calculated by the following equation: 100 %- [(negative control)/(positive control - negative control)]. The IC₅₀ values were obtained from the curves of percentage inhibition with eight concentrations of the compound. As the contaminants in the enzyme preparation are fairly low, the majority of the signal detected by the antiphosphotyrosine antibody is from EGFR.

Molecular docking modeling

Molecular docking of compound **2f** into the 3D EGFR complex structure was accomplished with the help of the AutoDock software package (version 4.0) which was implemented through the graphical user interface AutoDockTools (ADT 1.4.6). The 3D structures of the aforementioned compounds were constructed by means of ChemBio3D Ultra 12.0 software (Chemical Structure

Drawing Standard; Cambridge Soft corporation, USA 2009) then they were energetically minimized by MOPAC with 100 iterations and minimum RMS gradient of 0.10. The Gasteiger–Hückel charges of ligands were assigned. The crystal structures of EGFR (PDB code:1M17) complex were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All bound waters and ligands were eliminated from the protein and the polar hydrogens and the Kollman-united charges were added to the proteins.

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