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Discovery of benzimidazole derivatives as novel multi-target EGFR, VEGFR-2 and PDGFR kinase inhibitors

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ABSTRACT

Multi-target EGFR, VEGFR-2 and PDGFR inhibitors are highly useful anticancer agents with improved therapeutic efficacies. In this work, we used two virtual screening methods, support vector machines (SVM) and molecular docking, to identify a novel series of benzimidazole derivatives, 2-aryl benzimidazole compounds, as multi-target EGFR, VEGFR-2 and PDGFR inhibitors. 2-Aryl benzimidazole compounds were synthesized and their biological activities against a tumor cell line HepG-2 and specific kinases were evaluated. Among these compounds, compounds **5a** and **5e** exhibited high cytotoxicity against HepG-2 cells with IC_{50} values at ~2 μM. Further kinase assay study showed that compound **5a** have good EGFR inhibitory activity and moderate VEGFR-2 and PDGFR inhibitory activities, while **5e** have moderate EGFR inhibitory activity and slightly weaker VEGFR-2 and PDGFR inhibitory activities. Molecular docking analysis suggested that compound **5a** more tightly interacts with EGFR and PDGFR than compound **5e**. Our study discovered a novel series of benzimidazole derivatives as multi-target EGFR, VEGFR-2 and PDGFR kinases inhibitors.

1. Introduction

Receptor tyrosine kinases (RTKs), epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor-2 (VEGFR-2) and platelet-derived growth factor receptor (PDGFR) play very important roles in regulating tumor cell proliferation, differentiation, survival, angiogenesis and apoptosis.[1–4] These RTKs have been successfully explored as attractive targets for antitumor therapies, particularly for developing multi-target anticancer drugs with improved therapeutic efficacies.[5,6]

In addition to their individual roles in cancers, these RTKs also work synergistically in regulating tumor response to anticancer RTK inhibitor drug therapies. For instance, in certain circumstances, EGFR inhibition can lead to VEGFR-2 up-regulation which subsequently promotes tumor growth signaling independent of EGFR and thus contributes to the resistance of EGFR inhibitors.[7] The effect of EGFR inhibition can also be partially overcome by activation of PDGFR signaling and the subsequent transactivation of HER-3 signaling to promote alternative tumor growth signaling.[8] Therefore, in certain circumstances, multi-target RTK drugs that simultaneously inhibit EGFR, VEGFR-2 and PDGFR offer significantly improved anticancer therapeutic efficacy than drugs that inhibit individual RTKs.

In this work, we used two virtual screening methods, support vector machines (SVM)[9] and molecular docking,[10,11] to identify 2-aryl benzimidazole compounds, a novel series of benzimidazole derivatives, as multi-target EGFR, VEGFR-2 and PDGFR inhibitors. Benzimidazole derivatives have been reported to have various bioactivities, including antiviral,[12–15] antihypertensive,[16] antimicrobial,[17,18] antioxidant,[19] antiinflammatory[20] and anticancer[21–29] activities. There are several marketed benzimidazole based drugs, such as Astemizole (Janssen Pharmaceutica), Micardis (Boehringer Ingelheim), Omepraxole (AstraZeneca) and Albendazole (GlaxoSmithKline).[12] In particular, benzimidazole derivatives have been explored as anticancer inhibitors of Topoisomerase I,[21] PARP-1,[22,23] kinase Chk2,[24,25] Pgp and DNA synthesis,[26] and tyrosine kinases.[27–29] Nonetheless, to the best of our knowledge, although several benzimidazole series have been developed as tyrosine kinase inhibitors, the 2-aryl benzimidazole series have not been explored as multi-target EGFR, VEGFR-2 and PDGFR inhibitors in published reports.

Therefore, we designed and synthesized 2-aryl benzimidazole compounds. Their biological activities against tumor cell-line HepG-2 were evaluated. These compounds were able to induce tumor cell apoptosis. Kinase inhibition assay studies showed that

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compound **5a** had good EGFR inhibitory activity and fairly good VEGFR-2 and PDGFR inhibitory activities, whereas compound **5e** had good EGFR inhibitory activity and moderate VEGFR-2 and PDGFR inhibitory activities.

2. Results and discussion

2.1. High-throughput virtual screening

Virtual screening PubChem database was conducted using the similar procedure as recently published.[30] First, SVM models of EGFR inhibitors, VEGFR-2 inhibitors and PDGFR inhibitors[9,31] were used to screen the compounds. Then the initially selected SVM virtual hits were evaluated by Lipinsky's rule of five and those compounds which passed Lipinsky's rule of five were subject to further and more refined screening by using molecular docking.

In this study, we explored two virtual screening methods, support vector machines (SVM) and molecular docking to screen PubChem database for identification of novel multi-target EGFR, VEGFR-2 and PDGFR inhibitors. Three compounds containing a same 2-aryl benzimidazole scaffold (PubChem ID 47037197, 47037198 and 4175169), were identified by our virtual screening (Fig. 1). Specifically, compound 47037197 was identified as a virtual multi-target EGFR and VEGFR inhibitor, and compounds 47037198 and 4175169 as individual-target EGFR inhibitor and PDGFR inhibitor, respectively.

We synthesized the two compounds (PubChem ID 47037197, 47037198), which are similar in structure and easy to make quickly. The kinase assay result suggested that 2-aryl benzimidazole was a potential scaffold and can be modified in order to get novel compounds with better solubility and activity.

2.2. Chemistry

As shown in Scheme 1, a 4-step route was carried out to synthesize 2-phenyl-benzimidazole derivatives. Compound **3** was obtained from cyclization of commercially available compounds 4-nitro-*o*-phenylenediamine **1** and substituted benzaldehydes **2** in DMF using sodium metabisulfite as the catalyst under nitrogen atmosphere.[32] The nitro-group on compound **3** was then reduced to the amino group by $SnCl_2$ in ethanol solution to give compound **4**, followed by acetylation using chloracetyl chloride/3-chloropropionyl chloride to form compound **5**. Compound **6** with different heterocyclic functional groups were finally obtained by the reaction of **5** with corresponding secondary amines.

As shown in Scheme 2, 2-(1-methyl-1*H*-pyrrol-2-yl)-1*H*-benzimidazole derivatives were obtained using the similar procedure, but using *N*-methylpyrrole-2-carboxaldehyde instead, in order to explore the effect of other aromatic functional groups.

2.3. In vitro cell cytotoxicity assay

In vitro cell cytotoxicity of the 16 novel benzimidazole derivatives was initially evaluated against HepG-2 cells by MTT assay using colchicin as a positive control. As shown in Table 1, some compounds exhibited good antiproliferative activity with low μM IC_{50} values, among which compounds **5a** and **5e** showed the best activity with IC_{50} values at 2.0 and 1.8 μM, respectively. Comparing the data, we obtained some basic structure–activity relationships (SAR) as the following: (1) electron-withdrawing groups attached to the phenyl ring contributed more to the cytotoxicity, as suggested by the results of compounds **5b** and **5c**; (2) the length of the chain between the carbonyl and the end of the group obviously influenced the cytotoxicity. For example, compound **5b** was found to be inactive at concentration up to 50 μM, whereas compound **5a** containing a shorter ($n = 1$) chain showed much stronger inhibition effect with IC_{50} at 2.0 μM; (3) compounds containing some functional groups such as piperidine, pyrrole, etc., might cause a decrease in cytotoxicity in general except that for **6b1** and **6b3**.

2.4. In vitro kinase inhibition assay

The in vitro kinase assay was carried out to test the kinase inhibition activity of compounds **5a**, **5b**, and **5e** at the concentration of 50 μM, among which compounds **5a** and **5e** had good cytotoxicity, while **5b** was used as a negative control since it showed no obvious cytotoxicity. As shown in Table 2, at the concentration of 50 μM, compounds **5a** and **5e** inhibited 88.03% and 86.90% of EGFR activity, respectively, whereas compound **5b** had no obvious inhibition effect. This result was consistent with cell cytotoxicity activity. Furthermore, the inhibition rates of compound **5a** and **5e** against PDGFR-α, PDGFR-β and VEGFR-2 were found to be in the range of 33–42% and 16–18%, respectively. No obvious inhibition effect was observed for other kinases, including VEGFR-1, Abl-1 and PI3K-α. The IC_{50} values of compounds **5a** and **5e** against EGFR were further determined using Gefitinib as the positive control. Specifically, the IC_{50} values of compound **5a** and **5e** were 1.929 and 12.07 μM, respectively, with respect to the IC_{50} value of 4.2 nM for Gefitinib. Our results suggested that compound **5a** exhibited good EGFR inhibitory activity and moderate VEGFR-2 and PDGFR inhibitory activities, while compound **5e** showed moderate EGFR inhibitory activity and slightly weaker VEGFR-2 and PDGFR inhibitory activities.

2.5. Apoptosis test

It was reported that inhibition of EGFR usually led to cell cycle arrest at G0/G1 and apoptosis in tumor cell-lines.[33] Compounds **5a** and **5e** were chosen for further study on apoptosis using Annexin-V/PI binding assay based on their good cytotoxicity and kinase inhibitory effect. Annexin-V and PI (propidium iodide) stain phosphatidylserine residues and DNA, respectively.[34] As shown in Figure 2, the upper right-hand quadrant represents the late stage apoptotic cells (Annexin-V positive and PI positive) and the lower right-hand quadrant represents the early stage apoptotic (Annexin-V positive and PI negative). 28.6% of HepG-2 cells treated with compound **5a** were in early apoptosis stage and 16.6% in late apoptosis stage or dead; whereas compound **5e** showed better activity with 22.8% in early apoptosis stage and 18.7% in late apoptosis stage or dead. These results clearly demonstrated that compounds **5a** and **5e** induced HepG-2 cells apoptosis at low μM concentration.

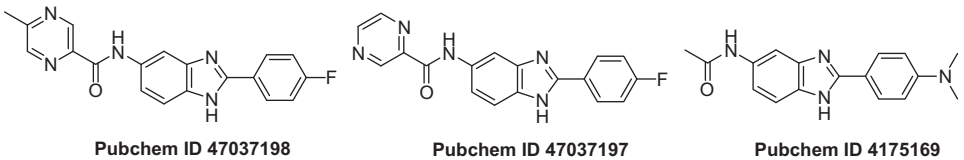
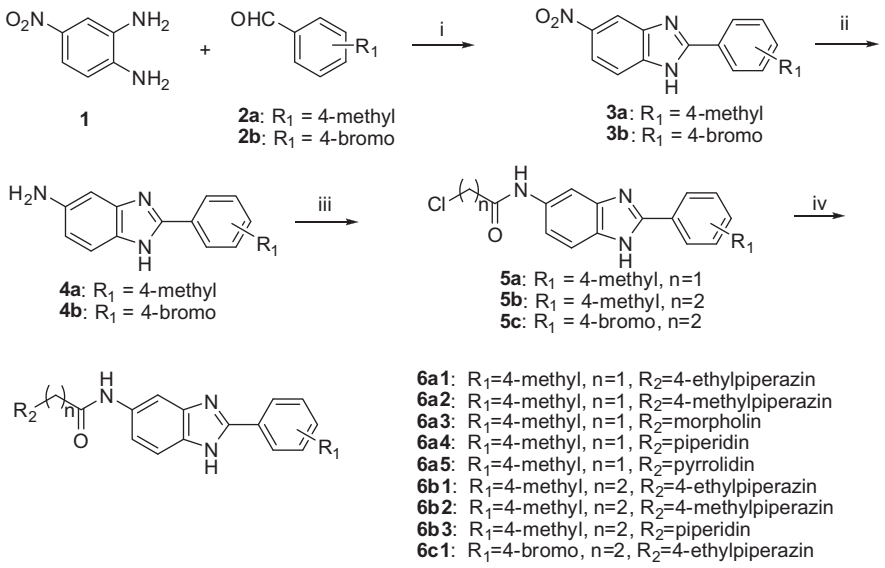
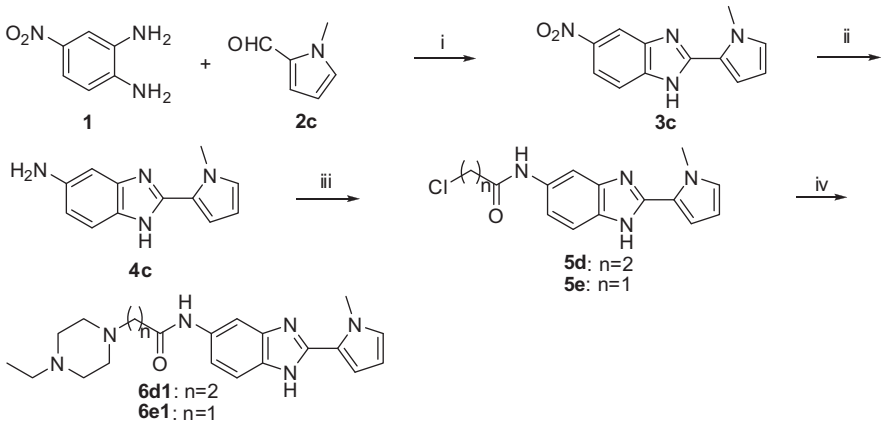


Figure 1. Structures of the three hit compounds which passed SVM screening.



Scheme 1. Reagents and conditions: (i) $Na_2S_2O_5$, DMF, 120 °C,15 h; (ii) $SnCl_2$, EtOH, reflux, 4 h; (iii) chloracetyl chloride/3-chloropropionyl chloride, reflux, 3 h; (iv) corresponding secondary amines, KI, THF, reflux, 3 h.



Scheme 2. Reagents and conditions: (i) $Na_2S_2O_5$, DMF, 120 °C, 15 h; (ii) $SnCl_2$, EtOH, reflux, 4 h; (iii) chloracetyl chloride/3-chloropropionyl chloride, reflux, 3 h; (iv) 1-ethylpiperazine, KI, THF, reflux, 3 h.

Table 1
Inhibitory activity of compounds against HepG-2 cell

Compound	IC_{50} (μM)	Compound	IC_{50}[a] (μM)
5a	2.0	**6b3**	11.9
6a1	31.9	**5c**	11.3
6a2	>50.0	**6c1**	27.4
6a3	>50.0	**5d**	>50.0
6a4	>50.0	**6d1**	>50.0
6a5	>50.0	**5e**	1.8
5b	>50.0	**6e1**	>50.0
6b1	14.6	Colchicin	0.05
6b2	>50.0		

[a] IC_{50} values were determined from MTT assays after incubation with test compounds for 72 h. All values are means of three experiments.

Table 2
Inhibitory activity of compounds with different kinases (inhibitory rate, %) at 50 μM

Kinase	**5a**	**5b**	**5e**	Staurosporine (%)
EGFR	88.0%	na[a]	86.9%	99.1
PDGFR-α	42.7%	17.0%	18.7%	92.6
PDGFR-β	52.6%	24.6%	17.8%	95.8
VEGFR-1(FLT1)	7.3%	3.3%	na[a]	93.4
VEGFR-2(KDR)	33.1%	na[a]	16.5%	92.9
ABL-1	1.0%	na[a]	na[a]	94.6
PI3K-α	na[a]	na[a]	3.7%	97.0

[a] No activity.

2.6. Molecular docking

In order to better understand the interaction between compounds and kinases, molecular docking studies on compounds **5a** and **5e** were performed using the Discovery Studio 2.5/CDOCKER protocol. Figure 3A demonstrates the compound **5a** docking into ATP binding site of EGFR kinase (PDB: 2J6M).[35] In this binding model, **5a** was nicely bound to the EGFR binding domain and forms a hydrogen bond with the crucial amino acid MET 793 (N–H···O:2.485 Å), which was proved to be an important binding site of EGFR inhibitor. As shown in Figure 3B, compound **5e** has a relatively weaker interaction with EGFR, which was consistent with the result that compound **5a** showed nearly 10-fold more potent than compound **5e** as an EGFR kinase inhibitor.

Comparing the molecular docking of compounds **5a** and **5e** with VEGFR-2 kinase (PDB: 1YWN)[36] shown in Figure 3C and D,

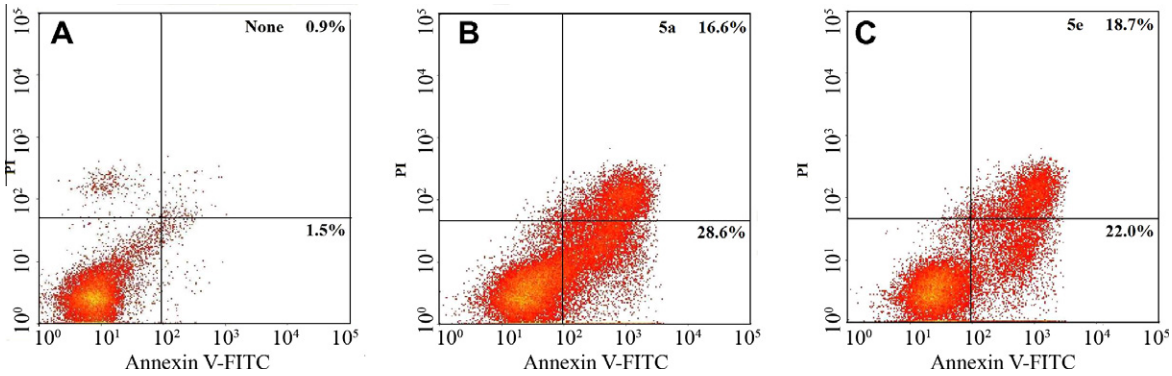


Figure 2. Flow cytometric analysis of phosphoatidylserine externalization (Annexin-V binding) and cell membrane integrity (PI staining). HepG-2 cells were treated with none, compound **5a** at 2 μM and compound **5e** at 2 μM, respectively, for 72 h.

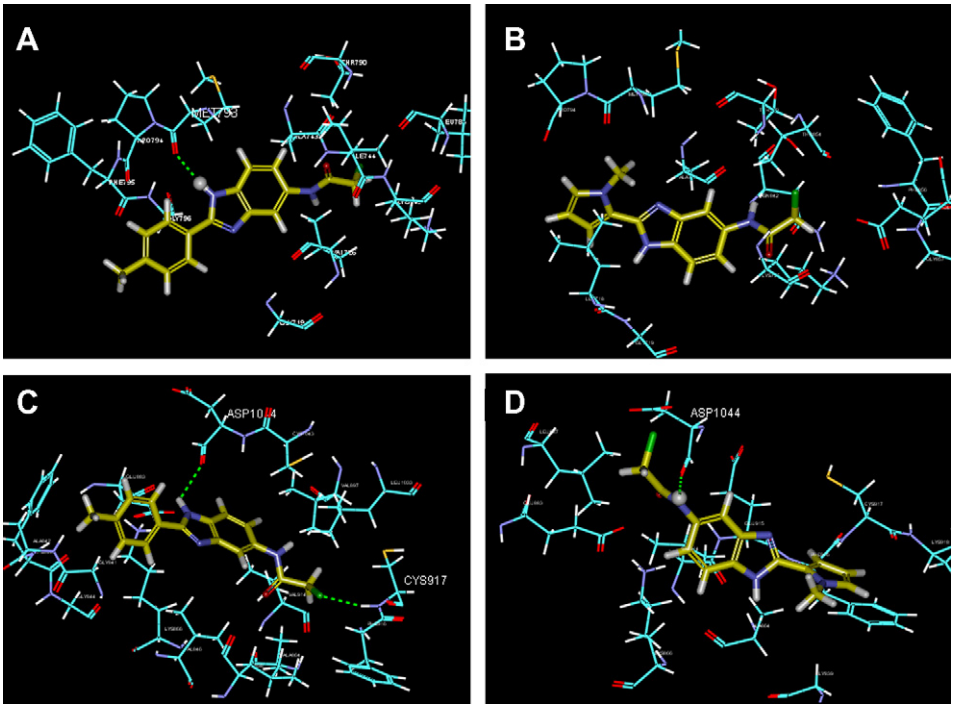


Figure 3. Molecular docking of benzimidazole derivatives with target proteins. (A) Compound **5a** with EGFR; (B) compound **5e** with EGFR; (C) compound **5a** with VEGFR-2; (D) compound **5e** with VEGFR-2.

compound **5a** formed two hydrogen bonds with CYS 917 (N–H···Cl:2.484 Å) and ASP 1044 (N–H···O:2.429 Å), whereas compound **5e** formed only one hydrogen bond with ASP 1044 (N–H···O:2.419 Å). This result was consistent with kinase assay data and thus further confirmed that compound **5a** is a better VEGFR-2 kinase inhibitor.

3. Conclusion

In conclusion 2-aryl benzimidazole compounds have been synthesized and discovered as multi-target RTK inhibitors that demonstrated good cytotoxicity activities against HepG-2 cells and exhibited inhibition against EGFR, PDGFR-α, PDGFR-β and VEGFR-2. The SAR indicated that electron-withdrawing substituent of 2-aryl ring and the shorter chain contributed much to the cytotoxicity activity. The results of kinase assay and molecular docking studying exhibited that 2-aryl benzimidazole compounds were novel EGFR, PDGFR and VEGFR-2 kinases inhibitors. Apoptosis test of these compounds were also consistent with their inhibitory actions against these kinases. Our study suggested that 2-aryl benzimidazole compounds were a series of novel benzimidazole derivatives for developing novel multi-target EGFR, PDGFR and VEGFR-2 kinase inhibitors. It is of interest to continue studying kinase activities of 2-aryl benzimidazole series.

4. Experimental

4.1. Chemistry

Unless otherwise noted, all solvents and reagents were commercially available and used without further purification. Nuclear magnetic resonance spectra were obtained using a Bruker 400 (400 MHz) spectrometer at room temperature. The mass spectra were obtained on a Waters Micromass Q-TOF Premier Mass Spectrometer. Melting points were determined with a SGW X-4 digital apparatus and were uncorrected.

4.1.1. Synthesis of 2-*p*-tolyl-1*H*-benzo[*d*]imidazol-5-amine (4a)

The reaction was performed under nitrogen atmosphere. 4-nitro-*o*-phenylenediamine **1** (4 g, 26.14 mmol) and *p*-tolualdehyde **2a** (3.77 g, 31.37 mmol) in 50 ml of DMF was added into a dried round-bottom flask. The mixture was stirred at room temperature for 5 min before sodium metabisulfite (12.4 g, 65.36 mmol) was added. The reaction mixture was then heated to 120 °C and kept at this temperature for 15 h. Once the starting materials were consumed (followed by TLC), the reaction was cooled down to room temperature, poured into water (200 mL), extracted with ethyl acetate (3 × 80 mL), and dried over anhydrous Na_2SO_4. The organic extracts were concentrated in vacuo and the crude product compound **3a** was obtained. Compound **3a**, Tin(II) chloride anhydrous (13.47 g, 71 mmol) and ethanol (100 mL) were added into a dried round-bottom flask and the mixture was refluxed for 4 h and then cooled to room temperature. Saturated NaOH solution was added to the reaction mixture until white precipitation produced. The slurry was filtered and the filtrate was extracted with ethyl acetate (3 × 80 mL). After the organic phase was dried over anhydrous Na_2SO_4, the crude product was purified by column chromatography (ethyl acetate/ammonium hydroxide, 100:1) to yield a brown solid. Yield: 55%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (d, J = 7.2 Hz, 2H), 7.47 (s, 1H), 7.28 (s, 2H), 6.86 (s, 1H), 6.69 (d, J = 7.6, 1H), 5.32 (s, 2H), 2.41 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 142.59, 139.65, 129.45, 127.12, 125.86, 112.55, 21.12; HR-MS(ESI): calcd for $C_{14}H_{14}N_3$ $[M+H]^+$ 224.1188; found: 224.1186.

4.1.2. Synthesis of 2-chloro-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]imidazol-5-yl)acetamide (5a)

Compound **4a** (200 mg) was refluxed in chloracetyl chloride (5 mL) for 4 h and the reaction was ended by adding 20 mL of water. Saturated NaOH solution was added until the pH was 8–9. The mixture was extracted with ethyl acetate (3 × 30 mL) and dried over anhydrous Na_2SO_4. The crude product was purified by column chromatography (ethyl acetate/ammonium hydroxide, 100:1) to yield a brown solid. Yield: 84%; mp 180–184 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.79 (s, 1H), 8.30 (s, 1H), 8.15 (d, J = 8.4 Hz, 2H), 7.74 (d, J = 8.8 Hz, 1H), 7.55 (dd, J = 8.8, 1.6 Hz, 1H), 7.51 (d, J = 8.4 Hz, 2H), 4.34 (s, 2H), 2.44 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.29, 149.07, 144.03, 136.92, 132.40, 130.41, 128.50, 128.06, 120.71, 118.55, 114.50, 103.67, 43.79, 21.45; HR-MS(ESI): calcd for $C_{16}H_{15}ClN_3O$ $[M+H]^+$ 300.0904; found: 300.0903.

4.1.3. General procedure for preparation of compounds 6a1–6a5

Compound **5a** (100 mg, 0.334 mmol) was dissolved in anhydrous THF (8 mL). Then, corresponding secondary amines (5.015 mmol) and KI (50 mg, 0.334 mmol) were added and the mixture was refluxed under nitrogen atmosphere for 3 h. Once the starting materials were consumed (followed by TLC), the solvent was removed by in vacuo and the crude solid was washed with water. The crude product was purified by column chromatography (ethyl acetate/methanol/ammonium hydroxide, 100:10:1).

4.1.3.1. 2-(4-Ethylpiperazin-1-yl)-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]imidazol-5-yl)acetamide (6a1). Yield: 56%; mp 233–237 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.35 (s, 1H), 8.36 (s, 1H), 7.99 (d, J = 8.0 Hz, 2H), 7.62 (d, J = 8.4 Hz, 1H), 7.24 (d, J = 8.0 Hz, 2H), 6.98 (d, J = 7.6 Hz, 1H), 3.19 (s, 2H), 2.82–2.45 (m, 10H), 2.39 (s, 3H), 1.13 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.35, 152.49, 139.98, 132.34, 129.33, 127.08, 126.42, 115.01, 61.68, 53.04, 52.52, 51.97, 21.18, 11.51; HR-MS(ESI): calcd for $C_{22}H_{28}N_5O$ $[M+H]^+$ 378.2294; found: 378.2292.

4.1.3.2. 2-(4-Methylpiperazin-1-yl)-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]-imidazol-5-yl)acetamide (6a2). Yield: 33%; mp 234–237 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.34 (s, 1H), 8.35 (s, 1H), 7.99 (d, J = 7.6 Hz, 2H), 7.61 (d, J = 7.6 Hz, 1H), 7.22 (d, J = 7.6 Hz, 2H), 6.99 (s, 1H), 3.18 (s, 2H), 2.80–2.60 (m, 4H), 2.60–2.40 (m, 4H), 2.40 (s, 3H), 2.36 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.40, 152.60, 139.94, 132.25, 129.31, 127.13, 126.46, 115.01, 61.66, 54.97, 53.14, 45.65, 29.44, 21.17; HR-MS(ESI): calcd for $C_{21}H_{26}N_5O$ $[M+H]^+$ 364.2137; found: 364.2129.

4.1.3.3. 2-Morpholino-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]imidazol-5-yl)-acetamide (6a3). Yield: 61%; mp 273–277 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.25 (s, 1H), 8.30 (s, 1H), 7.97 (d, J = 8.0 Hz, 2H), 7.61 (d, J = 8.4 Hz, 1H), 7.26 (d, J = 6.4 Hz, 2H), 7.05 (d, J = 7.6 Hz, 1H), 3.80 (t, J = 4.6 Hz, 4H), 3.19 (s, 2H), 2.65 (t, J = 4.6 Hz, 4H), 2.40 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.26, 132.53, 129.45, 126.31, 66.83, 62.28, 53.63, 21.19; HR-MS(ESI): calcd for $C_{20}H_{23}N_4O_2$ $[M+H]^+$ 351.1821; found: 351.1819.

4.1.3.4. 2-(Piperidin-1-yl)-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]imidazol-5-yl)acetamide (6a4). Yield: 27%; mp 245–249 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.57 (s, 1H), 8.33 (s, 1H), 7.99 (d, J = 8.0 Hz, 2H), 7.62 (d, J = 8.4 Hz, 1H), 7.26 (d, J = 4.8 Hz, 2H), 7.03 (d, J = 8.4 Hz, 1H), 3.17 (s, 2H), 2.61 (m, 4H), 2.40 (s, 3H), 1.68–1.66 (m, 4H), 1.51 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.62, 152.28, 139.99, 132.58, 129.34, 126.93, 126.42, 115.14, 62.39, 54.70, 29.45, 25.90, 23.29, 21.18; HR-MS(ESI): calcd for $C_{21}H_{25}N_4O$ $[M+H]^+$ 349.2028; found: 349.2023.

4.1.3.5. 2-(Pyrrolidin-1-yl)-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]imidazol-5-yl)acetamide (6a5). Yield: 22%; mp 229–233 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.36 (s, 1H), 8.34 (s, 1H), 7.98 (d, J = 8.4 Hz, 2H), 7.62 (d, J = 8.0 Hz, 1H), 7.27 (d, J = 5.6 Hz, 2H), 7.04 (s, 1H), 3.37 (s, 2H), 2.75 (m, 4H), 2.40 (s, 3H), 1.88 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.87, 152.20, 139.98, 132.67, 129.38, 127.00, 126.32, 115.20, 59.49, 58.20, 54.43, 23.87, 21.17, 18.19; HR-MS(ESI): calcd for $C_{20}H_{23}N_4O$ $[M+H]^+$ 335.1872; found: 335.1867.

4.1.4. Synthesis of 3-chloro-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]imidazol-5-yl)propanamide (5b)

Compound **5b** was synthesized in the same way as compound **5a**. Yield: 79%; mp 248–252 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.38 (d, J = 1.2 Hz, 1H), 8.17 (d, J = 8.4 Hz, 2H), 7.77 (d, J = 8.8 Hz, 1H), 7.59 (dd, J = 8.8, 1.6 Hz, 1H), 7.54 (d, J = 8.0 Hz, 2H), 3.93 (t, J = 6.2 Hz, 2H), 2.91 (t, J = 6.2 Hz, 2H), 2.45 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.63, 148.80, 143.97, 137.43, 132.38, 130.39, 128.05, 120.69, 118.41, 114.35, 103.31, 40.95, 21.44; HR-MS(ESI): calcd for $C_{17}H_{17}ClN_3O$ $[M+H]^+$ 314.1060; found: 314.1051.

4.1.5. General procedure for preparation of compounds 6b1, 6b2, 6b3

Compounds were synthesized in the same way as compounds **6a1–6a5**.

4.1.5.1. 3-(4-Ethylpiperazin-1-yl)-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]imidazol-5-yl)propanamide (6b1). Yield: 39%; mp 193–195 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.20 (s, 1H), 8.34 (s, 1H), 7.99 (d, J = 7.6 Hz, 2H), 7.60 (d, J = 8.0 Hz, 1H), 7.22 (d, J = 8.0 Hz, 2H), 6.98 (s, 1H), 2.81–2.43 (m, 14H), 2.38 (s, 3H), 1.12 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.83, 152.56, 140.04, 133.76, 129.54, 127.41, 126.66, 53.63, 52.85, 52.21, 52.10, 32.43, 21.44, 11.89; HR-MS(ESI): calcd for $C_{23}H_{30}N_5O$ $[M+H]^+$ 392.2450; found: 392.2444.

4.1.5.2. 3-(4-Methylpiperazin-1-yl)-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]-imidazol-5-yl)propanamide (6b2). Yield: 34%; mp 209–213 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.22 (s, 1H), 8.38 (s, 1H), 7.99 (d, J = 8.0 Hz, 2H), 7.61 (s, 1H), 7.22 (d, J = 7.6 Hz, 2H), 6.94 (s, 1H), 2.74–2.52 (m, 12H), 2.38 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.62, 152.37, 139.77, 133.50, 129.28, 127.24, 126.44, 54.98, 53.42, 51.99, 45.67, 32.19, 21.20; HR-MS(ESI): calcd for $C_{22}H_{28}N_5O$ $[M+H]^+$ 378.2294; found: 378.2285.

4.1.5.3. 3-(Piperidin-1-yl)-*N*-(2-*p*-tolyl-1*H*-benzo[*d*]imidazol-5-yl)propanamide (6b3). Yield: 42%; mp 188–191 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.72 (s, 1H), 8.53 (s, 1H), 8.02 (d, J = 8.0 Hz, 2H), 7.67 (s, 1H), 7.24 (d, J = 8.0 Hz, 2H), 6.86 (s, 1H), 2.70–2.54 (m, 8H), 2.39 (s, 3H), 1.72–1.70 (m, 4H), 1.55 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.99, 152.35, 139.83, 133.89, 129.42, 127.45, 126.54, 58.30, 54.25, 53.55, 32.40, 26.13, 24.12, 21.31, 18.33; HR-MS(ESI): calcd for $C_{22}H_{27}N_4O$ $[M+H]^+$ 363.2185; found: 363.2186.

4.1.6. Synthesis of 2-(4-bromophenyl)-1*H*-benzo[*d*]imidazol-5-amine (4b)

Compound **4b** was synthesized in the same way as compound **4a**. Yield: 33%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 7.44 (t, J = 8.2 Hz, 2H), 6.82 (d, J = 9.6 Hz, 1H), 6.68 (d, J = 8.4 Hz, 1H).

4.1.7. Synthesis of *N*-(2-(4-bromophenyl)-1*H*-benzo[*d*]imidazol-5-yl)-3-chloropropanamide (5c)

Compound **5c** was synthesized in the same way as compound **5b**. Yield: 51%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.55 (s, 1H), 8.35 (d, J = 1.2 Hz, 1H), 8.21 (d, J = 8.8 Hz, 2H), 7.94 (d, J = 8.8 Hz, 2H), 7.75 (d, J = 9.2 Hz, 1H), 7.58 (dd, J = 8.8, 1.6 Hz, 1H), 3.93 (t, J = 6.2 Hz, 2H), 2.90 (t, J = 6.2 Hz, 2H); HR-MS(ESI): calcd for $C_{16}H_{14}{}^{79}BrClN_3O$ $[M+H]^+$ 378.0009, for $C_{16}H_{14}{}^{81}BrClN_3O$ $[M+H]^+$ 379.9988; found: 378.0007, 379.9976.

4.1.8. *N*-(2-(4-bromophenyl)-1*H*-benzo[*d*]imidazol-5-yl)-3-(4-ethylpiperazin-1-yl) propanamide (6c1)

Compound **6c1** was synthesized in the same way as compounds **6a1**–**6a5**. Yield: 14%; mp 205–209 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.46 (s, 1H), 8.55 (s, 1H), 7.96 (d, J = 8.4 Hz, 2H), 7.68 (d, J = 4.4 Hz, 1H), 7.54 (d, J = 8.8 Hz, 2H), 6.82 (s, 1H), 2.75–2.46 (m, 14H), 1.13 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.75, 151.11, 133.79, 131.73, 129.07, 127.99, 123.83, 58.15, 53.40, 52.78, 52.10, 32.20, 11.81; HR-MS(ESI): calcd for $C_{22}H_{27}{}^{79}BrN_5O$ $[M+H]^+$ 456.1399, $C_{22}H_{27}{}^{81}BrN_5O$ $[M+H]^+$ 458.1379; found: 456.1396, 458.1377 .

4.1.9. Synthesis of 2-(1-methyl-1*H*-pyrrol-2-yl)-1*H*-benzo[*d*]imidazol-5-amine (4c)

Compound **4c** was synthesized in the same way as compound **4a**. Yield: 60%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (d, J = 8.4 Hz, 1H), 6.80 (s, 1H), 6.78 (t, J = 8.0 Hz, 1H), 6.64 (dd, J = 8.6, 2.4 Hz, 1H), 6.58 (dd, J = 4.0, 1.6 Hz, 1H), 6.19 (dd, J = 3.8, 2.6 Hz, 1H), 4.11 (s, 3H).

4.1.10. Synthesis of 3-chloro-*N*-(2-(1-methyl-1*H*-pyrrol-2-yl)-1*H*-benzo[*d*]-imidazol-5-yl)propanamide (5d)

Compound **5d** was synthesized in the same way as compound **5b**. Yield: 59%; mp 102–106 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.10 (s, 1H), 8.04 (d, J = 1.2 Hz, 1H), 7.47 (s, J = 8.4 Hz, 1H), 7.24 (dd, J = 8.6 Hz, J = 1.2 Hz, 1H), 6.99 (s, 1H), 6.84 (dd, J = 3.6 Hz, J = 1.6 Hz, 1H), 6.16 (t, J = 3.2 Hz, 1H), 3.92 (t, J = 6.2 Hz, 2H), 2.85 (t, J = 6.2 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.78, 146.72, 134.10, 127.36, 122.95, 114.66, 111.58, 108.11, 41.20, 36.68; HR-MS(ESI): calcd for $C_{15}H_{16}ClN_4O$ $[M+H]^+$ 303.1013; found: 303.1011.

4.1.11. Synthesis of 3-(4-ethylpiperazin-1-yl)-*N*-(2-(1-methyl-1*H*-pyrrol-2-yl)-1*H*-benzo [*d*]imidazol-5-yl)propanamide (6d1)

Compound **6d1** was synthesized in the same way as compounds **6a1–6a5**. Yield: 32%; mp 165–168 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (s, 1H), 7.60 (s, 1H), 6.78–6.77 (m, 2H), 6.72–6.71 (m, 1H), 6.19–6.17 (m, 1H), 4.12 (s, 3H), 3.06–2.20 (m, 14H), 1.13 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.83, 146.92, 133.64, 126.57, 123.58, 111.00, 108.04, 53.72, 53.01, 52.28, 36.65, 32.47, 12.04; HR-MS(ESI): calcd for $C_{21}H_{29}N_6O$ $[M+H]^+$ 381.2403; found: 381.2398.

4.1.12. Synthesis of 2-chloro-*N*-(2-(1-methyl-1*H*-pyrrol-2-yl)-1*H*-benzo[*d*] imidazol-5-yl)acetamide (5e)

Compound **5e** was synthesized in the same way as compound **5a**. Yield: 64%; mp 102–106 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.40 (s, 1H), 8.04 (d, J = 1.2 Hz, 1H), 7.50 (d, J = 8.4 Hz, 1H), 7.26 (dd, J = 8.6, 2.2 Hz, 1H), 7.03–7.02 (m, 1H), 6.88 (dd, J = 3.8, 1.8 Hz, 1H), 6.17 (dd, J = 3.8, 2.6 Hz, 1H), 4.29 (s, 2H), 4.08 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.55, 146.61, 133.74, 127.75, 122.46, 115.04, 112.14, 108.27, 43.92, 36.66; HR-MS(ESI): calcd for $C_{14}H_{14}ClN_4O$ $[M+H]^+$ 289.0856; found: 289.0855.

4.1.13. Synthesis of 2-(4-ethylpiperazin-1-yl)-*N*-(2-(1-methyl-1*H*-pyrrol-2-yl)-1*H*-benzo[*d*]imidazol-5-yl)acetamide (6e1)

Compound **6e1** was synthesized in the same way as compounds **6a1–6a5**. Yield: 61%; mp 104–107 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.33 (s, 1H), 8.38 (s, 1H), 7.61 (s, 1H), 6.86 (s, 1H), 6.77 (s, 1H), 6.72 (d, J = 3.6, 1H), 6.17 (s, 1H), 4.12 (s, 3H), 3.19 (s, 2H), 2.69–2.45 (m, 10H), 1.11 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.69, 147.19, 132.33, 126.72, 123.43, 111.20, 108.08, 61.89, 53.45, 52.97, 52.25, 36.67, 11.96; HR-MS(ESI): calcd for $C_{20}H_{27}N_6O$ $[M+H]^+$ 367.2246; found: 367.2236.

4.2. Molecular docking

The molecular docking of compounds **5a** and **5e** with kinases was carried out using Discovery Studio.2.5/CDOCKER protocol *(Accelrys Software Inc.)*. The protein crystallographic structure, EGFR (PDB: 2J6M) and VEGFR-2 (PDB: 1YWN), were downloaded from the Protein Data Bank (PDB). Hydrogen atoms were added and water molecules co-crystallized with the protein were removed from the original structure. The general procedure is as followed: (1) ligand and receptor preparation, (2) protocol generation, (3) docking and scoring. (4) analysis of the results.

4.3. Bioassay

4.3.1. Cell culture

HepG-2 (Hepatocellular carcinoma, human) was obtained from the Chinese Academy of Sciences Cell Bank. The cell was cultured in DMEM, including 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc.), 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37 °C with 5% CO_2.

4.3.2. Cytotoxicity assay

The cytotoxicity was measured by MTT assay. The cells (HepG-2) with a concentration of 3000 cells/well were seeded in 96-well plates containing 100 μL DMEM medium. After 12 h, the supernatant was replaced by fresh medium including various concentrations of compounds. The cells were incubated for 72 h, and then 10 μL of MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) solution (5 mg/mL) was added. After 4 h, the supernatant was discarded and formazan precipitates were dissolved in

100 μL DMSO. At 490 nm, the absorbance was measured by a Benchmark microplate reader (Molecular Devices Corporation).

4.3.3. Flow cytometry assay

Phosphatidylserine externalization was measured by Annexin V-FITC/PI apoptosis detection kit (Beyotime Company) according to the manufacturer's instructions.[37]

4.3.4. Kinase assay

In vitro kinase assay was tested by HD Biosciences Co., Ltd in Shanghai, China. The activities of compounds against EGFR, PDGFR, VEGFR, Abl-1, PI3K-α were tested using glo plus assay.

The general procedure for glo plus assay was as the following: Mix kinases, ATP, substrates and compounds in the reaction buffer of 25 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 0.01% Triton X-100, 100 μg/mL BSA, 2.5 mM DTT in 384-well plate. Total reaction volume was 10 μL. The assay plate was incubated at 30 °C for 1 h and reaction was stopped by the addition of equal volume of kinase glo plus reagent. The luminescence was read at envision. The signal was correlated with the amount of ATP remaining in the reaction and was inversely correlated with the kinase activity.

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Supplementary data

Supplementary data (general synthesis methods and kinase assay results of the two compounds (PubChem ID 47037197, 47037198), ^{1}H NMR and ^{13}C NMR spectrum) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.022.

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