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Graphical Abstract

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Synthesis and Biological Evaluation of Azole-diphenylpyrimidine Derivatives (AzDPPYs) as Potent T790M Mutant Form of Epidermal Growth Factor Receptor Inhibitors

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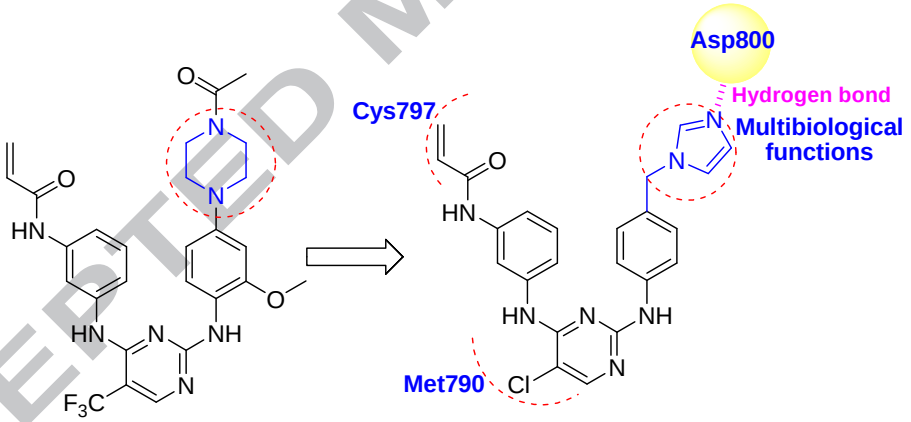

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ABSTRACT

A series of novel azole-diphenylpyrimidine derivatives (AzDPPYs) were synthesized and biologically evaluated as potent $EGFR^{T790M}$ inhibitors. Among these analogues, the most active inhibitor **6e** not only displayed high activity against $EGFR^{T790M/L858R}$ kinase (IC_{50} = 3.3 nmol), but also was able to repress the replication of H1975 cells harboring $EGFR^{T790M}$ mutation at a concentration of 0.118 μmol. In contrast to the lead compound rociletinib, **6e** slightly reduces the key EGFRT790M-minduced drug resistance. Significantly, inhibitor **6e** demonstrates high selectivity (SI = 299.3) for T790M-containing EGFR mutants over wild type EGFR, hinting that it will cause less side effects.

1. Introduction

The epidermal growth factor receptor (EGFR) protein, is over-expressed in up to 80% of non-small cell lung cancers (NSCLC), and has been a primary therapeutic target for NSCLC.[1-3] Inhibitors targeting the kinase domain of EGFR, such as gefitinib (**1**, **Fig.1**)[4,5] and erlotinib (**2**, **Fig.1**)[6], have shown promise in patients with activating mutations (i.e. in exons 18, 19 or 21) in EGFR. Unfortunately, drug resistance often occurs as a result of a secondary mutation such as the Thr^{790} to Met^{790} (T790M) mutation for most patients who initially respond to gefitinib and erlotinib.[7-9] Irreversible EGFR inhibitors containing a Michael acceptor functional group have been developed to circumvent the T790M mutation-related resistance. These inhibitors form a covalent bond with the Cys797 within the EGFR active site and have shown preclinical activity against T790M-containing mutants of EGFR.[10] Unfortunately, the family of the irreversible inhibitors always have skin rash, diarrhea, and other serious side effects,[15,16] with the exception of a few molecules, such as WZ4002[11], rociletinib[12], and osimertinib[13,14], Therefore, it is necessary to

develop new molecules for the development of novel drugs with better anti-NSCLC activity and less host toxicity.

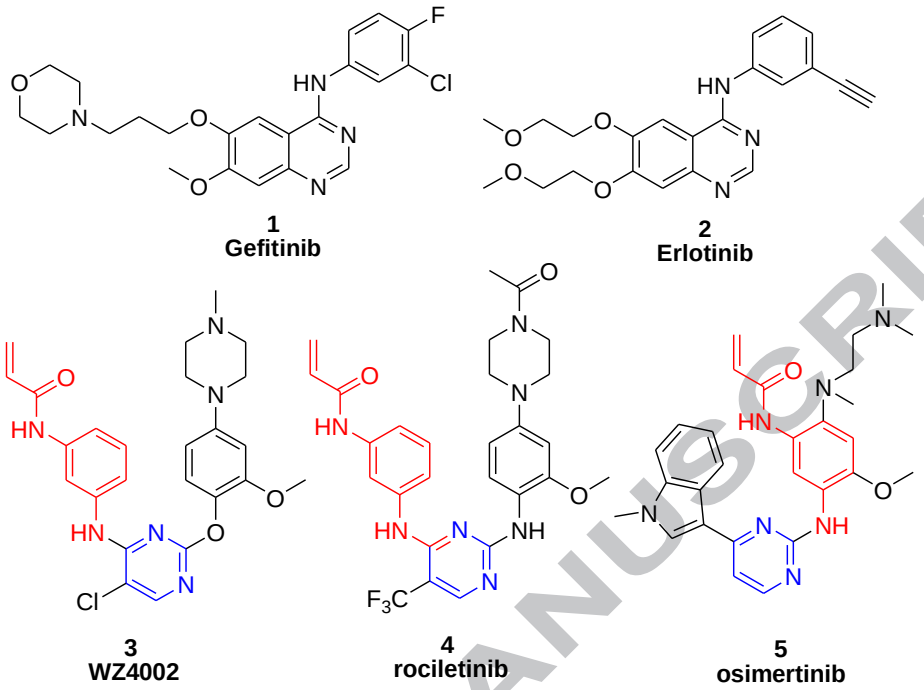


Figure 1. Chemical structures of novel EGFR inhibitors.

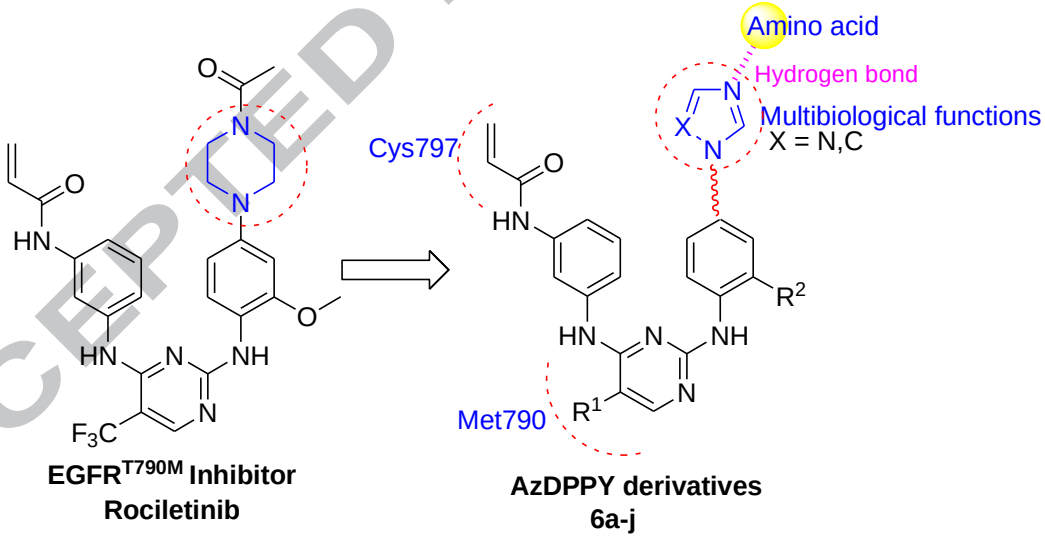


Figure 2. Designed strategy of the azole-diphenylpyrimidine derivatives.

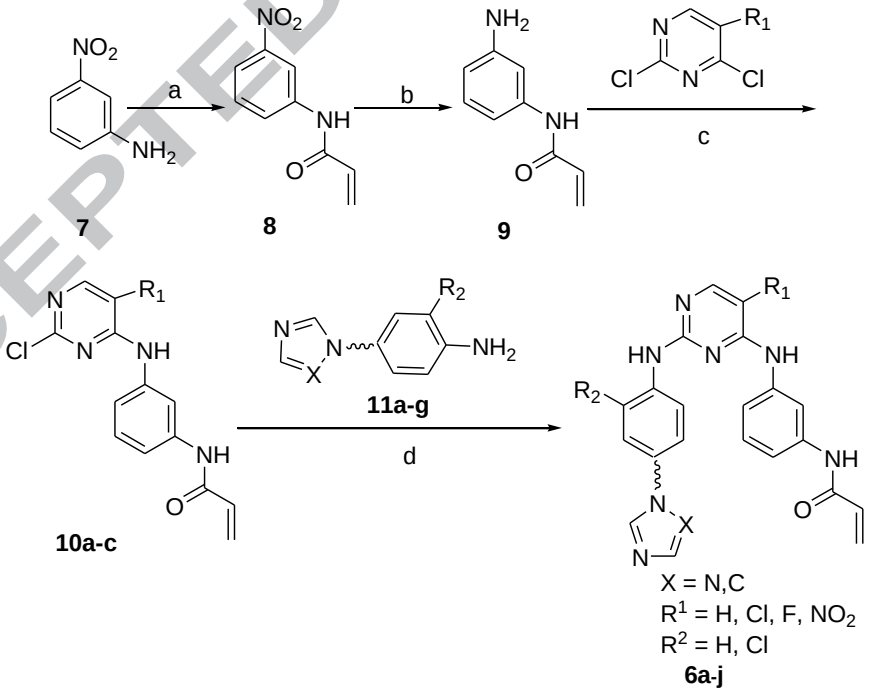
Generally, a common pyrimidine structure core is essential for these inhibitors to maintain the high anti-EGFR^{T790M} activity, as shown in figure 1. In addition, an acryloyl functionality is also important in forming a strong covalent bond with amino acid Cys797 in EGFR^{T790M}.[11] Azoles, particularly the bioisosteric 1, 3, 4-oxadiazoles, 1, 2, 4-triazoles, and 1, 3, 4-thiadiazoles, are well-known small heterocyclic templates that exhibit a broad-spectrum of biological activities such as antimalarial, antitubercular, antitumor, antibacterial, anticancer, CNS depressant, anticonvulsant, molluscicidal, analgesic, inflammatory, and anti-HIV. Typically, the azole functionality exhibited successful interactions with particular receptors on

enzyme active sites through polar interactions.[17-19] Herein, a series of pyrimidine derivatives bearing an azole group are designed and synthesized to enhance the binding affinity with $EGFR^{T790M}$ enzyme. Indeed, the molecular simulations shown in figure 5 indicates that the new azole substituent forms a new strong hydrogen bond with $EGFR^{T790M}$, suggesting that these molecules can improve the anti-$EGFR^{T790M}$ activity. Therefore, in this manuscript, a class of azole-diphenylpyrimidine derivatives (AzDPPYs) along with their activity against NSCLC were described.

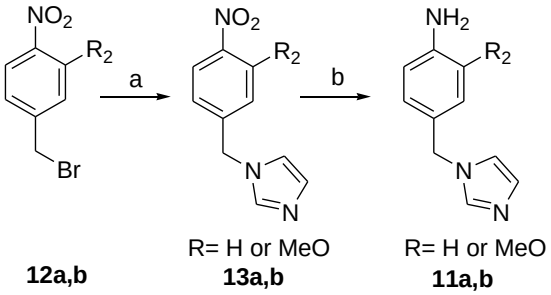
2. Results and discussion

2.1. Chemistry

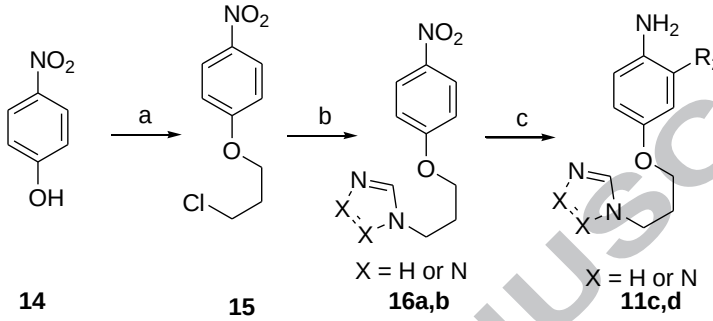
The designed compounds AzDPPYs **6a-j** were prepared according to the general strategy outlined in Scheme 1.[20,21] Commercially available reagent 3-nitroaniline (**7)** was reacted with acryloyl chloride to produce the nitro-substituted intermediate **8**. By reducing the nitro group in compound **8** using Fe-NH_4Cl conditions, the *N*-acryloyl-4-aminobenzamide (**9)** was produced in excellent yield (92%). Installation of the 4-aniline substituent **9** of the pyrimidine was achieved by direct regioselective displacement of 4-chloro in the presence of *N*,*N*-diisopropylethylamine (DIPEA). As specified in scheme 2, scheme 3 and scheme 4, the azole-substituted anilines **11a-g** were prepared by applying the reported synthetic methods,[22-25] including nucleophilic substitution reaction, acylation, and reaction reduction reaction. The desired azole-diphenylpyrimidine derivatives **6a-j** were conveniently synthesized when coupled by various anilines containing the azole group with the pyrimidine scaffold **10a-c**.



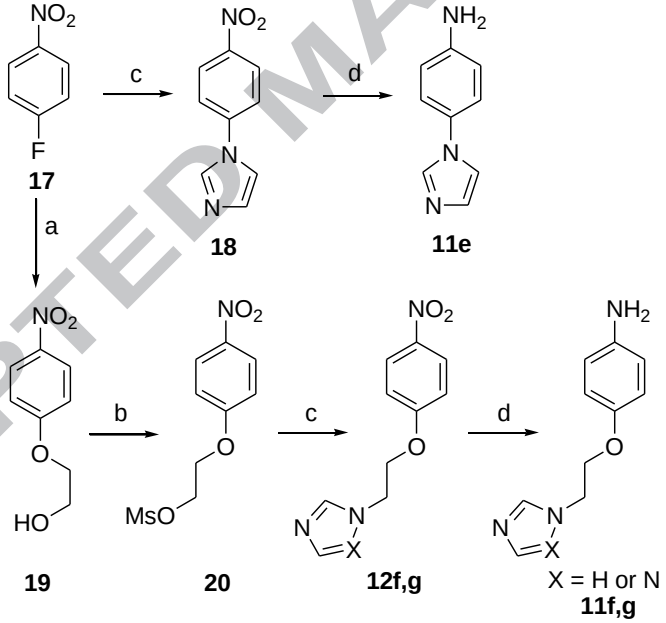
Scheme 1. Synthetic route of title compounds **6a-j**. Reagents and conditions: (a) acryloyl chloride, $NaHCO_3$, CH_3CN, rt, 0.5 h, 95%; (b) Fe-NH_4Cl, MeOH-H_2O, 2 h, 70 °C, 92%; (c) $ArNH_2$, DIPEA, 1,4-dioxane, rt, 2 h, 91%; (d) trifluoroacetic acid, 4-azole-substituted aniline, 2-BuOH, 100 °C, 12 h, 33–42%.



Scheme 2. Synthetic route of intermediates **11a-b**. (a) imidazole, K_2CO_3, CH_3CN, reflux, 5 h, 80–92%; (b) Fe-NH_4Cl, MeOH-H_2O, 2 h, 70 °C, 72–81%.



Scheme 3. Synthetic route of title intermediates **11c-d**. Reagents and conditions: (a) 1-bromo-3-chloropropane, K_2CO_3, CH_3CN, reflux, 12 h, 80–91%; (b) imidizole or tetrazole, K_2CO_3, CH_3CN, reflux, 5 h, 55–73%; (c) Fe-NH_4Cl, MeOH-H_2O, 2 h, 70 °C, 62–81%.



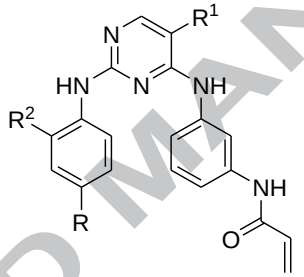
Scheme 4. Synthetic route of title intermediates **10a-m**. Reagents and conditions: (a) $HOCH_2CH_2OH$, K_2CO_3, 80 °C, 2 h, 72–87%; (b) $MeSO_2Cl$, $NaHCO_3$, CH_3CN, r.t, 2 h, 67–85%; (c) Azole, K_2CO_3, CH_3CN, reflux, 5 h, 47–71%; (d) Fe-NH_4Cl, MeOH-H_2O, 2 h, 70 °C, 48–62%.

2.2 Biological activity

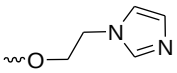
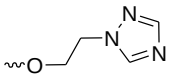
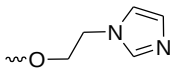
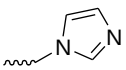
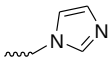
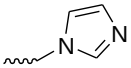
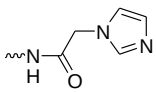
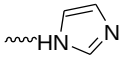
These compounds were evaluated by their inhibition ability for the autophosphorylation of wild-type EGFR and the T790M/L858R-mutated EGFR kinases using ADP-Glo™ Kinase Assay.[26-28] The novel inhibitors rociletinib and gefitinib were also evaluated as references using the same procedure. As seen in Table 1, the family of these molecules were able to interfer with the activity of EGFR T790M/L858R kinase within 100 nmol/L concentrations. Among them, inhibitors **6c**, **6e**, **6f**, possessing the IC_{50} values of 9.1 nmol, 3.3 nmol, and 9.0 nmol, were better than rociletinib (IC_{50} = 21.5 nmol) within EGFR T790M/

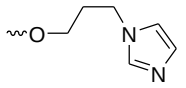
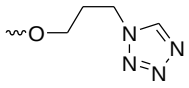
L858R kinase inhibition. In particular, the most active inhibitor **6e,** that contains an imidazolyl functionality, displyed approximately 8-fold higher potency against $EGFR^{T790M/L858R}$ than rociletinib. However, by simply installing a MeO substituent on the phenyl ring of compound **6e**, the inhibitor **6d** (IC_{50} = 109 nmol) is reduced and was able to reduce the potency of anti-EGFR T790M/L858R nearly 30-times. The addition of a long linker between the azole heterocycle and the phenyl ring also was not benefical. The example compunds **6a** and **6i**, which have the IC_{50} values of 106.0 nmol and 40.6 nmol, lost the anti-BTK capacitcy rapidly. These biological explortaions also demonstrated that addition one or two nitrogen atom to the imidazole ring was unfavorabe. For instances, molecules **6b**, and **6j,** featuring a triazole or tetrazole functionality, exhibited only moderate activity, with IC_{50} values aroud 100 nmol. Interestingly, the activity againt the wild-type EGFR found that these comounds are less sensitive to WT EGFR, with IC_{50} values being greater than 505.6 nmol. Note, the azo-DPPYs, possess high selectivity index, hinting that they will produce less side effects particularly the imidizle-substited compunds **6c** (SI >109.9) and **6e** (SI = 299.3).

Table 1 In vitro EGFR tyrosine kinases (wild type and L858R/T790M mutation) activities of the title compounds **6a-j** [a]



6a-j

Compd	R^1	R^2	R	EGFR (IC_{50},nM)[b]		
				WT	**L858R/T790M**	**SI (WT:L858R/T790M)**
6a	Cl	H		>1000	106.0	>9.434
6b	Cl	H		>1000	103.4	>9.671
6c	F	H		>1000	9.1	>109.9
6d	Cl	MeO		506	109.0	4.639
6e	Cl	H		1000	3.3	299.3
6f	F	H		987	9.0	110.2
6g	NO_2	H		>1000	118.7	>8.425
6h	NO_2	H		>1000	98.2	>10.19

6i	NO_2	H		>1000	40.6	>24.63
6j	NO_2	H		>1000	100.6	>9.940
rociletinib				460	21.5	21.4
gefitinib						

[a] Data represent the mean of at least three separate experiments. [b] Concentration needed to inhibit the autophosphorylation of the cytoplasmic domain of EGFR by 50%, as determined from the dose-response curve.

These newly synthesized compounds were also evaluated for their activity against the NSCLC cell lines ($A431^{WT}$, $H1975^{L858R/T790M}$, $A549^{k\text{-ras mutation}}$), as well as the normal lung cells HBE by using the MTT method. Gefitinib and rociletinib were also compared for cell viability, as seen in Table 2. Notably, molecule **6e**, was the most active inhibitor against $EGFR^{T790M}$ kinase, and also has the strongest capacity (IC_{50} = 0.118 μmol) for inhibiting the H1975 cell line harboring $EGFR^{T790M}$ mutation. This molecule possesses almost 10 times higher potency than gefitinib, and is equivalent to rociletinib in repression of the H1975 cell line replication. Although molecule **6a**, beared an additional carbon atom linker between the imidazolyl functionality and phenyl ring, has a moderate activity against $EGFR^{T790M/L858R}$ kinase (IC_{50} = 106.0 nmol), it could strongly inhibit H1975 cells at concentrations of 0.391 nmol. It is possible to form a new interaction mechanism to interfere with the T790M-mutated cells. In terms of their activity against A431 cell line harboring wild-type EGFR, compound **6f** (IC_{50} = 0.152 nmol), which has a fluorine atom at the *C*-5 position of pyrimidine core, was the most potent inhibitor within this class of inhibitors. When replacing the fluorine atom in compound **6f** with a chlorine substituent, the molecule **6e** yielded a 5 times increased activity, with an IC_{50} value of 0.733 nmol. It was apparent that the most active inhibitors were **6e** and **6f**, which displayed stronger potency than references against the A431 cells. Furthermore, the newly obtained AzDPPYs were effective in interference with the proliferation of the K-ras mutated A549 cell line within micromolar concentrations. Compounds **6e** and **6f** were the most effective inhibitors against A549 cells, having the IC_{50} values of 2.730 μmol and 3.563 μmol, respectively. Fortunately, most of these inhibitors were less sensitive to the normal HBE cell line (IC_{50} > 40μmol), indicating that they are less cytotoxic. Overall, the biological evaluations of AzDPPY derivatives, eventually led to the discovery of a promising inhibitor **6e** with high anti-$EGFR^{T790M}$ activity and low cytotoxicity.

Table 2 Cellular antiproliferative activities of the title compounds **6a-j.**

Compd	**Cellular antiproliferative activity (IC_{50}, μM)**[b]			
	H1975	**A431**	**A549**	**HBE**

6a	0.391±0.015	1.337±0.042	8.436±1.525	39.15±3.236
6b	2.366±0.098	5.950±0.266	10.93±0.663	>40
6c	1.814±0.056	2.476±0.102	8.366±0.554	39.95±3.241
6d	0.932±0.074	6.775±0.289	14.30±1.004	>40
6e	0.118±0.004	0.733±0.031	2.730±0.234	>40
6f	1.723±0.067	0.152±0.008	3.563±0.205	>40
6g	11.51±0.460	16.25±0.607	>40	>40
6h	12.62±0.510	12.35±0.507	>40	>40
6i	8.320±0.312	6.460±0.277	27.37±1.986	39.19±3.238
6j	8.050±0.303	7.960±0.299	19.35±0.822	>40
rociletinib	0.137±0.004	1.290±0.037	6.502±0.267	>40
gefitinib	10.89±0.960	3.308±0.106	29.43±2.506	23.76±2.368

[a] Data represent the mean of at least three separate experiments. [b] Dose-response curves were determined at five concentrations. The IC_{50} values are the concentrations in micromolar needed to inhibit cell growth by 50% as determined from these curves.

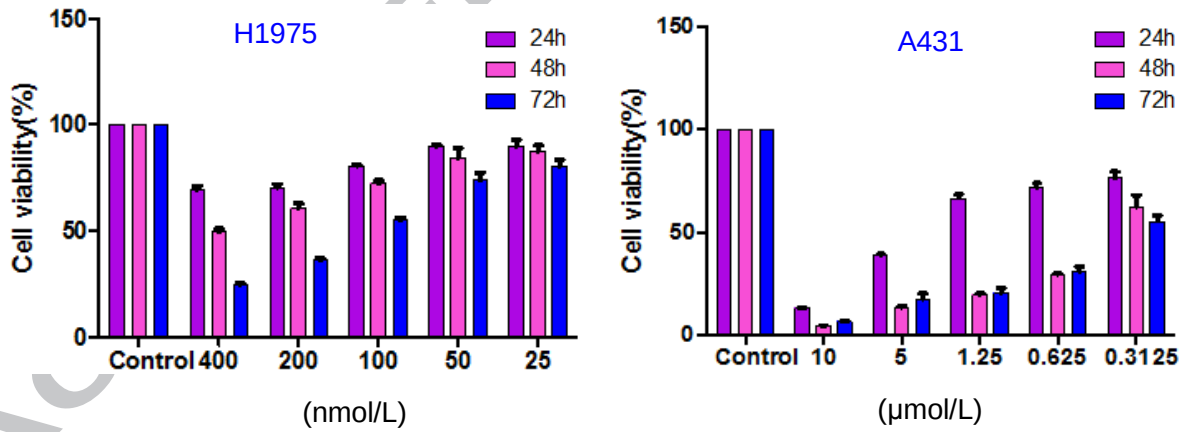


Figure 3. Various treating time of inhibitor **6e** on the affects of the ability against H1975 and A431 cell lines.

Additionally, the effects of treating time (24h, 48h, 72h) on the ability to inhibit H1975 and A431 cell lines was measured using the most active inhibitor, **6e.** Figure 1 confirms that the proliferations of the two cell lines were inhibited significantly by **6e** in dose-and time-dependent manners. Moreover, the effects of the inhibitor **6e** on apoptosis in H1975 cell line were also determined using flow cytometer analysis (FCMA). The result, shown in figure 4, displayed that the inhibitory effects of **6e** obviously increased with the increasing of drug concentration.

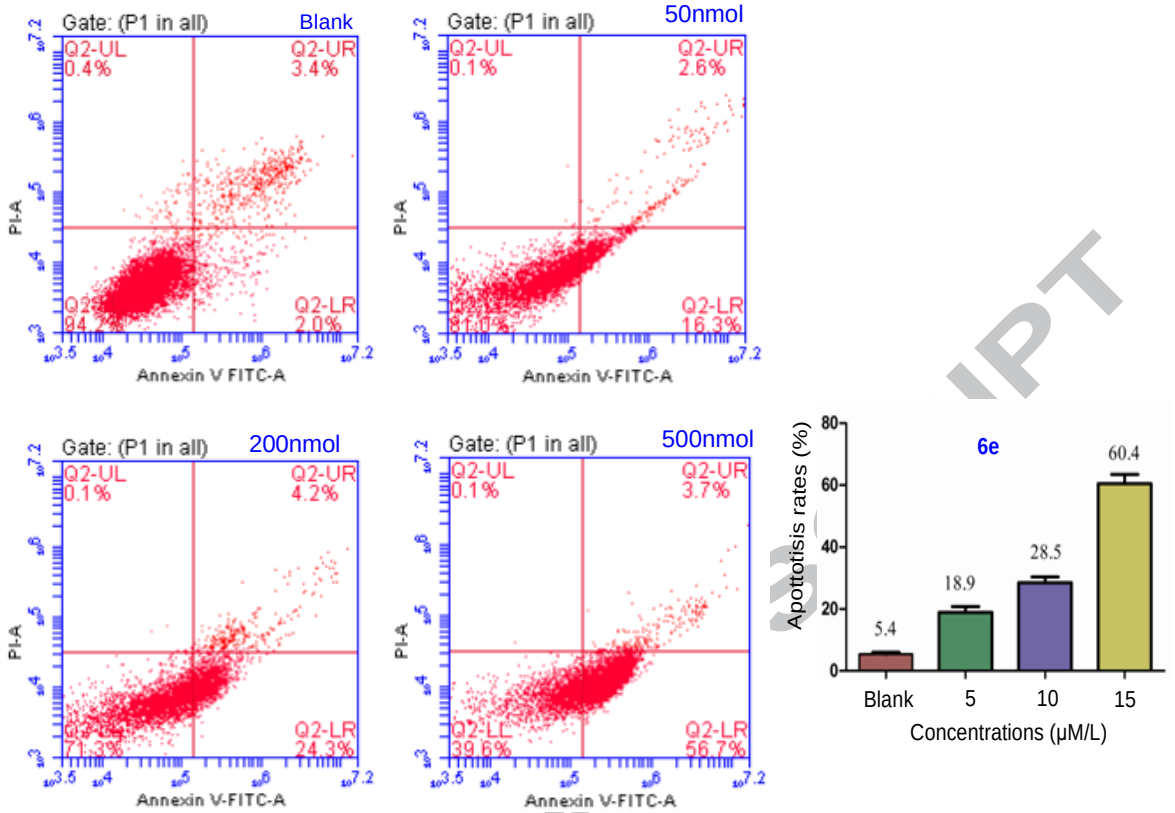


Figure 4. Compound **6e** induced H1975 cell apoptosis in vitro. The cells were incubated with the indicated concentrations of **6e** for 48 h, and the cells were stained with a annexin V/FTIC, followed by flow cytometry analysis. One representative experiment is shown. p <0.05 (n = 3)

2.3. Molecular modelling analysis

To explore the interaction mechanism of AzDPPYs with $EGFR^{T790M}$ enzyme, molecular simulations were performed by docking the representative compounds, specifically **6d**, **6e, 6f**, **6g** and **6i**, into the ATP binding pocket of $EGFR^{T790M}$ (PDB: 4I22).[29] For comparison, the lead compound WZ4002 was also performed using same procedure (Fig. 5a). The program AutoDock 4.2 with its default parameters was used.[30-32] As shown in figure 5a, WZ4002 forms several strong interactions with $EGFR^{T790M}$, including: (1) covalent bond between the acryl amide functionality with the amino acid Cys797; (2) strong contacts generated from the chlorine atom at the *C*-5 position of pyrimidine core with the mutant gatekeeper residue Met790; (3) hydrogen bond between the *N*-1 nitrogen atom of pyrimidine core and amino acid Met793; (4) polar forces formed by the piperazine ring with the outside of the ATP-binding pocket of $EGFR^{T790M}$. Evidently, the binding mode of the most active inhibitor **6e** (c, Fig.5b) with $EGFR^{T790M}$ is similar to that of WZ4002. For compound **6e**, it produced the excepted strong hydrogen bond between the newly introduced imidazole ring with the reside Asp803 through a water molecule. However, by only replacing the chlorine atom at the *C*-5 position of pyrimidine core with a fluorine atom, the binding sites of the obtained compound **6f** with $EGFR^{T790M}$ are different from those of **6e**. In terms of inhibitor **6f**, the covalent bond between the acryl amide and the amino acid Cys797 remains, but the desired hydrogen bond formed by the azole ring disappeared. Moreover, the *C*-5 fluorine atom of the pyrimidine core was removed far away from the mutant reside Met790, making apparent loss of contacts with this reside. While for the less active

inhibitors **6d** and **6f**, the important contacts of covalent bond with Cys797 or hydrogen bond with Met793 still appeared. But the less active molecule **6j** not only lost these important interaction forces, but also made the essential covalent bond disappeared. Overall, all these effects were in accordance with their activity data.

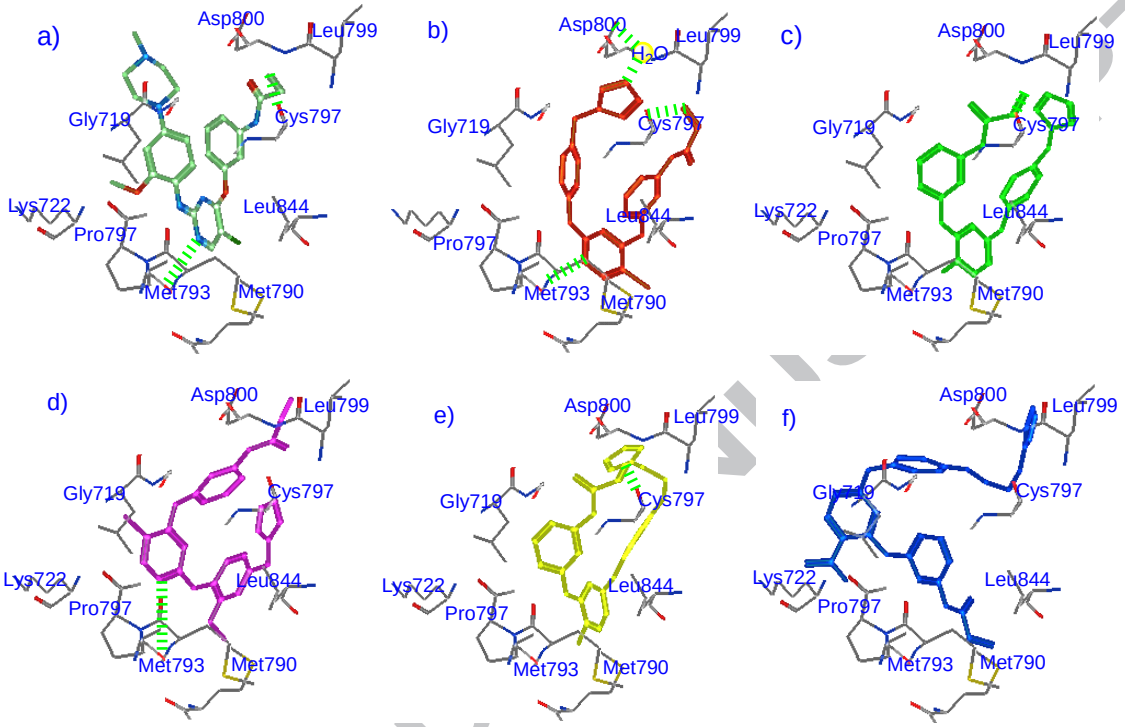


Figure 5. a) Binding site of WZ4002 within EGFR^{T790M} (PDB code: 4I22). b) Putative binding mode of **6e** within EGFR^{T790M} (PDB code: 4I22). c) Putative binding mode of **6d** within EGFR^{T790M} (PDB code: 4I22). d) Putative binding mode of **6d** within EGFR^{T790M} (PDB code: 4I22). d) Putative binding mode of **6g** within EGFR^{T790M} (PDB code: 4I22). d) Putative binding mode of **6j** within EGFR^{T790M} (PDB code: 4I22).

3. Conclusion

A series of novel azole-diphenylpyrimidine derivatives were designed and synthesized to strengthen the interactions with EGFR^{T790M} target. Biological evaluation studies in kinase and in NSCLC cell lines eventually led to the identification of a novel inhibitor **6e**, which not only displayed high activity against EGFR$^{T790M/L858R}$ kinase (IC_{50} = 3.30 nmol), but also were able to repress the replication of H1975 cell line harboring EGFR^{T790M} mutation at concentrations of 0.118 μmol. Moreover, this compound also high selectivity (SI = 299) for T790M-containing EGFR mutants over wild type EGFR, hinting that it will cause less side effects. Taken together, this exploration provided a promising DPPY derivative **6e** featuring a novel azole functionality with enhanced anticancer activity.

4. Experimental section

4.1. General Methods and Chemistry.

Solvents and reagents were obtained from commercial supplies and were used without further purification. High resolution ESI-MS were performed on an AB Sciex TripleTOF® 4600 LC/MS/MS system. ^{1}H NMR

and ^{13}C NMR spectra on a Brucker AV 400 MHz spectrometer were recorded in [*d*]DMSO. Coupling constants (*J*) are expressed in hertz (Hz). Chemical shifts (δ) of NMR are reported in parts per million (ppm) units relative to internal control (TMS). All reactions were monitored by TLC, using silica gel plates with fluorescence F254 and UV light visualization. Flash chromatography separations were obtained on Silica Gel (300–400 mesh) using dichloromethane/methanol as eluents.

4.2. General procedure for the synthesis of 6a–j.[20-25]

4-Azole-substituted aniline intermediates **11a-g** were prepared according to the reported literatures 22 to 25. While the intermediates *N*-(3-((2-chloro-5-substitutedpyrimidin-4-yl)oxy)phenyl)acrylamide **10a-b** were synthesized using the reported procedure described in references 20 to 21. All these intermediates were directly used without any purification and structural characterization. With these intermediates in hand, the newly obtained compounds were synthesized as described below.

A flask was charged with compound **10a-c** (0.70 mmol), **11a-g** (0.70 mmol), TFA (0.08 mL, 1.05 mmol), and 2-BuOH (10 mL).The slurry was heated to 100 °C for 5 h. The reaction mixture was allowed to cool to room temperature and was neutralized with a saturated aqueous sodium bicarbonate solution. The aqueous mixture was then extracted with CH_2Cl_2 (20 mL) three times. The crude product was purified using flash chromatography with dichloromethane/methanol (v/v, 1:1) as eluents.

***N*-[3-[[5-Chloro-2-[4-[2-(1*H*-imidazol-1-yl)ethoxy]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6a).**

Yield 46.1%; off-white solid. ^1H NMR (400 MHz, d_6-DMSO): δ 4.12 (s, 2H), 4.31 (s, 2H), 5.75 (d, *J* = 12.0 Hz, 1H), 6.24 (d, *J* = 16.8 Hz, 1H), 6.44 (dd, *J* = 12.0, 16.8 Hz, 1H) , 6.70 (s, 2H), 6.90 (s, 1H), 7.22 (s, 1H), 7.29-7.31 (m, 2H), 7.44-7.49 (m, 3H), 7.66 (s, 1H), 7.87 (s, 1H), 8.10 (s, 1H), 8.90 (s, 1H), 9.18 (s, 1H), 10.20 (s, 1H). HRMS (ESI) for $C_{24}H_{22}ClN_7O_2$, $[M+H]^+$ calcd: 476.1605, found: 476.1613.

***N*-[3-[[5-Chloro-2-[4-[2-(1*H*-triazol-1-yl)ethoxy]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6b).**

Yield 40.2%; off-white solid. ^1H NMR (400 MHz, d_6-DMSO): δ 4.07 (s, 2H), 4.26 (s, 2H), 5.70 (d, *J* = 12.0 Hz, 1H), 6.19 (d, *J* = 16.8 Hz, 1H), 6.39 (dd, *J* = 12.0, 16.8 Hz, 1H) , 6.64 (s, 2H), 6.84 (s, 1H), 7.17 (s, 1H), 7.24-7.29 (m, 2H), 7.44-7.48 (m, 2H), 7.61 (s, 1H), 7.82 (s, 1H), 8.05 (s, 1H), 8.84 (s, 1H), 9.13 (s, 1H), 10.15 (s, 1H). HRMS (ESI) for $C_{23}H_{21}ClN_9O_2$, $[M+H]^+$ calcd: 475.1517, found: 475.1563.

***N*-[3-[[5-Fluoro-2-[4-[2-(1*H*-imidazol-1-yl)ethoxy]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6c).**

Yield 36.7%; off-white solid. ^1H NMR (400 MHz, d_6-DMSO): δ 4.12 (t, *J* = 4.0 Hz, 2H)), 4.31 (t, *J* = 4.0 Hz, 2H), 5.75 (d, *J* = 12.0 Hz, 1H), 6.24 (d, *J* = 16.8 Hz, 1H), 6.44 (dd, *J* = 12.0, 16.8 Hz, 1H) , 6.74 (d, *J* = 8.0 Hz, 2H), 6.91(s, 1H), 7.24 (d, *J* = 6.0 Hz,1H), 7.29 (d, *J* = 12.0 Hz, 2H), 7.42 (d, *J* = 8.4 Hz, 1H), 7.53 (d, *J* = 8.4 Hz, 2H), 7.69 (s, 1H), 7.93 (s, 1H), 8.07 (s, 1H), 9.01 (s, 1H), 9.39 (s, 1H), 10.15 (s, 1H).

HRMS (ESI) for $C_{24}H_{22}FN_7O_2$, $[M+H]^+$ calcd: 460.1927, found: 460.1901.

***N*-[3-[[5-Chloro-2-[2-methoxyl-4-(1*H*-imidazol-1-ylmethyl)]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6d).**

Yield 46.5%; off-white solid. ^{1}H NMR (400 MHz, d_6-DMSO): δ 3.78 (s, 3H), 5.05 (s, 2H), 5.74 (dd, J = 2.4, 10.0 Hz, 1H), 6.24 (dd, J = 2.0, 16.8 Hz, 1H), 6.42 (dd, J = 10.0, 17.2 Hz, 1H), 6.66 (d, J = 8 Hz, 1H), 6.88 (s, 1H), 6.97 (s, 1H), 7.15 (s, 1H), 7.20 (d, J = 8.4 Hz, 1H), 7.25 (s, 1H), 7.41 (d, J = 8.4 Hz, 1H), 7.73 (s, 1H), 7.79 (s, 1H), 7.91 (d, J = 6.0 Hz, 1H), 7.92 (s, 1H), 8.11 (s, 1H), 8.95 (s, 1H), 10.14 (s, 1H).

HRMS (ESI) for $C_{24}H_{22}ClN_7O_2$, $[M+H]^+$ calcd: 476.1608, found: 476.1614.

***N*-[3-[[5-Chloro-2-[4-(1*H*-imidazol-1-ylmethyl)]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6e).**

Yield 56.5%; off-white solid. ^{1}H NMR (400 MHz, d_6-DMSO): δ 5.02 (s, 2H), 5.75 (dd, J = 2.4, 10.0 Hz, 1H), 6.25 (dd, J = 2.0, 16.8 Hz, 1H), 6.47 (dd, J = 10.0, 17.2 Hz, 1H), 6.86 (s, 1H), 7.04 (d, J = 8.4 Hz, 2H), 7.10 (s, 1H), 7.30 (d, J = 6.0 Hz, 2H), 7.50 (d, J = 6.0 Hz, 1H), 7.57 (d, J = 8.4 Hz, 2H), 7.67 (s, 1H), 7.85 (s, 1H), 8.14 (s, 1H), 8.97 (s, 1H), 9.37 (s, 1H), 10.18 (s, 1H).

HRMS (ESI) for $C_{23}H_{20}ClN_7O$, $[M+H]^+$ calcd: 446.1478, found: 446.1490.

***N*-[3-[[5-Fluoro-2-[4-(1*H*-imidazol-1-ylmethyl)]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6f).**

Yield 36.7%; off-white solid. ^{1}H NMR (400 MHz, d_6-DMSO): δ 5.04 (s, 2H), 5.76 (dd, J = 2.0, 10.0 Hz, 1H), 6.26 (dd, J = 2.0, 16.8 Hz, 1H), 6.50 (dd, J = 10.0, 16.8 Hz, 1H), 6.88 (s, 1H), 7.03 (d, J = 8.4 Hz, 2H), 7.09 (s, 1H), 7.27 (d, J = 6.0 Hz, 1H), 7.43 (d, J = 6.0 Hz, 1H), 7.51 (d, J = 6.0 Hz, 1H), 7.64 (s, 1H), 7.69 (s, 1H), 7.93 (s, 1H), 8.11 (s, 1H), 9.24 (s, 1H), 9.46 (s, 1H), 10.16 (s, 1H).

HRMS (ESI) for $C_{23}H_{20}FN_7O$, $[M+H]^+$ calcd: 430.1768, found: 430.1782.

***N*-[3-[[5-Nitro-2-[4-[2-(1*H*-imidazol-1-yl)acetylamino]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6g).**

Yield 66.1%; faint yellow solid. ^{1}H NMR (400 MHz, d_6-DMSO): δ 5.07 (s, 2H), 5.71 (d, J = 10.0 Hz, 1H), 6.21 (d, J = 16.8 Hz, 1H), 6.40 (dd, J = 10.0, 16.8 Hz, 1H), 6.88 (s, 1H), 7.01 (s, 1H), 7.11 (d, J = 8.4Hz, 2H), 7.27 (d, J = 6.0Hz, 1H), 7.44 (d, J = 6.0Hz, 1H), 7.52 (d, J = 6.0 Hz, 1H), 7.65 (d, J = 8.4Hz, 2H), 7.69 (s, 1H), 7.83 (s, 1H), 7.95 (s, 1H), 8.51(s, 1H), 9.04 (s, 1H), 10.32 (s, 1H).

HRMS (ESI) for $C_{24}H_{21}N_9O_4$, $[M+H]^+$ calcd: 500.1722, found: 500.1714.

***N*-[3-[[5-Nitro-2-[4-(1*H*-triazol)]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6h).**

Yield 60.2%; faint yellow solid. ^{1}H NMR (400 MHz, d_6-DMSO): δ 5.67 (d, J = 10.0 Hz, 1H), 6.19 (d, J = 16.8 Hz, 1H), 6.29 (dd, J = 10.0, 16.8 Hz, 1H), 6.39 (s, 1H), 6.64 (d, J = 8.4 Hz, 2H), 6.84 (s, 1H), 7.01 (s, 1H), 7.21 (d, J = 8.4Hz, 1H), 7.28 (d, J = 6.0 Hz, 1H), 7.38 (d, J = 6.0Hz, 1H), 7.41 (s, 1H), 7.43 (s, 1H), 7.86 (s, 1H), 8.02 (s, 1H), 8.96 (s, 1H), 9.23 (s, 1H), 10.29 (s, 1H).

HRMS (ESI) for $C_{22}H_{18}N_9O_3$, $[M+H]^+$ calcd: 443.1531, found: 443.1574.

***N*-[3-[[5-Nitro-2-[4-[3-(1*H*-imidazol-1-yl)propoxy]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6i).**

Yield 61.0%; faint yellow solid. ^{1}H NMR (400 MHz, d_6-DMSO): δ 3.86 (m, 2H), 4.07 (t, J = 4.0 Hz, 2H), 4.57 (t, J = 4.0 Hz, 2H), 5.72 (d, J = 10.0 Hz, 1H), 6.17 (d, J = 16.8 Hz, 1H), 6.21 (dd, J = 10.0, 16.8 Hz, 1H), 6.39 (s, 1H), 6.64 (d, J = 8.4 Hz, 2H), 6.84 (s, 1H), 7.17 (d, J = 8.4Hz, 2H), 7.24 (d, J = 6.0 Hz, 1H), 7.44 (d, J = 8.4Hz, 2H), 7.61 (s, 1H), 7.82 (s, 1H), 8.05 (s, 1H), 9.03 (s, 1H), 9.45 (s, 1H), 10.27 (s, 1H). HRMS (ESI) for $C_{25}H_{24}N_9O_4$, $[M+H]^+$ calcd: 501.1987, found: 501.2006.

***N*-[3-[[5-Nitro-2-[4-[3-(1*H*-tetrazol-1-yl)propoxy]phenylamino]-4-pyrimidinyl]amino]phenyl]-2-propenamide (6j).**

Yield 56.8%; faint yellow solid. ^{1}H NMR (400 MHz, d_6-DMSO): δ 3.87 (m, 2H), 4.10 (t, J = 4.0 Hz, 2H), 4.62 (t, J = 4.0 Hz, 2H), 5.75 (d, J = 10.0 Hz, 1H), 6.18 (d, J = 16.8 Hz, 1H), 6.25 (dd, J = 10.0, 16.8 Hz, 1H), 6.45 (s, 1H), 6.67 (d, J = 8.4 Hz, 1H), 6.87 (s, 1H), 7.20 (d, J = 8.4Hz, 1H), 7.28 (d, J = 6.0 Hz, 1H), 7.49 (d, J = 8.4Hz, 2H), 7.71 (s, 1H), 7.88 (s, 1H), 8.15 (s, 1H), 9.13 (s, 1H), 9.55 (s, 1H), 10.26 (s, 1H). HRMS (ESI) for $C_{23}H_{22}N_{10}O_4$, $[M+Na]^+$ calcd: 525.1787, found: 525.1739.

4.3. In vitro kinase enzymatic assay

The wild-type EGFR enzyme, mutant EGFR T790M/L858R enzyme, and the ADP-Glo™ Kinase Assay system that measures ADP formed from a kinase reaction were purchased from Promega Corporation (USA). Concentrations consisting of suitable levels from 0.1 to 1000 nM were used for all of the tested compunds. For the ADP-Glo kinase assay, ADP is converted into ATP, which is converted into light by Ultra-Glo™ Luciferase. The experiments were performed according to the instructions of the manufacturer.[26-29] Plate was measured on TriStar2 LB 942 Multimode Microplate Reader (BERTHOLA) to detect the Luminescence. Curve fitting and data presentations were performed using Graph Pad Prism version 5.0.

4.4. Cellular activity assay

H1975 cells were obtained from Fuheng Biology Company (Shanghai, China). Cells were seeded in 96-well plates at a density of 2,000 to 3,000 cells/well and were maintained at 37 °C in a 5% CO_2 incubator in DMEM or RPMI1640 containing 10% fetalbovine serum (FBS, Gibico). Cell viability was then assessed with MTT reagent (Thiazolyl blue tetrazolium bromide; Sigma, Oakville, ON). Cells were esposed to treatment for 48 h, and the number of cells used per experiment for each cell lines was adjusted to obtain an absorbance of 0.5 to 1.2 at 570 nm. Compounds were tested at appropriate concentrations (0.05 to 40 μM), with each concentration duplicated five times. The data were calculated using GraphPad Prim version 5.0. Dose-response curves were fitted using a nonlinear regression model with a sigmoidal dose-response.

4.5. Cell Apoptosis Assay

Cells were treated with solvent control (DMSO), or compound **6e** in medium containing 5% FBS for 48 hours. Both non-adherent and adherent cells were collected and pooled, washed twice with PBS and

resuspended in 70% ethanol for permeabilization. Cell suspensions were then treated with propidium iodide solution (48 μg/ml propi-dium iodide, 40 μg/ml RNase A) for 30 minutes at room temperature. A total of 3 × 10^5 cells were evaluated using the Beckman Coulter Epics XL flow cytometer (Beckman-Coulter,Mississauga, ON) and the percentage of apoptotic cells was calculated from cells with less than 2NDNA content using FCS Express flow cytometry analysis software (ModFit LT 3.1).

4.6. Molecular docking study

Docking studies were carried out on AutoDock 4.2. The crystal structure (PDB: 4I22) of the kinase domain of $EGFR^{T790M}$ bound to inhibitor **1** was used in the docking studies. [29] The enzyme preparation and the hydrogen atoms adding was performed in the prepared process.The whole EGFR enzyme was defined as a receptor and the site sphere was selected on the basis of the binding location of gefitinib. By moving gefinitib and the irrelevant water, molecule **6d**, **6e**, **6f**, **6g**, and **6j** was placed, respectively The binding interaction energy was calculated to include van der Waals, electrostatic, and torsional energy terms defined in the tripos force field. The structure optimization was performed using a genetic algorithm, and only the best-scoring ligand protein complexes were kept for analyses.[30-32]

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