Bioorganic Chemistry 89 (2019) 102994

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Identification of 2(1*H*)-pyrimidinones as potential EGFR T790M inhibitors for the treatment of gefitinib-resistant non-small cell lung cancer

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ARTICLE INFO

Keywords:
NSCLC
EGFR T790M
Resistant
Inhibitor
Pyrimidinone

ABSTRACT

A new class of 2(1*H*)-pyrimidinone derivatives was identified as potential EGFR T790M inhibitors against TKI-resistant NSCLC. These novel compounds inhibited the EGFR T790M kinase activity at concentrations in the range of 85.3 to 519.9 nM. In particular, compound **7e** exhibited the strongest activity against both $EGFR^{WT}$ (IC_{50} = 96.9 nM) and $EGFR^{T790M}$ (IC_{50} = 85.3 nM) kinases in the cells. Compared with inhibitor **7e**, compound **7b** displayed enhanced antiproliferative activity against gefitinib-resistant H1975 cells harboring the EGFR T790M mutation. In addition, compound **7b** also has low toxicity against the normal human liver cells LO2, with an IC_{50} of 11.1 μM. Moreover, both the AO/EB and DAPI staining assays also demonstrated the inhibitory efficacy of **7b** against the resistant H1975 cells. This contribution provides a new scaffold 2(1*H*)-pyrimidinone as potential EGFR T790M inhibitor against drug-resistant NSCLC.

1. Introduction

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, and accounts for 85% of all lung cancer patients [1]. The epidermal growth factor receptor (EGFR, also known as ErbB1 and HER1) is expressed in 20–80% of NSCLC tumors and is recognized as one of the most promising drug targets for the treatment of NSCLC [2]. The first generation of small-molecule EGFR inhibitors include gefitinib (**1**, Fig. 1) and erlotinib (**2**, Fig. 1), which compete with ATP in a reversible manner to bind the kinase domain of their targets [3,4]. Unfortunately, all patients who experience marked improvement on these drugs eventually develop progression of disease after a median of 12 months due to the acquisition of drug resistance [5,6]. About half of the cases with acquired resistance to first-generation EGFR inhibitors can be accounted for by a second-site mutation in exon 20 of the EGFR kinase domain, which results in the substitution of methionine for threonine at position 790 (T790M). The bulkier methionine residue at position 790 sterically hinders binding of gefitinib and erlotinib to the ATP-binding pocket [7,8]. Additional research has indicated that the T790M mutation also increases the affinity of EGFR for ATP, therefore outcompeting ATP-competitive tyrosine-kinase inhibitors (TKIs) and restoring enzymatic activity in their presence [9].

To overcome the T790M-mediated acquired resistance to EGFR TKIs, many trials have studied the intensification of EGFR inhibition through use of second- and third- generation inhibitors, such as afatinib (**3**) [10], WZ4002 (**4**) [11], rociletinib (**5**) [12],and osimertinib (**6**) [13,14] (Fig. 1). These inhibitors overcome T790M resistance mainly through covalent inhibition of EGFR by binding to a cysteine side chain (Cys797) through Michael addition with a suitable electrophile attached to the inhibitor molecule [15]. Despite of their excellent clinical efficacy in the treatment of EGFR mutant NSCLC patients, the development of resistance to these new third-generation EGFR inhibitors still occurs 14–20 months later [16]. Therefore, identifying of more potent EGFR T790M inhibitors to overcome TKI resistance is still very urgent.

The co-crystal structure (PDB code: 3IKA) of the EGFR T790M kinase domain in complex with WZ4002 (**4**) [11] indicated that a "U-shaped" configuration of a pyrimidine core, together with an aniline ring bearing a hydrophilic group and an acrylamide moiety, is propitious to bind with EGFR target (Fig. 2) [5]. Accordingly, a class of

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https://doi.org/10.1016/j.bioorg.2019.102994
Received 16 November 2018; Received in revised form 5 May 2019; Accepted 17 May 2019
Available online 18 May 2019

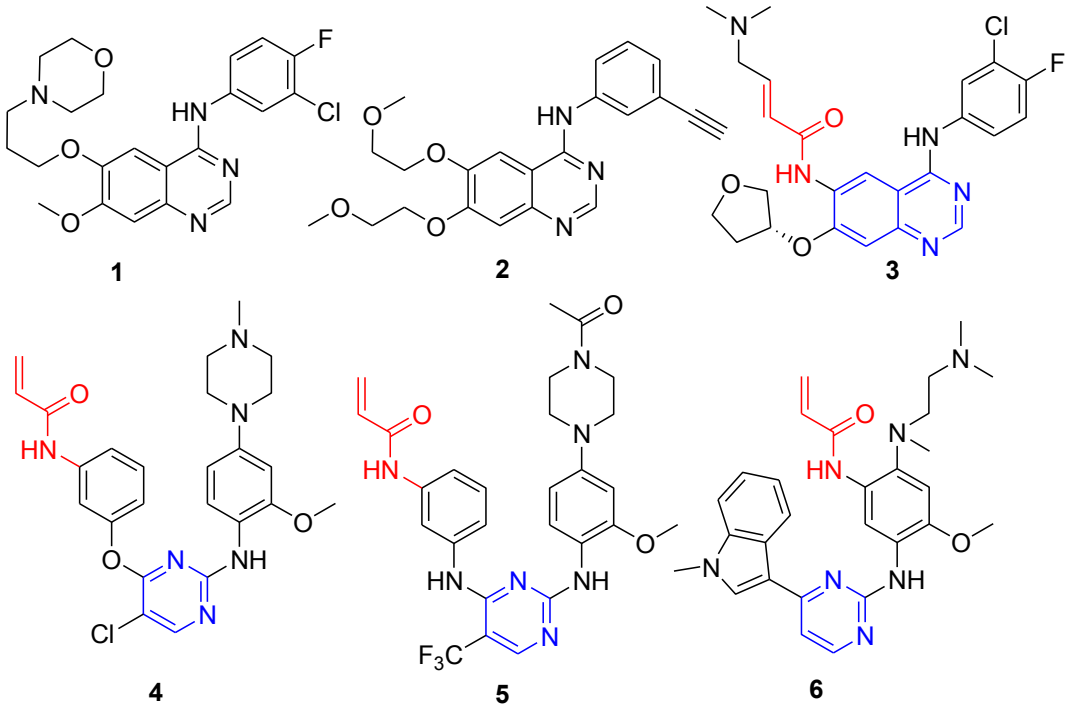


Fig. 1. Structures of novel EGFR inhibitors.

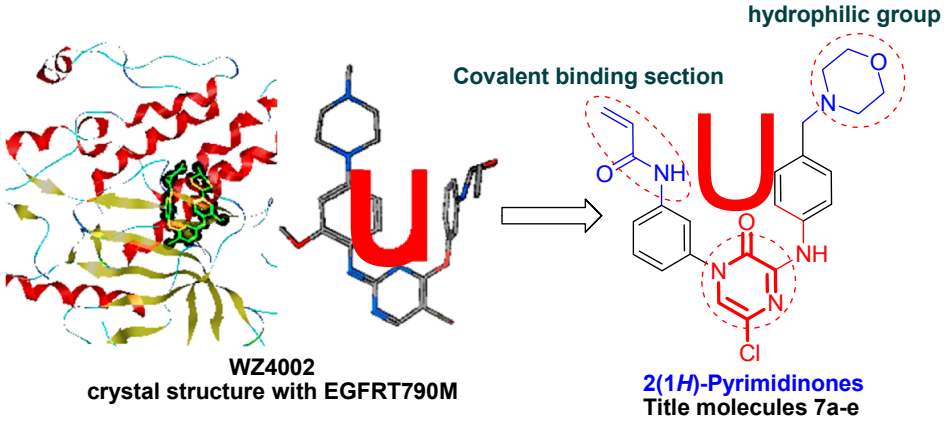


Fig. 2. Discovery strategy of the desired 2(1*H*)-pyrimidinone analogues.

2(1*H*)-pyrimidinone derivatives were described as potential EGFR T790M inhibitors to overcome TKI resistance in this manuscript (see Figs. 3 and 4).

2. Results and discussion

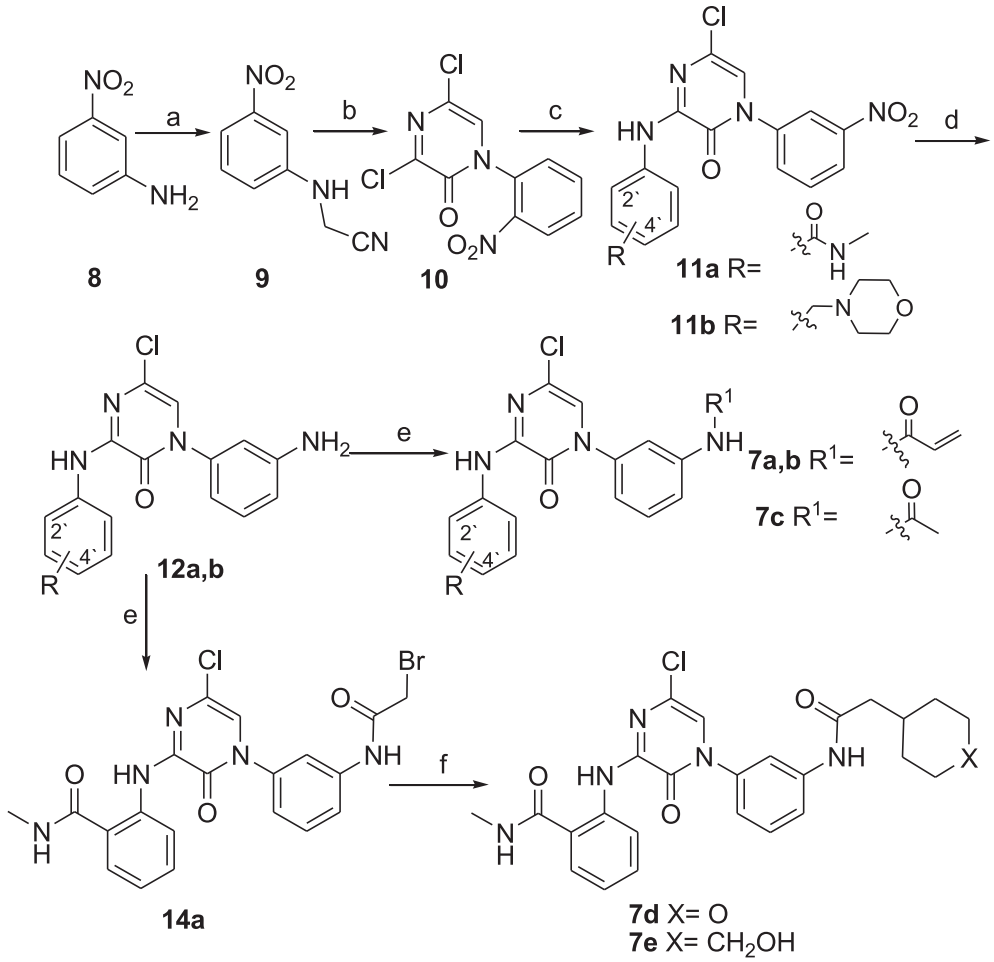
2.1. Synthesis of the title molecules

The synthetic methods for the preparation of the title molecules **7a–e** are depicted in Scheme 1 [17–19]. 3-Nitroaniline **8** was reacted with bromoacetonitrile to produce compound **9**. By reacting compound **9** with oxalic dichloride, the 2(1*H*)-pyrimidinone scaffold (**10**) was formed. Compounds **11a,b** were synthesized by a nucleophilic substitution reaction between intermediate **10** and anilines. After reducing the nitro group in compound **11**, the aniline derivatives **12a,b** were generated. Finally, the title molecules **7a-c** and intermediate **14a** were respectively synthesized by an acylation of compounds **12a,b** with acylation reagent, comprised of acetyl chloride, acryloyl chloride and bromoacetyl bromide. The title molecules 1**3d,e** were also prepared through an additional nucleophilic substitution reaction between **14a** and *N*-heterocycle.

2.2. Inhibition of kinase activity and cancer cell viability

2.2.1. Enzymatic activity

All the title molecules were evaluated for their effect on the enzymatic activity of wild type EGFR ($EGFR^{WT}$) and mutant EGFR T790M ($EGFR^{T790M}$) kinases using the ADP-Glo™ kinase assay system [20,21]. Gefitinib was also evaluated as a positive control. The assay results, listed in Table 1, indicated that these 2(1*H*)-pyrimidinone analogues inhibited the kinase activity at concentrations in the range of 85.3 to 519.9 nM. In particular, among these derivatives, compound **7e** exhibited the strongest inhibitory activity against both $EGFR^{WT}$ (IC_{50} = 96.9 nM) and $EGFR^{T790M}$ kinases (IC_{50} = 85.3 nM). The results of the kinase-based assay clearly revealed that compounds (**7a** and **7b**) bearing an acryloyl group are more active than those without this substituent (**7c** and **7d**).



Scheme 1. Synthetic route of the title molecules. Reagents and conditions: (a) K_2CO_3, KI, CH_3CN, 48 h, 100 °C, 54%; (b) $(COCl)_2$, DCM, r.t, 68%; (c) TFA, 2-BuOH, 100 °C, 56–67%; (d) Fe-NH_4Cl, MeOH-H_2O, 2 h, 70 °C, 68–75%; (e) $NaHCO_3$, CH_3CN, r.t., 53–76%; (f) K_2CO_3, KI, CH_3CN, 100 °C, 48 h, 66–70%.

2.2.2. Antiproliferative activity

In addition, all these molecules were also explored their effect on the proliferation of various cancer cells, including lung cancer cell line (H1975 cells, A549 cells, A431 cells), and lymphoma cell line (Ramos cells and Raji cells). Moreover, normal hepatocyte cell line (LO2) was also used as normal control to evaluate their cytotoxicity on non-cancerous cells. The results shown in Table 2 indicated that most of the prepared compounds remarkably inhibited the proliferation of lung cancer cells at micromolecular concentrations. On the other hand, these compounds were ineffective in blocking the proliferation of lymphoma cells (IC_{50} > 22.9 μM). Among these 2(1*H*)-pyrimidinones, compound **7e**, the strongest $EGFR^{T790M}$ inhibitor, was also a very potent inhibitor of the proliferation of H1975 cells harboring EGFR T790M mutation (IC_{50} = 7.69 μM). However, compound **7e** had certain cytotoxic effect on normal LO2 cells (IC_{50} = 1.79 μM). In contrast, another potent $EGFR^{T790M}$ inhibitor **7b**, not only displayed stronger antiproliferative activity against H1975 cells than **7e**, but it also exhibited very low toxicity to LO-2 cells (IC_{50} > 11.1 μM). Thus, compound **7b** may serve as a new lead compound for the discovery of anti-TKI-resistant agent.

2.3. Effect on the proliferation of H1975 cells

2.3.1. Wound healing and transwell assays

A wound healing assay was performed to determine whether **7b** can inhibit H1975 cell migration. As shown in Fig. 5, compound **7b** strongly inhibited migration of H1975 cells at a drug concentration range from 1 to 20 μM. In addition, the AO/EB and DAPI staining assays also indicated that compound **7b** remarkably induced apoptosis in H1975 cells with the increase of the treatment time and drug concentration.

2.3.2. AO/EB and DAPI staining

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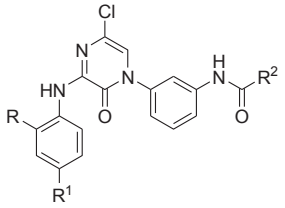
2.4. Molecular simulation

To investigate the putative interaction mechanism of pyrimidinones with the $EGFR^{T790M}$ -mutated kinase (PDB code: 3IKA), two representative molecules, namely **7b** and **7e** were separately docked into the ATP-binding pocket. For comparison, the novel $EGFR^{T790M}$ inhibitor WZ4002 was also analyzed as well. The software AutoDock 4.2 its default parameters [22,23] was used for docking performance analysis. Apparently, both compounds **7b** (Fig. 5a) and **7e** (Fig. 5b) can come into tight contact with the $EGFR^{T790M}$ binding pocket. Also, as anticipated, compound **7b** formed a very strong covalent bond between its introduced acryloyl group and the Cys797 reside in the $EGFR^{T790M}$ protein. Instead, compound **7e** formed a hydrogen-bond between the carbonyl group in the *C*-2 aniline side chain and amino acid Met793. In contrast, the pyrimidinone core in **7b** mainly comes to contact with resides Asn842 and Val726, while the core in compound **7e** mainly interacts with resides Asp800 and Phe795. It is possible that these contacts produce are responsible for the inhibitory of these compounds against the kinase activity of the mutated $EGFR^{T790M}$ enzyme. By contrast, WZ4002 forms several stronger 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

Table 1
Anti-EGFR^{WT} and EGFR^{T790M} Enzymatic activity of the title molecules **7a-e**[a]



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Compd.	R	R^1	R^2	Enzymatic activity[b] (IC_{50}, nM)[b]	
				$EGFR^{L858R/T790M}$	$EGFR^{WT}$
7a		H		230	227.4
7b	H			266	298.1
7c		H	CH_3	504	337.2
7d		H		285	519.9
7e		H		85.3	96.9
Gefitinib				832.3	15.5

[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 calculated using GraphPad Prim version 5.0.

Table 2
Antiproliferative activity of the title molecules[a.]

Compd.	Antiproliferative activity[b] (IC_{50}, μM)					
	H1975	A431	A549	Raji	Ramous	LO2
7a	8.31	5.51	3.40	42.7	> 40	14.3
7b	6.60	10.3	6.09	22.9	> 40	11.1
7c	> 10.0	4.50	12.1	> 40	> 40	15.4
7d	12.4	> 16.0	> 20	> 40	> 40	23.9
7e	7.69	24.4	> 20	> 40	> 40	1.79
Gefitinib	10.89	3.308	10.07	> 40	> 40	> 10

[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 calculated using GraphPad Prim version 5.0.

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 $EGFR^{T790M}$. Accordingly, their binding contacts are in accord with the biological activity against $EGFR^{T790M}$ kinase.

3. Conclusion

A novel anti-$EGFR^{T790M}$ kinase scaffold, namely 2(1*H*)-pyrimidinone, was identified in this work to act against the gefitinib-resistant NSCLC cancer cells. These 2(1*H*)-pyrimidinone derivatives could significantly inhibited both $EGFR^{WT}$ and $EGFR^{T790M}$ kinase activity at concentrations in the range of 85.3 to 519.9 nM. Among these derivatives, compound **7e** exhibited the strongest activity against the $EGFR^{WT}$ (IC_{50} = 96.9 nM) and $EGFR^{T790M}$ (IC_{50} = 85.3 nM) cells, while compound **7b** displayed the highest antiproliferative activity against $EGFR^{T790M}$-mutated H1975 cells. Noteworthy, compound **7b** also had low toxicity against the normal LO2 cells (IC_{50} > 11.1 μM). Moreover, further analysis with the AO/EB and DAPI staining assays also indicated its efficiency against gefitinib-resistant H1975 cells. Thus, compound **7b** may serve as a new scaffold to discover more potent EGFR T790M inhibitors.

4. Materials and methods

4.1. Chemistry

4.1.1. General information

Commercial available solvents and reagents were directly used without further purifications. High resolution ESI-MS was performed on an AB Sciex TripleTOF® 4600 LC/MS/MS system. ^{1}H NMR and ^{13}C NMR spectra, which were recorded in [*d*] DMSO, were respectively performed on Brucker AV 100 and 400 MHz spectrometer. Coupling constants (*J*) are expressed in hertz (Hz). Chemical shifts (δ scale) are reported in parts per million (ppm) relative to the central peak of the solvent. All reactions were monitored by TLC, using silica gel plates with fluorescence GF254 and UV light visualization. Preparative TLC separations were obtained on Silica Gel (300–400 mesh) using dichloromethane/methanol (20:1) as developing solvents.

4.1.2. General procedure for the synthesis of 3, 5-dichloropyrazin-2(1H)-one 10

Oxalyl chloride (3.4 mL, 40 mmol) was added dropwise to the solution of compound **9** (1.77 g, 10 mmol) in dry toluene (20 mL). After stirring for 45 min, triethylamine hydrochloride (2.1 mL, 15 mmol) and DMF (3d) was added by small portions to the mixture, and was then stirred for 2 days. The reaction mixture was concentrated and the residue was purified by silica gel column chromatography (from 10% to 30% EtOAc in petroleum ether) to afford substituted 3, 5-dichloropyrazin-2(1*H*)-one **10**.

4.1.3. General procedure for the synthesis of the intermediates 11a,b

A flask was charged with 3, 5-dichloropyrazin-2(*1H*)-one **10** (2.85 g, 10 mmol), anilines (12 mmol), TFA (0.08 mL, 1.05 mmol), and 2-BuOH (10 mL). The slurry was heated to 100 °C and maintained at this temperature for 12 h. Then, the reaction mixture was allowed to cool to room temperature and was neutralized with a saturated aqueous sodium bicarbonate solution. The aqueous mixture was extracted with CH_2Cl_2 (20 mL) three times and concentrated to obtain the crude product. The crude product was directly used in the next step without further purification.

4.1.4. General procedure for the synthesis of the intermediates 12a,b

Compound **11** (10 mmol) was dissolved in methanol (30 mL) and water (30 mL). Iron powder (1.68 g, 30 mmol) and ammonium chloride (2.67 g, 50 mmol) were added, and the resulting mixture was heated to 70 °C and stirred for 3 h. The reaction mixture was cooled to room temperature and filtered through Celite. The methanol solvent was removed in vacuo, and the resulting residue was basified with sodium bicarbonate and extracted with dichloromethane (20 mL) three times. The organic layer was separated, dried using anhydrous sodium sulfate, concentrated, and directly used in the subsequent step.

4.1.5. General procedure for the synthesis of the title compounds 7a-c and intermediate 13a

Acetyl chloride (0.257 g, 12 mmol) or acryloyl chloride (1.09 g, 12 mmol) was added dropwise to a solution of compound **12a,b** (0.814 g, 3.18 mmol) and triethylamine (2.42 g, 24 mmol) in methylene chloride (20 mL) at 0 °C. The reaction was stirred for 1 h, and then was concentrated. The residue was purified by silica gel preparative TLC using dichloromethane/methanol (20:1) as developing solvents to prepare the title molecules **7a-c**.

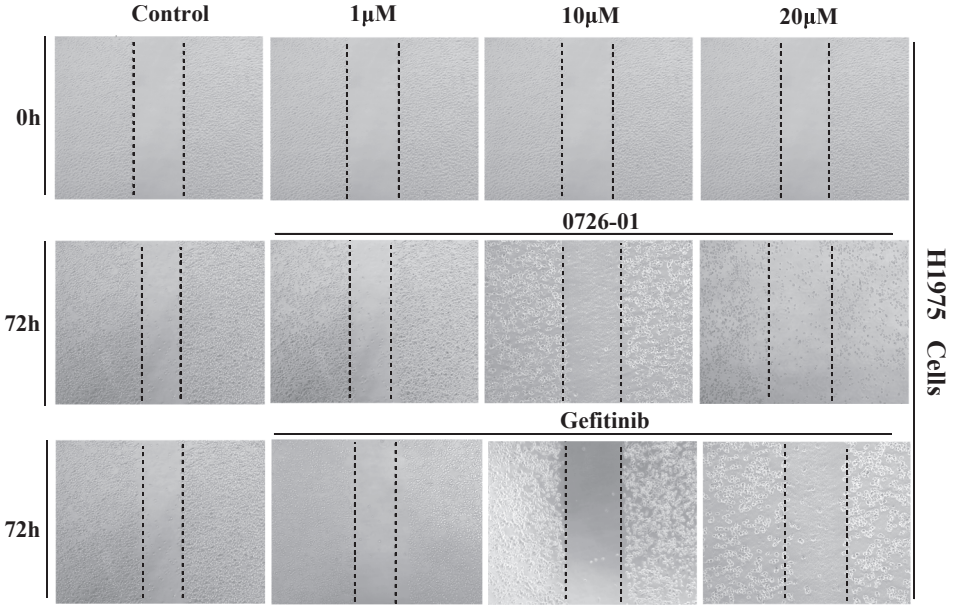

Fig. 3. Representative images of H1975 cells treated with different concentrations of **7b** for 0 and 72 h by the wound-healing assay.

4.1.6. General procedure for the synthesis of the title compounds 7a,b and 7d,e

Compound **14a** (490 mg, 10 mmol), potassium carbonate (2.76 g, 20 mmol), and morpholine (1.31 g, 15 mmol) were added to 20 mL of acetone. The resulting mixture was heated to reflux for 4 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was collected and concentrated in vacuo. The residue was purified by silica gel preparative TLC using dichloromethane/methanol (20:1) as developing solvents to produce the desired compounds **7d,e**.

4.1.6.1. (7a)N-(3-(3-(2-(acetylamino)phenylamino-5-chloro-2-oxo-1(2H)-pyrazinyl)phenyl)acrylamide. Yield: 53.1%; off-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.84 (s, 1H), 10.14 (s, 1H), 8.51 (d, J = 8.2 Hz, 1H), 8.41 (d, J = 4.4 Hz, 1H), 7.63 (s, 1H), 7.45 (d, J = 8.0 Hz, 2H), 7.29 (s, 1H), 7.19 (d, J = 8.0 Hz, 1H), 7.03 (s, 1H), 6.94 (d, J = 8.0 Hz, 1H), 6.85 (t, J = 7.4 Hz, 1H), 6.26 – 6.09 (m, 1H), 6.00 (dd, J = 17.0, 1.8 Hz, 1H), 5.50 (dd, J = 10.0, 1.8 Hz, 1H), 3.10 – 3.04 (m, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 168.94, 163.87, 150.62, 147.53, 140.13, 139.76, 139.34, 132.30, 132.07, 130.01, 128.62, 127.91, 124.21, 122.56, 121.60, 121.49, 119.84, 119.78, 117.53, 116.84, 26.65; HRMS (ESI) for $C_{21}H_{18}ClN_5O_3$, [M + H] + calcd: 424.1098; found: 424.1114.

4.1.6.2. (7b)N-(3-(3-(4-(1-morpholino)methyl)phenylamino-5-chloro-2-oxo-1(2H)-pyrazinyl) phenyl)acrylamide. Yield: 68.5%; off-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.44 (s, 1H), 9.65 (s, 1H), 8.01 – 7.77 (m, 3H), 7.70 (d, J = 7.6 Hz, 1H), 7.50 (t, J = 8.0 Hz, 1H), 7.34 – 7.21 (m, 4H), 6.55 – 6.37 (m, 1H), 6.29 (d, J = 16.6 Hz, 1H), 5.89 – 5.69 (m, 1H), 3.59 (m, 4H), 2.39 (m, 4H), 1.23 (s, 2H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 163.88, 150.43, 147.77(2C), 140.24, 139.91, 138.43, 132.08, 130.07, 129.82, 127.94, 124.60, 121.73, 120.53(4C), 119.83, 117.50, 116.14, 66.54, 62.34, 53.49, 29.50; HRMS (ESI) for $C_{24}H_{24}ClN_5O_3$, [M + H] + calcd: 466.1568; found:466.1713.

4.1.6.3. (7c)N-(3-(3-(2-(acetylamino)phenylamino-5-chloro-2-oxo-1(2H)-pyrazinyl) phenyl) acetamide. Yield: 75.9%; off-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.12 (s, 1H), 10.21 (s, 1H), 8.79 (d, J = 8.2 Hz, 1H), 8.70 (d, J = 4.4 Hz, 1H), 7.82 (s, 1H), 7.74 (dd, J = 7.8, 1.2 Hz, 1H), 7.63–7.54 (m, 2H), 7.46 (s, 1H), 7.30 (s, 1H), 7.20–7.12 (m, 2H), 2.79 (d, J = 4.6 Hz, 3H), 2.07 (s, 3H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 171.93, 169.10, 168.93, 150.61, 147.52, 140.43, 139.72, 139.33, 132.29, 129.90, 128.61, 124.16, 121.49, 121.10, 119.77, 119.46, 117.10, 116.88, 26.65, 22.98; HRMS (ESI) for $C_{20}H_{18}ClN_5O_3$, [M + H] + calcd:412.1098; found:412.1115.

4.1.6.4. (7d)N-(3-(3-(2-(acetylamino)phenylamino-5-chloro-2-oxo-1(2H)-pyrazinyl) phenyl) -(1-morpholino)acetamide. Yield: 70.1%; off-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.06 (s, 1H), 8.22 (d, J = 7.8 Hz, 1H), 7.90 (d, J = 11.2 Hz, 4H), 7.70 (s, 2H), 7.55–7.40 (m, 2H), 7.27–7.16 (m, 2H), 4.61 (s, 2H), 4.07 (d, J = 4.8 Hz, 3H), 3.64 (s, 4H), 1.23 (s, 4H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 170.68, 158.73, 154.78, 145.70, 142.30, 139.91, 139.54, 135.74, 135.24, 129.95, 129.18, 128.68, 126.93, 121.76, 121.67, 120.36, 119.76, 117.78, 108.75, 66.45, 60.50, 53.57, 44.58, 42.11; HRMS (ESI) for $C_{24}H_{25}ClN_6O_4$, [M + H] + calcd:497.1626; found:497.1675.

4.1.6.5. (7e)N-(3-(3-(2-(acetylamino)phenylamino-5-chloro-2-oxo-1(2H)-pyrazinyl) phenyl) - (4-hydroxy-1-piperidinyl)acetamide. Yield: 65.6%; off-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.95 (s, 1H), 8.23 (s, 1H), 8.21 (s, 1H), 7.97 (s, 2H), 7.92 (s, 2H), 7.88 (s, 1H), 7.70 (s, 1H), 7.50 (s, 1H), 7.25 (s, 1H), 7.20 (d, J = 7.8 Hz, 2H), 4.02 (d, J = 5.2 Hz, 2H), 3.54 (s, 4H), 1.75 (s, 4H), 1.23 (s, 3H), 0.84 (d, J = 6.6 Hz, 1H); ^{13}C NMR (400 MHz, DMSO-d_6): δ 158.73, 154.78, 145.70(2C), 142.32, 139.83, 139.56, 135.74(2C), 130.00, 129.19, 128.68, 126.93, 121.67, 120.36, 119.69, 117.74, 108.75(2C), 66.21, 60.64, 51.36, 42.60, 32.53, 29.49; HRMS (ESI) for $C_{25}H_{27}ClN_6O_4$, [M + H] + calcd:511.1782; found:511.1420.

4.2. Biological evaluation method

4.2.1. Cell culture and reagents

NSCLC cells (H1975, A431, and A549), and human normal hepatocyte cells (LO2) were obtained from Fuheng Biology Company (Shanghai, China). The MTT was obtained from Biotool Company (Switzerland). The wild-type EGFR, and mutant EGFR L858R/T790M kinase assay systems were purchased from Promega Corporation (USA). H1975, HCC827 and A549 cells were grown in RPMI-1640 (Gibco®, USA) supplemented with 10% FBS (Gibco®, USA), 1% penicillin-streptomycin (Beyotime Company, China). A431 and LO2 cells were grown in DMEM (Gibco®, USA) supplemented with 10% FBS (Gibco®, USA), 1% penicillin-streptomycin (Beyotime Company, China). All cells were

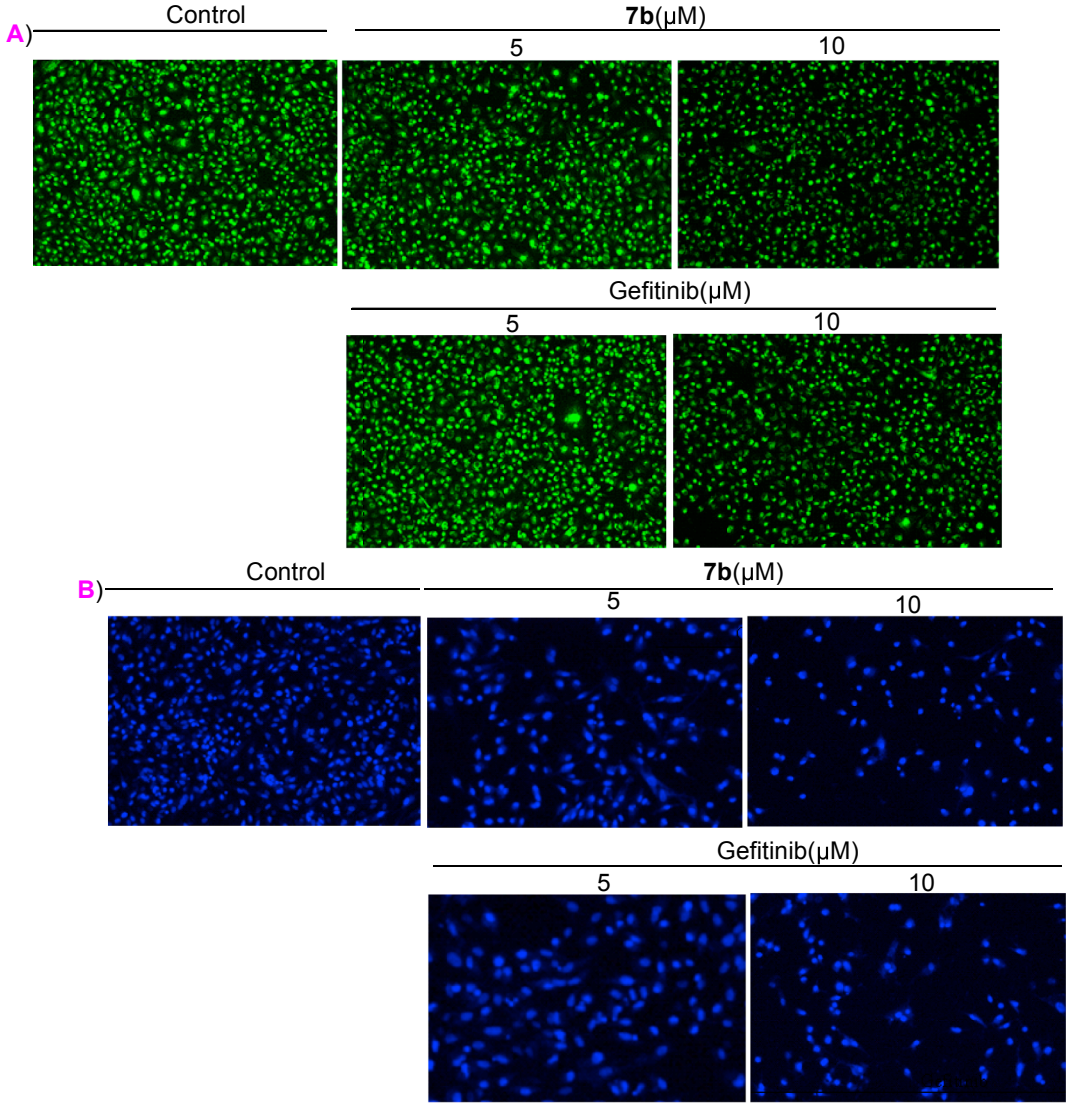

Fig. 4. Morphological changes of H1975 cells (100 ×, final magnification, (**A**): AO/EB (Acridine Orange/Ethidium Bromide) double fluorescent staining of H1975 cells treated with different concentrations of gefitinib and **7b** for 72 h; (**B**): DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) staining of H1975 cells treated with different concentrations of gefitinib and **7b** for 72 h.

maintained and propagated as monolayer cultures at 37 °C in humidified 5% CO_2 incubator.

4.2.2. Kinase enzymatic assays

The experiments were generally performed according to the instructions of the manufacturer. Concentrations consisting of suitable levels from 10 to 1000 nM were used for all of the tested compunds. The more detailed and complete protocols, see the ADP-Glo™ kinase Assay Technical Manual #313, and the active kinase datasheet available at: http://www.promega.com/tbs/tm313/tm313/tm313.html and http://www.promega.com/KESProtocol (or http://www.promega.com/tbs/signaling.htm), respectively. The test was performed in a 384-well plate, and includes the major steps below: (1) perform a 5 μL kinase reaction using 1 × kinase buffer (e.g., 1 × reaction buffer A), (2) incubate at room temperature for 60 min, (3) add 5 μL of ADP-Glo™ Reagent to stop the kinase reaction and deplete the unconsumed ATP, leaving only ADP and a very low background of ATP, (4) incubate at room temperature for 40 min, (5) add 10 μL of Kinase Detection, (6) reagent to convert ADP to ATP and introduce luciferase and luciferin to detect ATP, (7) incubate at room temperature for 30 min, (8) plate was measured on TriStar® LB942 Multimode Microplate Reader (BERTHOLA) to detect the luminescence (Integration time 0.5–1 s). Curve fitting and data presentations were performed using Graph Pad Prism version 5.0.

4.2.3. Cell growth inhibitory activity

4.2.3.1. MTT assay. The cells were seeded at a density of 1 to 5 × 10^3 cells/mL in 96-well plates in growth medium supplemented with 10% serum at 37 °C with 5% CO_2 for one day. After 12 h of incubation, 100 μL of medium was removed, and 100 μL of sample solution with different concentrations of inhibitor was added and then the cells were incubated for 48 or 72 h. Subsequently, 10 μL of MTT reagent (Biotool Company, 5.0 mg/mL) dissolved in phosphate-buffered saline (PBS) was added and the cells were incubated for another 4 h. The absorbance was read at 570 nm with a microplate reader (Thermo, USA). The data were calculated using GraphPad Prim version 5.0.

4.2.3.2. Wound-healing assay. The cancer cells were cultured in 6-well plates for 48 h at 37 °C. Wounds were created in the cell monolayer and washed with PBS to remove cell debris, then the cells were treated with different concentrations of inhibitor for 72 h. After that, the dead cells were washed away with PBS, and the images were taken by the inverted microscope (OLYMPUS, Japan).

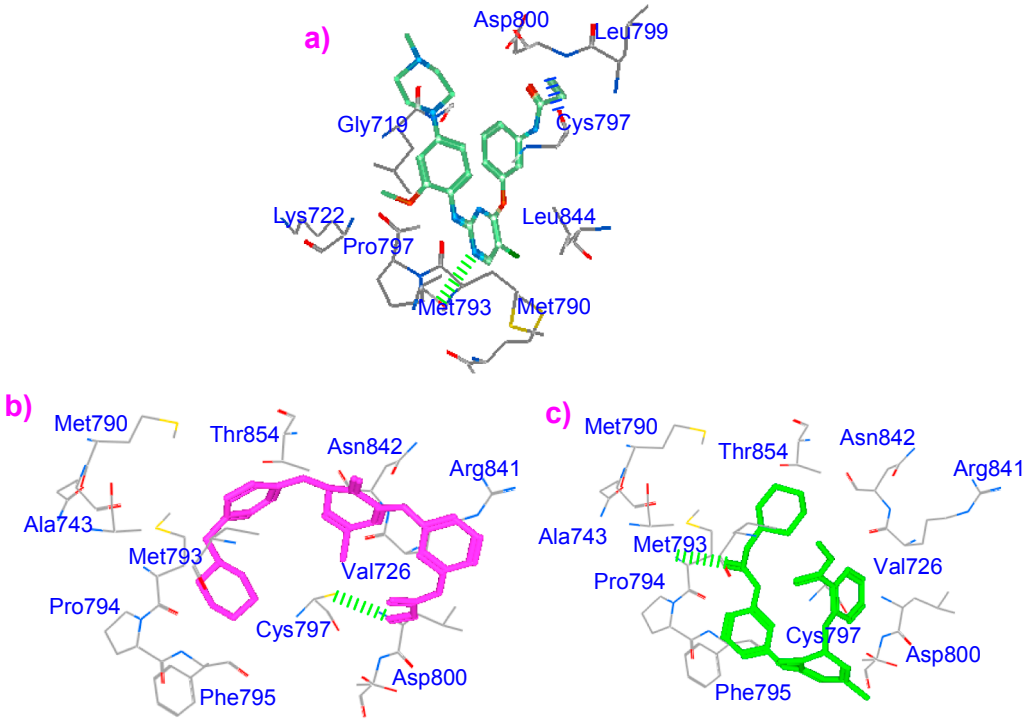

Fig. 5. (a) Binding sites of WZ4002 within EGFR^{T790M} (PDB code: 4I22) [11]; (b) Putative binding modes of pyrimidinone **7b**; (c) Putative binding modes of pyrimidinone **7e** with EGFR^{T790M} enzyme.

4.2.3.3. AO/EB and DAPI staining assay. Approximately 2×10^5 cells/well of H1975 cells in 6-well plate were incubated in an incubator for 12 h, then treated with different concentrations of inhibitors for 72 h. After incubation, the cells were washed with PBS for two times. Then, total of 20 μL of the solution containing the AO/EB dye mix (1.0 μg/mL of AO and 1.0 μg/mL of EB in PBS) was added to the cells. The apoptotic, necrotic and live cells were observed and counted under the fluorescent inverted microscope (OLYMPUS, Japan). DAPI staining was performed after being treated as mentioned above. The cells plated in 6-well plates were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min, then washed with PBS for three times. Cells were subsequently incubated in DAPI (1.0 μg/mL) solution at room temperature for 15 min, then examined under the fluorescent inverted microscope (OLYMPUS, Japan).

4.3. Molecular modeling

AutoDock 4.2 software was used for docking studies. Most of the -parameters were seted as default. The crystal structure (PDB: 3IKA) of the kinase domain of EGFR^{T790M} bound to inhibitor **4** was used in the docking studies. The enzyme preparation and the hydrogen atoms adding was performed in the prepared process. The binding interaction energy was calculated to include van der Waals, electrostatic, and torsional energy terms defined in the tripos force field. The WWW site also includes many resources for use of AutoDock, including detailed Tutorials that guide users through basic AutoDock usage, docking with flexible rings, and virtual screening with AutoDock. Tutorials may be found at: http://autodock.scripps.edu/faqs-help/tutorial.

Acknowledgements

We are grateful to the National Natural Science Foundation of China, China (No. 81672944), Research Fund of Higher Education of Liaoning province, China (LQ2017008, LQ2017039), the National Natural Science Foundation of Liaoning province, China (20180530066), the Cultivation Project of Youth Techstars, Dalian, China (No. 2016RQ043, 2017RQ066), the Natural Science Foundation of Science and Technology Department of Jiangxi Province, China (No. 20171BBE50027, 20171BBG70029), the Natural Science Foundation of Education Department of Jiangxi Province, China (No. GJJ170275), and the Natural Science Foundation of Nanchang City, China (No. 2018CXTD014) for the financial support of this research.

References

[1] J.R. Molina, P. Yang, S.D. Cassivi, S.E. Schild, A.A. Adjei, Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship, Mayo. Clin. Proc. 83 (2008) 584–594.
[2] S.V. Sharma, D.W. Bell, J. Settleman, D.A. Haber, Epidermal growth factor receptor mutations in lung cancer, Nat. Rev. Cancer 7 (2007) 169–181.
[3] J. Vansteenkiste, Gefitinib (Iressa): a novel treatment for non-small cell lung cancer, Expert. Rev. Anticancer. Ther. 4 (2004) 5–17.
[4] P. Bonomi, Erlotinib: a new therapeutic approach for non-small cell lung cancer, Expert. Opin. Investig. Drugs. 12 (2003) 1395–1401.
[5] Z.D. Song, Y. Ge, C.Y. Wang, S.S. Huang, X.H. Shu, K.X. Liu, Y.W. Zhou, X.D. Ma, Challenges and perspectives on the development of small-molecule EGFR inhibitors against T790M-mediated resistance in non-small-cell lung cancer, J. Med. Chem. 59 (2016) 6580–6594.
[6] S. Kobayashi, T.J. Boggon, T. Dayaram, P.A. Jänne, O. Kocher, M. Meyerson, B.E. Johnson, M.J. Eck, D.G. Tenen, B. Halmos, EGFR mutation and resistance of non-small-cell lung cancer to gefitinib, N. Engl. J. Med. 352 (2005) 786–792.
[7] W. Pao, V.A. Miller, K.A. Politi, G.J. Riely, R. Somwar, M.F. Zakowski, M.G. Kris, H. Varmus, Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain, Plos. Med. 2 (2005) e73.
[8] E.L. Kwak, R. Sordella, D.W. Bell, N. Godin-Heymann, R.A. Okimoto, B.W. Brannigan, P.L. Harris, D.R. Driscoll, P. Fidias, T.J. Lynch, S.K. Rabindran, J.P. McGinnis, A. Wissner, S.V. Sharma, K.J. Isselbacher, J. Settleman, D.A. Haber, Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib, Proc. Natl. Acad. Sci. USA 102 (2005) 7665–7670.
[9] C.H. Yun, K.E. Mengwasser, A.V. Toms, M.S. Woo, H. Greulich, K.K. Wong, M. Meyerson, M.J. Eck, The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP, P. Natl. Acad. Sci. USA 105 (2008) 2070–2075.
[10] N. Minkovsky, A. Berezov, BIBW-2992, a dual receptor tyrosine kinase inhibitor for the treatment of solid tumors, Curr. Opin. Investig. Drugs 9 (2008) 1336–1346.
[11] W. Zhou, D. Ercan, L. Chen, C.H. Yun, D. Li, M. Capelletti, A.B. Cortot, L. Chirieac, R.E. Iacob, R. Padera, J.R. Engen, K.K. Wong, M.J. Eck, N.S. Gray, P.A. Jänne, Novel mutant-selective EGFR kinase inhibitors against EGFR T790M, Nature 462 (2009) 1070–1074.
[12] A.O. Walter, R.T. Sjin, H.J. Haringsma, K. Ohashi, J. Sun, K. Lee, A. Dubrovskiy,

M. Labenski, Z. Zhu, Z. Wang, M. Sheets, T. St Martin, R. Karp, D. Van Kalken, P. Chaturvedi, D. Niu, M. Nacht, R.C. Petter, W. Westlin, K. Lin, S. Jaw-Tsai, M. Raponi, T. Van Dyke, J. Etter, Z. Weaver, W. Pao, J. Singh, A.D. Simmons, T.C. Harding, A. Allen, Discovery of a mutant-selective covalent inhibitor of EGFR that overcomes T790M-mediated resistance in NSCLC, Cancer. Discov. 3 (2013) 1404–1415.

[13] M.R. Finlay, M. Anderton, S. Ashton, P. Ballard, P.A. Bethel, M.R. Box, R.H. Bradbury, S.J. Brown, S. Butterworth, A. Campbell, C. Chorley, N. Colclough, D.A. Cross, G.S. Currie, M. Grist, L. Hassall, G.B. Hill, D. James, M. James, P. Kemmitt, T. Klinowska, G. Lamont, S.G. Lamont, N. Martin, H.L. McFarland, M.J. Mellor, J.P. Orme, D. Perkins, P. Perkins, G. Richmond, P. Smith, R.A. Ward, M.J. Waring, D. Whittaker, S. Wells, G.L. Wrigley, Discovery of a potent and selective EGFR inhibitor (AZD9291) of both sensitizing and T790M resistance mutations that spares the wild type form of the receptor, J. Med. Chem. 57 (2014) 8249–8267.

[14] D.A. Cross, S.E. Ashton, S. Ghiorghiu, C. Eberlein, C.A. Nebhan, P.J. Spitzler, J.P. Orme, M.R. Finlay, R.A. Ward, M.J. Mellor, G. Hughes, A. Rahi, V.N. Jacobs, M.R. RedBrewer, E. Ichihara, J. Sun, H. Jin, P. Ballard, K. Al-Kadhimi, R. Rowlinson, T. Klinowska, G.H. Richmond, M. Cantarini, D.W. Kim, M.R. Ranson, W. Pao, AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer, Cancer. Discov. 4 (2014) 1046–1061.

[15] L. Wang, J. Zhao, Y. Yao, C. Wang, J. Zhang, X. Shu, X. Sun, Y. Li, K. Liu, H. Yuan, X. Ma, Covalent binding design strategy: a prospective method for discovery of potent targetedanticancer agents, Eur. J. Med. Chem. 142 (2017) 493–505.

[16] T. Grabe, J. Lategahn, D. Rauh, C797S resistance: the undruggable EGFR mutation in non-small cell lung cancer? ACS. Med. Chem. Lett. 9 (2018) 779–782.

[17] Z.D. Song, S.S. Huang, H.Q. Yu, Y. Jiang, C.Y. Wang, Q. Meng, X.H. Shu, H.J. Sun, K.X. Liu, Y.X. Li, X.D. Ma, Synthesis and biological evaluation of morpholine-substituted diphenylpyrimidine derivatives (Mor-DPPYs) as potent EGFR T790M inhibitors with improved activity toward the gefitinib-resistant nonsmall cell lung cancers (NSCLC), Eur. J. Med. Chem. 133–329 (2017).

[18] I.E. Francois, B.P. Cammue, S. Bresseleers, H. Fleuren, G. Hoornaert, V.P. Mehta, S.G. Modha, E.V. Van der Eycken, K. Thevissen, Synthesis and fungicidal activity of 3,5-dichloropyrazin-2(1*H*)-one derivatives, Bioorg. Med. Chem. Lett. 19 (2009) 4064–4066.

[19] J. Gising, P. Ortqvist, A. Sandström, M. Larhed, A straightforward microwave method for rapid synthesis of *N*-1, *C*-6 functionalized 3,5-dichloro-2(1*H*)-pyrazinones, Org. Biomol. Chem. 7 (2009) 2809–2815.

[20] S. Goueli, K. Hsiao, G. Vidugiris, J. Hennek, H. Zegzouti, ADP-GloTM: a one platform ideal for assay development, screening and profiling of all phosphotransferases, J. Bone. Joint. Surg. Am. 4 (2006) 122–137.

[21] H. Zegzouti, M. Zdanovskaia, K. Hsiao, S.A. Goueli, ADP-Glo: a bioluminescent and homogeneous ADP monitoring assay for kinases, Assay. Drug. Dev. Technol. 7 (2009) 560–572.

[22] S.A. Wildman, X. Zheng, D. Sept, J.T. Auletta, T.L. Rosenberry, G.R. Marshall, Drug-like leads for steric discrimination between substrate and inhibitors of human acetylcholinesterase, Chem. Biol. Drug Des. 78 (2011) 495–504.

[23] G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D.S. Goodsell, A.J. Olson, AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility, J. Comput. Chem. 30 (2009) 2785–2791.