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Original article

Synthesis and evaluation of 2-anilinopyrimidines bearing 3-aminopropamides as potential epidermal growth factor receptor inhibitors

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ABSTRACT

Novel compounds **12a**–**i** were synthesized and biologically evaluated. Several ones exhibited stronger inhibitory activity than gefitinib against EGFR L858R/T790M and antiproliferative effects on H1975 and HCC827 cells. The 3-aminopropamide in compounds like **12h** could be converted to the active acrylamide in the presence of arginine. Importantly, **12h** showed improved stability relative to compound **1** whose structure is same to **12h** excepting an acrylamide moiety. Interestingly, **12i**, a NO donating compound of **12h**, showed more potent and selective inhibition than **12h** on H1975 cells. Significantly, **12i** produced high levels of NO in H1975 cells but not in non-tumorous 16HBE cells, and its inhibition was diminished by NO scavenger. Furthermore, **12i** dose-dependently produced inhibitory effects on EGFR downstream signaling in H1975 cells.

1. Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related death worldwide [1]. The NSCLC patients at large present with advanced, unresectable or metastatic disease, and a 5-year survival rate of less than approximately 15% [2]. Therefore, novel therapeutic strategies including safe and potent anti-NSCLC drugs are urgently needed.

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptors [3], which is involved in various events of cancer cell proliferation, survival, adhesion, migration, and differentiation through downstream signaling pathway activation by autophosphorylation of several tyrosine residues after EGFR dimerization [4]. Hyperactivation of EGFR signaling pathway has been found in more than half of NSCLCs due to gene amplification or EGFR mutation [5] and plays an indispensable role in the pathogenesis of NSCLC [6]. In this context, EGFR tyrosine kinase inhibitors (TKIs) have been emerging as one of the effective and targeting treatment strategies for NSCLC [6].

Gefitinib and erlotinib (Fig. 1), the first generation reversible EGFR TKIs, are initially effective in the treatment of NSCLC patients with a short in-frame deletion of exon 19 (del E746_A750) and the L858R point mutation in exon 21 in the EGFR kinase domain [7]. However, the development of resistance has limited their clinical efficacy, which is believed to be associated with a conversion of the threonine gatekeeper to a methionine (T790M) in the catalytic domain of EGFR in about 50% of cases [8,9]. This mutation restores the affinity for ATP similar to that of wide type (WT) EGFR, and prevents reversible inhibitors from binding at higher ATP concentrations [10]. The second generation irreversible EGFR TKIs include

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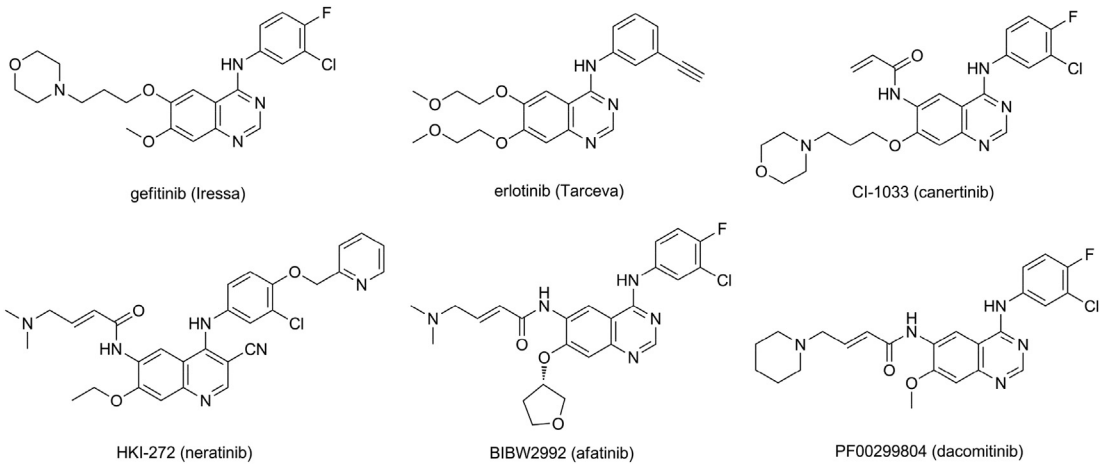


Fig. 1. Chemical structures of EGFR TKIs with a quinazoline or quinoline core scaffold.

CI-1033 [11], BIBW2992 [12], HKI-272 [13], PF00299804 [14] (Fig. 1), etc, which are able to inhibit T790M-mutant EGFR in vitro since an electrophilic functionality (acrylamide group) in the molecules could undergo a Micheal addition reaction with a conserved cysteine residue present in EGFR (Cys797) to achieve occupancy greater than that of the reversible inhibitors [15]. Nevertheless, the dose-limiting and off-target toxicity such as diarrhea and skin rash, which are caused by unselective inhibition of WT EGFR, limit the clinical efficacy of these second generation EGFR TKIs [11–14,16].

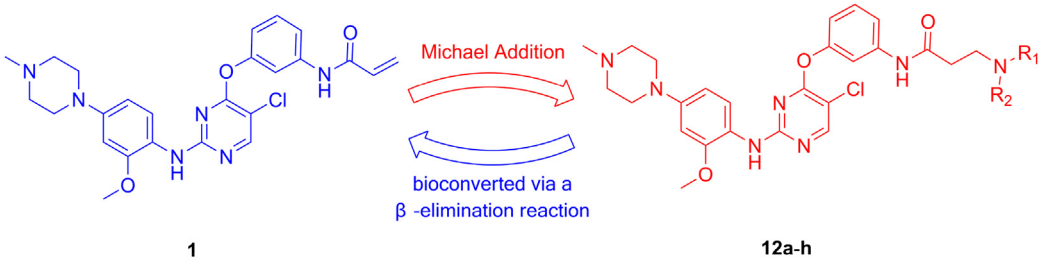
To circumvent this problem, the third generation of irreversible EGFR TKIs such as WZ4002 (**1**, Scheme 1) with better selectivity against mutant EGFR T790M over WT EGFR, was investigated [17]. Compound **1** is built based on an anilinopyrimidine driving portion which is different from the first and second generation EGFR TKIs with a quinazoline or quinoline core scaffold. Accordingly, the anilinopyrimidine is supposed to be an attracting scaffold for the design of small molecule inhibitors against EGFR mutants. Like the second generation inhibitors, compound **1** contains an acrylamide group which could covalently bind to the Cys797 of EGFR T790M to generate inhibitory activity [17]. However, the high reactivity of the acrylamide group may cause rapid metabolism or nonspecific reaction with off targets, lowering the bioavailability and efficiency [18,19]. In this regard, a protection strategy over the warhead acrylamide could be beneficial. It is known that the β-aminoethyl ketones could be bioconverted via a β-elimination reaction to the α,β-unsaturated ketones, covalently modifying their biological targets [20,21]. Accordingly, Carmi et al. masked the acrylamide warhead of the irreversible inhibitors containing a quinazoline or quinoline driving portion to produce a Mannich base, 3-aminopropamide. The resulting compounds exhibited enhanced stability towards small thiols in vitro, and importantly, could undergo a β-elimination (retro-Michael addition) reaction to liberate the acrylamide which can react with thiols in the intracellular environment [20].

Nitric oxide (NO), a signaling and effector molecule, plays a pivotal role in diverse and important physiological and pathophysiological processes [22]. It is generally believed that high levels of NO generated from NO-donors can not only inhibit tumor cell proliferation and survival, and induce apoptosis in sensitive tumor cells, but also sensitize resistant tumor cells to chemotherapy, radiation and immunotherapy as well as inhibit metastasis in vitro and vivo [23]. Recently, we investigated phenylsulfonylfuroxan-based NO-releasing compounds as potent and selective EGFR inhibitors for intervention of NSCLC [24,25].

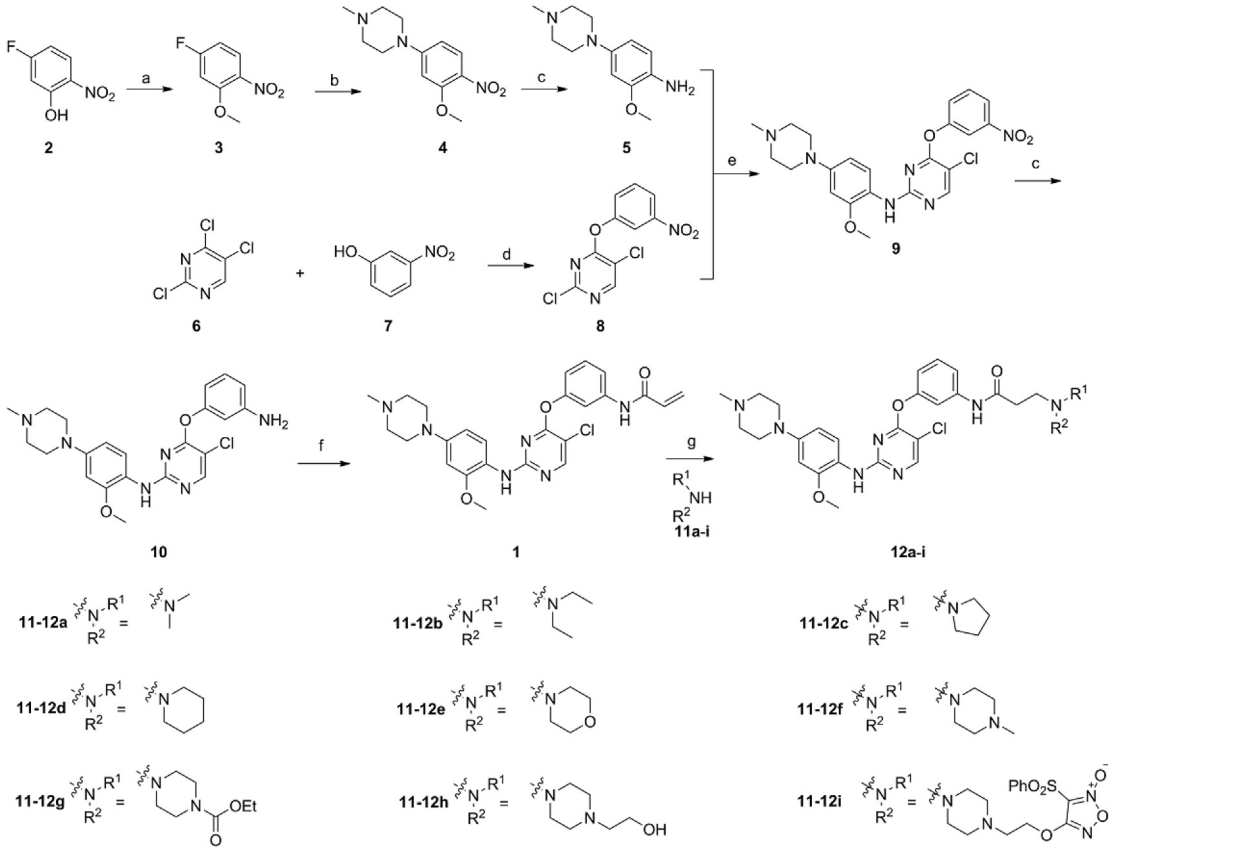
As part of our ongoing program to study the novel irreversible EGFR TKIs, we designed and synthesized a group of irreversible EGFR inhibitors **12a**–**h** containing 2-anilinopyrimidine as a scaffold and 3-aminopropamide as the "protected" warhead (Scheme 1). Additionally, the NO-donating modification strategy was applied for compound **12h** yielding **12i** as comparison in efficacy. Biological studies including kinase inhibitory activity, antiproliferative activity against several NSCLC cells, possible mechanism of action, and NO release property for **12i**, were conducted and discussed.

2. Chemistry

Compounds **12a**–**i** were synthesized as depicted in Scheme 2. Starting from compound **2**, a methylation reaction using methyl iodide and a following amination at 5-position by N-methylpiperazine furnished methylphenyl ether **4**, which underwent a nitro-reduction to produce aniline **5**. 3-Nitrophenol **7** was



Scheme 1. Rationale for the design of 2-anilinopyrimidines **12a**–**h**.



Scheme 2. Synthesis of the compounds **12a**–**i**. *Reagents and conditions*: (a) CH_3I, K_2CO_3, acetone, reflux, 4 h. (b) *N*-methyl-piperazine, K_2CO_3, DMSO, rt, overnight. (c) iron powder, NH_4Cl, THF/H_2O (1/1, v/v), reflux, 3 h. (d) K_2CO_3, DMF, rt, 2 h. (e) **5**, TFA, 2-BuOH, reflux, 3 h. (f) Acryloyl chloride, Et_3N, anhydrous CH_2Cl_2, 0 °C, 1 h. (g) **11a**–**i**, THF/MeOH, reflux, 4 h.

regioselectivity coupled to the 4-position of 2,4,5-trichloropyrimidine **6** to afford aryl ether **8** at the room temperature. Coupling reaction of **5** with **8** was accomplished in the presence of trifluoroacetic acid under reflux, offering anilinopyrimidine **9**. The nitro group in **9** was reduced by iron powder/NH_4Cl to produce arylamine **10**. Acylation of anilino group of **10** using acryloyl chloride gave compound **1**. The Compounds **12a**–**i** were obtained though Michael addition reaction of **1** with various secondary amine in THF/MeOH under reflux.

3. Results and discussion

3.1. Biological evaluation of *12a–h*

3.1.1. Kinase inhibitory activity

The enzyme inhibitory activity against different types of EGFR (EGFR WT, EGFR L858R, EGFR L858R/T790M) of the target compounds **12a**–**h**, and positive controls gefitinib and **1,** were evaluated using a well established FRET-based Z′-Lyte assay (Table 1) [26,27]. Among them, compounds **12a**–**c** and **12g** exhibited reasonable and selective inhibitory activity against EGFR L858R/T790M over WT EGFR and EGFR L858R as compound **1** did. However, their ability to inhibit EGFR L858R/T790M (IC_{50} = 0.184, 0.181, 0.088 and 0.537 μM, respectively) was lower than **1** (IC_{50} = 0.0007 μM), but superior to gefitinib (IC_{50} = 0.941 μM).

3.1.2. Inhibitory activity on gefitinib-sensitive HCC827 and resistant H1975 cells

The antiproliferative effects of **12a**–**h** on EGFR mutation NSCLC cell lines, including gefitinib-resistant H1975 cells harboring EGFR L858R/T790M and gefitinib-sensitive HCC827 cells bearing EGFR del E746_A750, were next determined by a MTT assay using gefitinib and **1** as positive controls. As shown in Table 2, compounds **12a**–**h** (IC_{50}s = 0.156–1.598 μM) produced stronger antiproliferative activity against H1975 cells than gefitinib (IC_{50} = 6.917 μM), but lower than **1** (IC_{50} = 0.046 μM). Among them, compounds **12a**–**d** showed stronger inhibitory activity against H1975 cells (IC_{50} = 0.156, 0.166, 0.268 and 0.178 μM, respectively) and HCC827 cells (IC_{50}s = 0.210–0.337 μM). One plausible explanation is that the capability of different 3-aminopropamides to

Table 1
In vitro enzymatic inhibitory activity of compounds **12a**–**i** against different types of EGFR.

Compound	EGFR IC_{50} (μM)[a]		
	WT	L858R	L858R/T790M
gefitinib	0.0001 ± 0.0002	0.0002 ± 0.0001	0.941 ± 0.073
1	0.0044 ± 0.0006	0.0048 ± 0.0009	0.0007 ± 0.0001
12a	0.638 ± 0.125	0.804 ± 0.264	0.184 ± 0.087
12b	0.877 ± 0.276	0.818 ± 0.348	0.181 ± 0.056
12c	0.525 ± 0.256	0.427 ± 0.198	0.088 ± 0.024
12d	10 ± 2.176	4.527 ± 0.241	2.177 ± 0.687
12e	>10	>10	>10
12f	7.442 ± 2.043	10 ± 1.923	4.706 ± 0.134
12g	0.698 ± 0.324	2.385 ± 0.645	0.537 ± 0.198
12h	2.388 ± 0.973	5.866 ± 0.003	2.419 ± 0.876
12i	0.201 ± 0.099	0.262 ± 0.075	0.263 ± 0.103

[a] EGFR activity assays were performed using the FRET-based Z′-Lyte assay according to the manufacturer's instructions. The compounds were incubated with the kinase reaction mixture for 1.5 h before measurement. The data were means ± SD of three independent experiments.

Table 2
Antiproliferative activity of compounds **12a**–**i** and **11i** against cells harboring a different status of EGFR.

Compound	IC_{50} (μM)[a]					
	H1975[b]	HCC827[c]	A431[d]	A549[e]	BEAS-2b[f]	16HBE[g]
gefitinib	6.917 ± 0.657	0.006 ± 0.002	4.485 ± 0.295	15.58 ± 2.143	13.94 ± 1.992	12.71 ± 3.675
1	0.046 ± 0.011	0.008 ± 0.003	1.108 ± 0.213	2.102 ± 0.584	1.811 ± 0.253	1.355 ± 0.321
12a	0.156 ± 0.034	0.266 ± 0.015	0.545 ± 0.078	0.923 ± 0.102	0.615 ± 0.058	1.143 ± 0.258
12b	0.166 ± 0.021	0.249 ± 0.009	0.291 ± 0.054	1.003 ± 0.032	0.433 ± 0.106	0.620 ± 0.182
12c	0.268 ± 0.043	0.210 ± 0.023	0.804 ± 0.137	1.213 ± 0.046	1.888 ± 0.782	1.129 ± 0.318
12d	0.178 ± 0.029	0.337 ± 0.098	0.410 ± 0.298	1.119 ± 0.092	0.365 ± 0.069	0.970 ± 0.047
12e	1.018 ± 0.125	0.899 ± 0.078	3.910 ± 0.329	2.622 ± 0.135	2.269 ± 0.831	3.596 ± 1.216
12f	1.042 ± 0.243	1.497 ± 0.328	0.548 ± 0.092	1.030 ± 0.289	0.412 ± 0.145	1.753 ± 0.152
12g	1.598 ± 0.296	0.883 ± 0.067	4.534 ± 0.296	5.283 ± 1.231	5.779 ± 1.039	3.705 ± 0.139
12h	0.931 ± 0.039	0.799 ± 0.049	0.632 ± 0.089	2.816 ± 0.498	1.657 ± 0.394	2.056 ± 0.284
12i	0.108 ± 0.029	0.130 ± 0.008	4.469 ± 0.541	8.251 ± 0.992	3.368 ± 1.290	2.876 ± 0.217
11i	5.656 ± 0.059	>10	3.058 ± 0.284	>10	19.77 ± 2.018	7.150 ± 1.219

[a] The inhibitory effects of individual compounds on the proliferation of cancer cell lines were determined by the MTT assay. The data were means ± SD from at least three independent experiments.
[b] H1975 is a human lung cancer cell line (EGFR L858R/T790M).
[c] HCC827 is a human lung cancer cell line (EGFR del E746_A750).
[d] A431 is human epithelial carcinoma cell line (overexpressed WT EGFR).
[e] A549 is a human lung cancer cell line (WT EGFR/*k*-RAS dependent).
[f] BEAS-2B are normal human bronchial epithelial cell lines.
[g] 16HBE are normal human bronchial epithelial cell lines.

liberate the acrylamide moiety in the physiological conditions might be correlated to their anti-proliferation activity. For example, **12a**–**d** with small secondary amine moieties showed stronger inhibitory effects than **12f**–**h** with bigger heterocycle amines, since the formers are more likely to undergo a β-elimination reaction to liberate the acrylamide moiety than latters.

3.1.3. Effects on the cell lines harboring WT EGFR

To examine how the target compounds **12a**–**h** affect both cancer cells and normal cells harboring WT EGFR, we determined their antiproliferative activity against following cell lines: human epithelial carcinoma cells A431 with overexpressed WT EGFR; NSCLC cells A549 possessing WT EGFR and *k*-Ras mutation; human normal bronchial epithelial cell lines BEAS-2B and 16HBE harboring WT EGFR. As shown in Table 2, most of compounds displayed less inhibition on the growth of the cell lines harboring WT EGFR than inhibition on that of H1975 and HCC827 cells.

3.1.4. Ability to liberate compound *1*

To verify whether the 3-aminopropamide moieties in the target compounds could be converted to the active acrylamide group, compounds **12a**, **12g** and **12h** were selected to test the ability to liberate compound **1** in the mimetic physiological conditions. After the three compounds (50 μM) were respectively incubated with arginine (1 mM) in PBS (pH = 7.4) for 72 h at 37 °C, the percentages of compound **1** were detected and determined using an HPLC-based method. As illustrated in Table 3, all three compounds could release compound **1** in the presence of arginine, demonstrating that the 3-aminopropamide could be converted to acrylamide via a β-elimination reaction induced by a natural basic amino acid under physiological conditions. Notably, treatment with more active compound **12a** produced higher levels of compound **1**, whereas less active compound **12g** produced lower levels of **1**, indicating that the ability of the tested compounds to liberate the free warhead was positively correlated with the inhibitory activity against H1975 cells in vitro ($R = 0.955$, $p < 0.05$, determined by Pearson's correlation analysis).

Table 3
Percentages of **1** liberated from **12a**, **12g** and **12h**.

Compound	Percent (%) of compound **1**[a]
12a	18.52 ± 0.42
12g	2.41 ± 0.80
12h	5.44 ± 0.37

[a] The compound **1** was detected by LC-UV after incubation in the presence of arginine (1 mM) at 37 °C for 72 h. Means ± SD, $n = 3$. The peak corresponding to **1** was collected and verified by HRMS.

3.1.5. Stability and reactivity of *12h*

Compound **12h** was selected as the prototype to test for its chemical (buffered solutions at pH 7.4 and 9.0) and biological (50% (v/v) rat plasma) stability, as well as for the activity against thiol nucleophiles with low molecular weight (LMW) [20]. As shown in Table 4, both of compounds **1** and **12h** were stable at pH 7.4, with over 99% of the parent compounds remaining after 24 h incubation at 37 °C in the buffer. Compound **1** was undetectable under the alkaline pH conditions (pH 9.0) after 24 h, while 81% of **12h** was recovered, with **1** (2.4%) as one of the degradation products. In the presence of rat plasma, **12h** showed much higher stability than **1** after incubation. The reactivity of **1** and **12h** towards thiol nucleophiles was assessed and the results are summarized in Table 5. While **1** was rapidly adducted by L-cysteine, glutathione (GSH) and dithiothreitol (DTT), compound **12h** kept its integrity under the same conditions, suggesting that these compounds with 3-aminopropamides might possess enhanced stability and reduced reactivity towards small thiol nuclephiles as compared with **1** in vitro.

3.2. Rationale for the design of NO-donating compound *12i*

Compound **12i** mentioned above was designed and synthesized based on the following reasons: i) Among the compounds **12a**–**h**, only **12h** with a free hydroxyl group was suitable for further NO-

Table 4
In vitro stability studies on **1** and **12h**.

Condition	Percent (%) of compound remaining (50 μM, 24 h)[a]	
	1	12 h
pH = 7.4	101.02 ± 1.42	99.48 ± 2.93
pH = 9.0	Below the limit of detection	81.39 ± 1.59
50% (v/v) rat plasma	Below the limit of detection	104.9 ± 3.07

[a] Percentages of test compounds remaining, detected by LC-UV after incubation for 24 h at 37 °C (see the Experimental Section). Means ± SD, $n = 3$.

Table 5
In vitro reactivity studies on compounds **1** and **12h**.

Condition	Percent (%) of compound remaining (50 μM, 1 h) [a]	
	1	12 h
GSH	73.52 ± 3.80	100.07 ± 0.23
DTT	11.32 ± 2.03	100.92 ± 1.96
L-cysteine	12.21 ± 2.67	101.19 ± 2.19

[a] Percentages of test compounds remaining, detected by LC-UV after incubation in the presence of LMW thiols (2 mM) for 1 h at pH 7.4, 37 °C (see the Experimental Section). Means ± SD, $n = 3$.

donating modification; ii) Although **12h** did not exhibit the strongest antiproliferative activity against H1975 and HCC827 cell lines relative to other compounds, it showed relatively low cell growth inhibitory effects on normal cells with WT EGFR (Table 2). Given that cancer cells are more sensitive to the high levels of NO compared to normal cells [28–31], we hypothesized such furoxan-based 3-aminopropamide **12i** may display strong antiproliferative activity selectively against H1975 and HCC827 cell lines.

3.3. Biological evaluation of *12i*

3.3.1. Kinase and cellular inhibitory activities of *12i*

Kinase and cellular inhibitory activities of **12i** were assayed and the results are summarized in Tables 1 and 2, respectively. It exhibited more potent enzyme inhibition over EGFR L858R/T790M (IC_{50} = 0.263 μM) than gefitinib (IC_{50} = 0.941 μM) and **12h** (IC_{50} = 2.419 μM). Furthermore, **12i** displayed stronger activity (IC_{50} = 0.108 and 0.130 μM) than its precursor **12h** (IC_{50} = 0.931 and 0.799 μM) against H1975 and HCC827 cell lines. Interestingly, **12i** exhibited lower cell growth inhibitory activity against cells with WT EGFR relative to **12h** (IC_{50}s range 2.876–8.251 μM for **12i**, 0.632–2.816 μM for **12h**, respectively), suggested that **12i** might possess a higher safety index.

3.3.2. Effects of NO on antiproliferative activity

From Table 2 it can be seen that **12h** (IC_{50} = 0.931 and 0.799 μM) and **11i** (IC_{50} = 5.656 and >10 μM), which are two moieties of **12i**, exhibited much less inhibitory activity than **12i** (IC_{50} = 0.108 and 0.130 μM) over H1975 and HCC827 cells, respectively, suggesting the synergic effects of anilinopyrimidine and NO donor moieties. To verify the contribution of NO to the inhibitory activity, the levels of NO released by compounds **12i**, **11i** and gefitinib were detected in vitro. NSCLC cells H1975 and normal 16HBE cells were respectively exposed to 100 μM of **12i**, **11i** or gefitinib for the same incubation time. The levels of NO produced by individual compounds in the cells are presented as that of nitrite, which was produced in the lysates of these cells and determined by the Griess assay (Fig. 2a) [32]. As expected, treatment with gefitinib resulted in little NO in H1975 and 16HBE cells. Treatment with **12i** promoted high levels of NO in H1975 cells, which was even greater than the group of furoxan compound **11i**. In contrast, NO was almost not detected in the cell lysates of 16HBE cells treated with **12i**. One plausible explanation was that better recognition capacity of **12i** to the EGFR mutation relative to **11i** could be helpful for cell penetration, resulting in selective and high NO production in H1975 cells.

Next, **12i** was tested on its antiproliferative effects in the presence or absence of a NO scavenger, hemoglobin. H1975 and HCC827 cells were pre-treated with, or without, different concentrations of hemoglobin for 1 h and then treated with 5 μM of **12i**. The effects of different treatments on the growth of H1975 and HCC827 cells were determined by the MTT assay (Fig. 2b). It was observed that treatment with **12i** alone remarkably inhibited the growth of H1975 and HCC827 cells and this inhibitory effect was diminished by pretreatment with hemoglobin in a dose-dependent manner. These results indicated that NO produced by **12i** played a significant role in the inhibition on NSCLC cell growth in vitro.

3.3.3. Effects on EGFR activation and downstream signaling

To investigate the mechanisms underlying the activity of NO-releasing 3-aminopropamide-based EGFR inhibitor, we examined the inhibitory effects of **12i** on EGFR activation and the downstream signal in H1975 cell line harboring EGFR L858R/T790M using gefitinib as a control. H1975 cells were treated with vehicle control, indicated doses of **12i**, or gefitinib. The expression and activation of EGFR-related signal events, Akt and ERK were determined by immunoblotting assays, and the results are shown in Fig. 3. Compound **12i** inhibited the phosphorylation of EGFR in a dose-dependent manner, and the treatment of **12i** at 0.1 μM

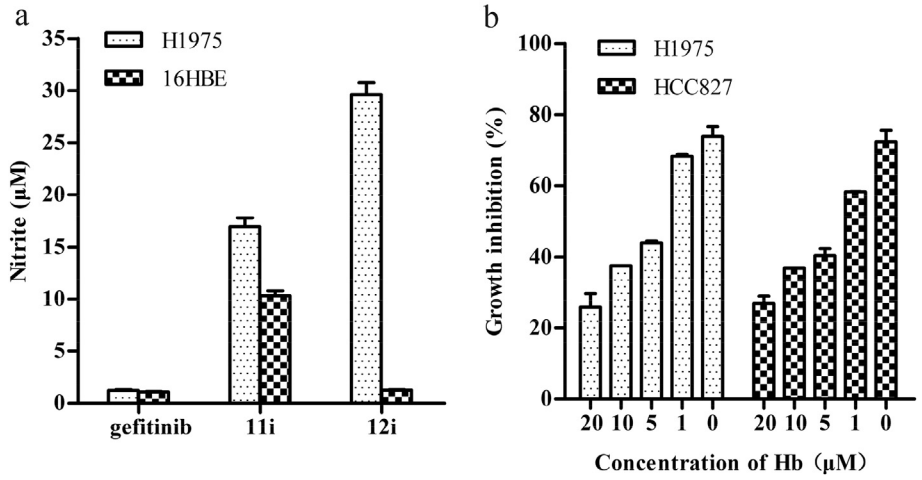


Fig. 2. Effects of NO production by the target compounds on NSCLC cell proliferation. (a) Variable levels of NO (presented as nitrite) produced by the indicated compounds. H1975 and 16HBE cells were treated with individual compounds at 100 μM for 150 min, and the concentrations of nitrite in the cell lysates were determined by Griess assay. The individual values were determined by measuring absorbance at 540 nm and calculated according to the standard curve. (b) Effects of hemoglobin on the antiproliferative effect of **12i**. H1975 and HCC827 cells were pretreated with the indicated concentrations of hemoglobin (Hb) (0, 1, 5, 10 or 20 μM) for 1 h and treated with 5 μM of **12i** for 24 h. The results were expressed as percent of cell growth inhibition relative to control cells. Data were expressed by means ± SD.

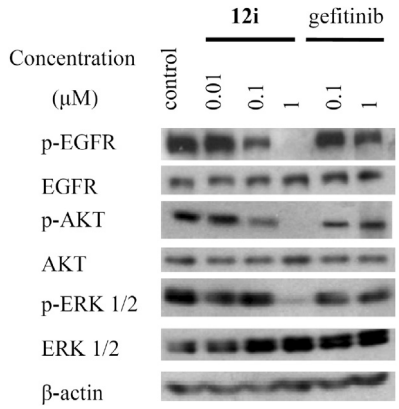


Fig. 3. Compound **12i** inhibit the activation of EGFR and downstream signaling in H1975 NSCLC cells harboring EGFR L858R/T790M.

significantly reduced the expression of phosphor-EGFR in our experimental condition. Importantly, the treatment of **12i** (1 μM) completely suppressed the expression of phosphor-EGFR, -Akt and -Erk, while gefitinib (1 μM) didn't show significant suppressive effects as compared to both vehicle control and **12i**.

4. Conclusions

In summary, a series of irreversible EGFR inhibitors **12a**–**i** containing 2-anilinopyrimidine as a scaffold and 3-aminopropamide as a "protected" warhead have been designed and synthesized. Several compounds exhibited stronger enzyme inhibitory activity against EGFR L858R/T790M and antiproliferative effects on H1975 and HCC827 cells than gefitinib. It was observed that compounds **12a**, **12g** and **12h** could be converted to the active compound **1** in the presence of natural basic amino acid arginine under physiological PBS conditions, and the amounts of compound **1** liberated by these compounds were positively correlated with their inhibitory effects on H1975 cells in vitro. Importantly, **12h** showed improved stability towards small thiol nuclephiles relative to **1** whose structure is same to **12h** excepting an acrylamide moiety. Interestingly, **12i**, a NO donating analogue of **12h**, showed more potent and selective inhibition than **12h** on EGFR mutant H1975 cells but only moderate anti-proliferation activity against cells with WT EGFR (A431, A549, BEAS-2B and 16HBE cells), suggesting that it might possess better selectivity and safety index. Significantly, **12i** produced high levels of NO in H1975 cells but not in non-tumorous 16HBE cells, and its antiproliferative activity was diminished by NO scavenger hemoglobin. Furthermore, **12i** dose-dependently produced inhibitory effects on EGFR and downstream signaling in H1975 cells. Therefore, our novel findings provide a proof of principle in design of new EGFR TKIs. Further investigation on the in vivo growth inhibitory effects of human cancer cells and the mechanisms underlying the action of these hybrids are undergoing, and the results will be reported in the due course.

5. Experimental protocols

5.1. Chemistry

^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance 300 MHz spectrometer at 300 K, using TMS as an internal standard. MS spectra were recorded on a Mariner Mass Spectrum (ESI) and HRMS on Agilent technologies LC/MSD TOF. Melting points of individual compounds were determined on a Mel-TEMP II melting point apparatus and uncorrected. TLCs and preparative TLC were performed on silica gel GF/UV 254, and the chromatograms were conducted on silica gel (100–200 mesh) and visualized under UV light at 254 and 365 nm. All solvents were reagent grade and, when necessary, were purified and dried by standards methods. Organic solutions were dried over anhydrous sodium sulfate. Compounds secondary amines and alkylol amines were commercially available. Compound **1** was synthesized as described in literature [17]. **11i** were synthesized as previously described [28–31].

5.1.1. General procedure for the preparation of *12a–i*

Compound **1** was dissolved in MeOH/THF (5/30 mL), and the required amine base (2–3.6 equiv) was added. The mixture was heated under reflux for 4 h prior to the solvent being removed, 5% NH_3 (aq) (50 mL) was added and the mixture was extracted with CH_2Cl_2 (50 × 3 mL). The organic layers were collected, washed with brine and dried over anhydrous sodium sulfate. After removal all solvent, the residue was purified using flash chromatography with dichloromethane- methanol- triethylamine to obtain the target compounds.

*5.1.1.1. N-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl) phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-3-(dimethylamino)propanamide (**12a**).* The title compound was obtained starting from **11a** and **1.** As a yellow solid, 50% yield. mp 95–97 °C. Analytical data for **12a**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.37 (s, 9H, 3 × NCH_3), 2.53 (t, 2H, $COCH_2$, J = 6.06 Hz), 2.62 (t, 4H, 2 × NCH_2CH_2N, J = 4.41 Hz), 2.68 (t, 2H, NCH_2, J = 5.82 Hz), 3.13 (t, 4H, 2 × NCH_2CH_2N, J = 4.5 Hz), 3.81 (s, 3H, OCH_3), 6.23 (dd, 1H, ArH, J = 7.2 Hz), 6.45 (s, 1H, ArH), 6.93 (d, 1H, ArH, J = 8.07 Hz), 7.31 (s, 1H, ArH), 7.39 (t, 1H, ArH, J = 8.1 Hz), 7.44 (brs, 1H, ArH), 7.62 (d, 1H, ArH, J = 8.16 Hz), 7.65 (brs, 1H, PhNH), 8.22 (s, 1H, ArH), 11.01 (s, 1H, PhNHCO); ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.05, 163.88, 158.57, 158.01, 152.13, 151.50, 148.38, 140.39, 129.68, 122.91, 119.52, 116.36, 116.07, 112.28, 106.40, 103.93, 99.86, 55.48, 54.59, 54.52, 48.48, 45.60, 44.48, 34.13; ESI-MS: m/z 540 $[M + H]^+$; ESI-HRMS (m/z): $[M + H]^+$ calcd for $C_{27}H_{34}ClN_7O_3$, 540.2490; obsd 540.2493.

*5.1.1.2. N-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl) phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-3-(diethylamino)propanamide (**12b**).* The title compound was obtained starting from **11b** and **1.** As a yellow solid, 55% yield. mp 79–81 °C. Analytical data for **12b**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.11 (t, 6H, 2 × NCH_2CH_3, J = 7.14 Hz), 2.37 (s, 3H, NCH_3), 2.61 (t, 4H, 2 × NCH_2CH_2N, J = 4.83 Hz), 2.69 (t, 4H, 2 × NCH_2CH_3, J = 7.14 Hz), 2.83 (t, 4H, $COCH_2CH_2N$, J = 6.09 Hz), 3.13 (t, 4H, 2 × NCH_2CH_2N, J = 4.5 Hz), 3.81 (s, 3H, OCH_3), 6.22 (dd, 1H, ArH, J = 7.11 Hz), 6.45 (d, 1H, ArH, J = 2.25 Hz), 6.92 (d, 1H, ArH, J = 7.92 Hz), 7.37 (t, 1H, ArH, J = 8.13 Hz), 7.40 (s, 2H, ArH), 7.57 (d, 1H, ArH, J = 7.95 Hz), 7.64 (brs, 1H, PhNH), 8.22 (s, 1H, ArH), 11.29 (s, 1H, PhNHCO); ^{13}C NMR (75 MHz, DMSO-d_6): δ 175.42, 168.91, 163.49, 163.19, 157.41, 156.50, 153.24, 145.66, 134.94, 128.46, 124.80, 121.58, 121.29, 117.51, 111.66, 109.20, 105.12, 60.75, 59.78, 53.75, 53.20, 51.41, 50.85, 38.69, 16.33; ESI-MS: m/z 568 $[M + H]^+$; ESI-HRMS (m/z): $[M + H]^+$ calcd for $C_{29}H_{38}ClN_7O_3$, 568.2803; obsd 568.2805.

*5.1.1.3. N-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl) phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-3-(pyrrolidin-1-yl)propanamide (**12c**).* The title compound was obtained starting from **11c** and **1.** As a yellow solid, 60% yield. mp 129–131 °C. Analytical data for **12c**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.93 (m, 4H, 2 × CH_2), 2.43 (s, 3H, NCH_3), 2.71 (m, 4H, 2 × NCH_2CH_2N), 2.84 (t, 2H, $COCH_2CH_2N$, J = 5.97 Hz), 2.98 (m, 4H, 2 × NCH_2), 3.15 (m, 2H, $COCH_2CH_2N$), 3.17 (m, 4H, 2 × NCH_2CH_2N), 3.81 (s, 3H, OCH_3), 6.22 (dd, 1H, ArH, J = 7.8 Hz), 6.43 (s, 1H, ArH), 6.92 (d, 1H, ArH, J = 7.86 Hz), 7.37 (t, 1H, ArH, J = 8.04 Hz), 7.44 (s, 1H, ArH), 7.56 (d, 3H, 2 × ArH, PhNH,

J = 7.95 Hz), 8.21 (s, 1H, ArH), 10.78 (s, 1H, PhNHCO); ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.06, 163.65, 158.26, 157.92, 152.11, 151.90, 148.24, 140.30, 129.68, 122.91, 119.60, 116.50, 116.17, 112.35, 106.45, 103.94, 99.90, 55.50, 54.30, 53.15, 50.37, 48.26, 45.26, 33.84, 22.86; ESI-MS: *m/z* 566 $[M + H]^+$; ESI-HRMS (*m/z*): $[M + H]^+$ calcd for $C_{29}H_{36}ClN_7O_3$, 566.2646; obsd 566.2644.

*5.1.1.4. N-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl) phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-3-(piperidin-1-yl)propanamide (**12d**).* The title compound was obtained starting from **11d** and **1.** As a yellow solid, 61% yield. mp 190–192 °C. Analytical data for **12d**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.54 (m, 2H, CH_2), 1.73 (m, 4H, 2 × CH_2), 2.42 (s, 3H, NCH_3), 2.71 (m, 10H, 2 × NCH_2CH_2N, $COCH_2CH_2N$, 2 × NCH_2), 2.91 (m, 2H, $COCH_2CH_2N$), 3.16 (m, 4H, 2 × NCH_2CH_2N), 3.81 (s, 3H, OCH_3), 6.22 (dd, 1H, ArH, J = 6.96 Hz), 6.44 (s, 1H, ArH), 6.92 (d, 1H, ArH, J = 7.59 Hz), 7.38 (m, 1H, ArH), 7.46 (s, 1H, ArH), 7.57 (m, 3H, 2 × ArH, PhNH), 8.21 (s, 1H, ArH), 11.08 (s, 1H, PhNHCO); ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.98, 163.65, 158.23, 157.92, 152.13, 151.89, 148.40, 140.41, 129.68, 123.31, 119.57, 116.34, 116.07, 112.30, 106.42, 103.94, 99.86, 55.49, 54.44, 53.68, 53.25, 48.41, 45.47, 33.26, 24.78, 23.31; ESI-MS: *m/z* 580 $[M + H]^+$; ESI-HRMS (*m/z*): $[M + H]^+$ calcd for $C_{30}H_{38}ClN_7O_3$, 580.2803; obsd 580.2806.

*5.1.1.5. N-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl) phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-3-morpholinopropanamide (**12e**).* The title compound was obtained starting from **11e** and **1.** As a yellow solid, 64% yield. mp 103–105 °C. Analytical data for **12e**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.37 (s, 3H, NCH_3), 2.53–2.60 (m, 10H, 2 × NCH_2CH_2N, $COCH_2CH_2N$, 2 × NCH_2CH_2O), 2.71 (m, 2H, $COCH_2CH_2N$), 3.11 (m, 4H, 2 × NCH_2CH_2N), 3.72 (m, 4H, 2 × OCH_2CH_2N), 3.82 (s, 3H, OCH_3), 6.21 (dd, 1H, ArH, J = 6.96 Hz), 6.45 (s, 1H, ArH), 6.95 (d, 1H, ArH, J = 7.8 Hz), 7.33 (s, 1H, ArH), 7.41 (t, 2H, ArH, J = 8.1 Hz), 7.63 (d, 2H, ArH, PhNH, J = 7.11 Hz), 8.23 (s, 1H, ArH), 10.93 (s, 1H, PhNHCO); ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.30, 163.66, 158.23, 157.91, 152.14, 151.90, 148.38, 140.43, 129.69, 122.91, 119.50, 116.32, 116.08, 112.32, 106.39, 103.93, 99.79, 66.12, 55.46, 54.60, 54.06, 53.00, 48.57, 45.72, 33.95; ESI-MS: *m/z* 582 $[M + H]^+$; ESI-HRMS (*m/z*): $[M + H]^+$ calcd for $C_{29}H_{36}ClN_7O_4$, 582.2596; obsd 582.2599.

*5.1.1.6. N-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl) phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-3-(4-methylpiperazin-1-yl)propanamide (**12f**).* The title compound was obtained starting from **11f** and **1.** As a yellow solid, 55% yield. mp 173–175 °C. Analytical data for **12f**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.29 (s, 3H, NCH_3), 2.38 (s, 3H, NCH_3), 2.52 (t, 8H, 4 × NCH_2CH_2N, J = 5.76 Hz), 2.61 (t, 6H, $COCH_2CH_2N$, 2 × NCH_2CH_2N, J = 4.41 Hz), 2.71 (t, 2H, $COCH_2CH_2N$, J = 5.7 Hz), 3.13 (t, 4H, 2 × NCH_2CH_2N, J = 4.62 Hz), 3.82 (s, 3H, OCH_3), 6.23 (dd, 1H, ArH, J = 7.92 Hz), 6.45 (s, 1H, ArH), 6.95 (d, 1H, ArH, J = 8.07 Hz), 7.40 (t, 2H, ArH, J = 8.16 Hz), 7.46 (s, 1H, ArH), 7.62 (d, 2H, ArH, PhNH, J = 7.41 Hz), 8.23 (s, 1H, ArH), 11.06 (s, 1H, PhNHCO); ^{13}C NMR (75 MHz, DMSO-d_6): 170.33, 163.66, 158.23, 157.92, 152.14, 151.11, 148.31, 140.46, 129.67, 122.81, 119.55, 116.27, 116.05, 112.29, 106.05, 103.94, 99.84, 55.49, 54.52, 54.41, 53.49, 51.97, 48.49, 45.59, 45.27, 34.10; ESI-MS: *m/z* 595 $[M + H]^+$; ESI-HRMS (*m/z*): $[M + H]^+$ calcd for $C_{30}H_{39}ClN_8O_3$, 595.2912; obsd 595.2914.

*5.1.1.7. Ethyl-4-(3-((3-((5-chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)phenyl) amino)-3-oxopropyl)piperazine-1-carboxylate (**12g**).* The title compound was obtained starting from **11g** and **1.** As a yellow solid, 59% yield. mp 98–100 °C. Analytical data for **12g**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.27 (t, 3H, $COONCH_2CH_3$, J = 7.11 Hz), 2.36 (s, 3H, NCH_3), 2.52 (t, 6H, 2 × NCH_2CH_2N, $COCH_2CH_2N$, J = 4.02 Hz), 2.58 (t, 4H, 2 × NCH_2CH_2N, J = 4.08 Hz), 2.72 (t, 2H, $COCH_2CH_2N$, J = 5.7 Hz), 3.11 (t, 4H, 2 × NCH_2CH_2N, J = 4.53 Hz), 3.52 (t, 4H, 2 × NCH_2CH_2N, J = 4.53 Hz), 3.82 (s, 3H, OCH_3), 4.29 (q, 2H, $COONCH_2CH_3$, J = 7.08 Hz), 6.22 (dd, 1H, ArH, J = 7.77 Hz), 6.45 (s, 1H, ArH), 6.95 (d, 1H, ArH, J = 7.26 Hz), 7.30 (s, 1H, ArH), 7.40 (t, 2H, ArH, J = 8.07 Hz), 7.62 (d, 2H, ArH, PhNH, J = 7.44 Hz), 8.23 (s, 1H, ArH), 10.7 (s, 1H, PhNHCO); ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.25, 163.66, 158.22, 157.92, 154.51, 152.13, 151.10, 148.39, 140.41, 129.68, 122.56, 119.50, 116.32, 116.09, 112.32, 106.39, 103.92, 99.80, 60.63, 55.47, 54.61, 53.60, 52.12, 48.59, 45.73, 43.27, 34.09, 14.52; ESI-MS: *m/z* 653 $[M + H]^+$; ESI-HRMS (*m/z*): $[M + H]^+$ calcd for $C_{32}H_{41}ClN_8O_5$, 653.2967; obsd 653.2969.

*5.1.1.8. N-(3-((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl) phenyl)amino)pyrimidin-4-yl)oxy)phenyl)-3-(4-(2-hydroxyethyl) piperazin-1-yl)propanamide (**12h**).* The title compound was obtained starting from **11h** and **1.** As a yellow solid, 49% yield. mp 99–101 °C. Analytical data for **12h**: ^{1}H NMR (300 MHz, DMSO): δ 2.38 (s, 3H, NCH_3), 2.51–2.66 (m, 16H, 6 × NCH_2CH_2N, $COCH_2CH_2N$), 3.15 (t, 2H, NCH_2, J = 3.87 Hz), 3.17 (brs, 4H, 2 × NCH_2CH_2N), 3.56 (brs, 2H, $HOCH_2$), 3.74 (s, 3H, OCH_3), 6.20 (brs, 1H, ArH), 6.38 (s, 1H, ArH), 6.93 (d, 1H, ArH, J = 7.17 Hz), 7.26 (d, 1H, ArH, J = 7.25 Hz), 7.38 (t, 1H, ArH, J = 7.35 Hz), 7.47 (d, 1H, ArH, J = 8.01 Hz), 7.58 (s, 1H, ArH), 8.10 (s, 1H, PhNH), 8.34 (s, 1H, ArH), 10.35 (s, 1H, PhNHCO); ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.97, 164.45, 159.00, 158.70, 152.89, 151.90, 148.86, 141.26, 130.42, 123.65, 120.48, 117.08, 116. 89, 113.10, 107. 29, 104.76, 100.73, 60.22, 58.32, 56.31, 54.94, 54.09, 53.22, 52.19, 48.89, 46.19, 34.65; ESI-MS: *m/z* 625 $[M + H]^+$; ESI-HRMS (*m/z*): $[M + H]^+$ calcd for $C_{31}H_{41}ClN_8O_4$, 625.3018; obsd 625.3020.

*5.1.1.9. 4-(2-(4-(3-((3-(((5-Chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)oxy)phenyl) amino)-3-oxopropyl)piperazin-1-yl)ethoxy)-3-(phenylsulfonyl)-1,2,5-oxadiazole 2-oxide (**12i**).* The title compound was obtained starting from **11i** and **1.** As a yellow solid, 60% yield. mp 132–134 °C. Analytical data for **12i**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.35 (s, 3H, NCH_3), 2.57 (m, 14H, 6 × NCH_2CH_2N, $COCH_2CH_2N$), 2.69 (t, 2H, $COCH_2CH_2N$, J = 5.1 Hz), 2.82 (t, 2H, NCH_2, J = 5.28 Hz), 3.09 (brs, 4H, 2 × NCH_2CH_2N), 3.76 (s, 3H, OCH_3), 4.51 (t, 2H, OCH_2, J = 5.22 Hz), 6.23 (d, 1H, ArH, J = 6.54 Hz), 6.45 (s, 1H, ArH), 6.94 (d, 1H, ArH, J = 7.95 Hz), 7.31(s, 1H, ArH), 7.40 (t, 2H, ArH, J = 8.16 Hz), 7.60 (t, 2H, ArH, J = 7.41 Hz), 7.67 (d, 1H, ArH, J = 6.93 Hz), 7.75 (t, 2H, ArH, PhNH, J = 7.41 Hz), 8.05 (d, 2H, ArH, J = 7.32 Hz), 8.23 (s, 1H, ArH), 11.05 (s, 1H, PhNHCO); ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.89, 164.15, 159.87, 159.21, 158.44, 152.65, 151.90, 140.94, 137.82, 136.57, 130.50, 130.21, 128.65, 127.82, 123.65, 119.99, 116.79, 116.53, 112.77, 106.86, 104.76, 100.32, 69.84, 56.06, 55.98, 55.12, 54.13, 53.28, 52.99, 49.08, 46.24, 34.69; ESI-MS: *m/z* 849 $[M + H]^+$; ESI-HRMS (*m/z*): $[M + H]^+$ calcd for $C_{39}H_{45}ClN_{10}O_8S$, 849.2909; obsd 849.2911.

5.2. In vitro stability and reactivity assays

Chemical stability was tested under physiological (0.01 M PBS, pH 7.4), and alkaline (0.01 M borate buffer, pH 9.0) pH conditions, at 37 °C. 1 mM Stock solutions of **12h** and **1** were prepared in DMSO, and each sample was incubated at a final concentration of 50 μM in prewarmed buffered solutions (final concentration of DMSO: 5% v/v). At the regular time points, solutions were sampled with same volume of acetonitrile and immediately injected into the HPLC system. For rat plasma stability assays, quickly-thawed rat plasma was diluted to 50% (v/v) with 0.1 M PBS, pH 7.4. And then compound stock solution in DMSO was added (final compound concentration: 50 μM, DMSO concentration: 5% v/v) and maintained at 37 °C. At specific time, two volumes of acetonitrile were added and

the mixture was centrifuged (4 °C, 14,000 g, 15 min) and analyzed by RP HPLC. The reactivity of **12h** and **1** toward dithiothreitol (DTT), L-cysteine and reduced GSH was evaluated by adding a freshly prepared thiol solution (2 mM) to PBS (pH 7.4) containing the test compound (1 μM). Formation of conjugates with LMW thiols after 1 h of incubation at 37 °C was measured by HPLC–UV.

*5.2.1. Releasing ability of compound **1***

The releasing ability of parent compound **1** was tested in PBS (0.01 M, PH 7.4) solution with arginine (1 mM) at 37 °C. 1 mM Stock solutions of **12a**, **12g** and **12h** were prepared in DMSO. Each sample with a final concentration of 50 μM was incubated in prewarmed buffered solutions (final concentration of DMSO: 5% v/v). At the regular time points, solutions were mixtured with same volume of acetonitrile and immediately injected into HPLC system.

The HPLC analysis was performed on a Shimadzu 2010-20AT HPLC system on a C18 reversed-phase column (5 μm, 150 mm × 4.6 mm) by a gradient elution using (A) 0.05 M of phosphate buffer (pH = 3); (B) acetonitrile as the mobile phase 0 min (27% B) →12.5 min (55% B) →15 min (90% B), and then returning to initial conditions after 4 min, followed by 5 min re-equilibration time. Injection volume: 10 μL. The flow rate was 1.0 mL/min, and ultraviolet detection was at 281 nm.

5.2.2. Cell lines and reagents

H1975 (NSCLC, EGFR L858R/T790M), HCC827 (NSCLC, EGFR del E746_A750), A431 (epidermoid carcinoma, EGFR overexpression), A549 (NSCLC, EGFR wild type), BEAS-2B and 16HBE (human bronchial epithelial cells) cells were obtained from ATCC. The cells were maintained at 37 °C in a 5% CO_2 incubator in DMEM or RPMI 1640 (Hyclone) containing 10% fetal bovine serum (FBS, Biochrom, AG).

5.2.3. In vitro enzymatic activity assay

Wild type and EGFR mutants (L858R, L858R/T790M) and the Z′-Lyte Kinase Kit were purchased from Invitrogen. Ten concentration gradients were set for all the tested compounds. The experiments were performed according to the instructions of the manufacturer.

5.2.4. MTT assay

1000 cells/well of H1975, 2000 cells/well of HCC827, HCC827 GR, A431, A549, 16HBE and BEAS-2B were cultured in 8% FBS respective growth medium in 96-well microplates overnight. The cells were then treated in triplicate with various concentrations of each compound and cultured in 5% FBS medium for 72 h. Control cells were treated with vehicle alone. During the last 4 h of incubation, the cells were exposed to tetrazolium dye (MTT) solution (5 mg/mL, 20 μL per well). The generated formazan crystals were dissolved in 100 μL of dimethyl sulfoxide (DMSO), and the absorbance was read spectrophotometrically at 570 nm using an enzyme-linked immunosorbent assay plate reader. The data was calculated using Graph Pad Prism version 5.0. The IC_{50}s were fitted using a non-linear regression model with a sigmodial dose response.

5.2.5. Nitrate/nitrite measurement in vitro

The levels of nitrate/nitrite produced by individual compounds in the cells were determined by the colorimetric assay using the nitrate/nitrite colorimetric assay kit (Beyotime, China), according to the manufacturer's instructions. Briefly, H1975 and 16HBE cells (1×10^6/well) were treated with 100 μM of each compound for 150 min. Subsequently, the cells were harvested and their cell lysates were prepared, then mixing with Griess for 10 min, followed by measuring at 540 nm. The cells treated with diluent were used as negative controls for the background levels of nitrate/nitrite production, while sodium nitrate at different concentrations was used as the positive control for the standard curve.

5.2.6. Western blotting analysis

The H1975 cells were incubated with 0.01, 0.1 or 1 μM **12i** or vehicle control (0.1% DMSO) and 0.1 or 1 μM gefitinib for 24 h. Following incubation, the cells were harvested and lyzed. The cell lysates (50 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% gel) and transferred onto nitrocellulose membranes. After they were blocked with 5% fat-free milk, the target proteins were probed with anti-EGFR, anti-phospho-EGFR (Tyr1068), anti-Akt, antiphospho-Akt (Ser473), anti-ERK, antiphospho-ERK (Thr202/Tyr204), and anti-β-actin antibodies (Cell Signaling, Boston, MA), respectively. The bound antibodies were detected by horseradish peroxidase (HRP)-conjugated second antibodies and visualized using the enhanced chemiluminescent reagent.

References

[1] R. Siegel, D. Naishadham, A. Jemal, Cancer statistics, 2013, CA: a Cancer Journal for Clinicians 63 (2013) 11–30.
[2] A.G. Pallis, L. Serfass, R. Dziadziusko, J.P. van Meerbeeck, D. Fennell, D. Lacombe, J. Welch, C. Gridelli, Targeted therapies in the treatment of advanced/metastatic NSCLC, European Journal of Cancer 45 (2009) 2473–2487.
[3] H. Zhang, A. Berezov, Q. Wang, G. Zhang, J. Drebin, R. Murali, M.I. Greene, ErbB receptors: from oncogenes to targeted cancer therapies, Journal of Clinical Investigation 117 (2007) 2051–2058.
[4] M.A. Olayioye, R.M. Neve, H.A. Lane, N.E. Hynes, The ErbB signaling network: receptor heterodimerization in development and cancer, The EMBO Journal 19 (2000) 3159–3167.
[5] C. Mayo, J. Bertran-Alamillo, M.A. Molina-Vila, A. Gimenez-Capitan, C. Costa, R. Rosell, Pharmacogenetics of EGFR in lung cancer: perspectives and clinical applications, Pharmacogenomics 13 (2012) 789–802.
[6] K. Gately, J. O'Flaherty, F. Cappuzzo, R. Pirker, K. Kerr, K. O'Byrne, The role of the molecular footprint of EGFR in tailoring treatment decisions in NSCLC, Journal of Clinical Pathology 65 (2012) 1–7.
[7] M. Ladanyi, W. Pao, Lung adenocarcinoma: guiding EGFR-targeted therapy and beyond, Modern Pathology 21 (Suppl. 2) (2008) S16–S22.
[8] S. Kobayashi, T.J. Boggon, T. Dayaram, P.A. Janne, 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, New England Journal of Medicine 352 (2005) 786–792.
[9] 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 Medicine 2 (2005) 225–235.
[10] 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, Proceedings of the National Academy of Sciences of the United States of America 105 (2008) 2070–2075.
[11] P.A. Janne, J. von Pawel, R.B. Cohen, L. Crino, C.A. Butts, S.S. Olson, I.A. Eiseman, A.A. Chiappori, B.Y. Yeap, P.F. Lenehan, K. Dasse, M. Sheeran, P.D. Bonomi, Multicenter, randomized, phase II trial of CI-1033, an irreversible pan-ERBB inhibitor, for previously treated advanced non small-cell lung cancer, Journal of Clinical Oncology 25 (2007) 3936–3944.
[12] F.A. Eskens, C.H. Mom, A.S. Planting, J.A. Gietema, A. Amelsberg, H. Huisman, L. van Doorn, H. Burger, P. Stopfer, J. Verweij, E.G. de Vries, A phase I dose escalation study of BIBW 2992, an irreversible dual inhibitor of epidermal growth factor receptor 1 (EGFR) and 2 (HER2) tyrosine kinase in a 2-week on, 2-week off schedule in patients with advanced solid tumours, British Journal of Cancer 98 (2008) 80–85.
[13] S.K. Rabindran, C.M. Discafani, E.C. Rosfjord, M. Baxter, M.B. Floyd, J. Golas, W.A. Hallett, B.D. Johnson, R. Nilakantan, E. Overbeek, M.F. Reich, R. Shen, X. Shi, H.R. Tsou, Y.F. Wang, A. Wissner, Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase, Cancer Research 64 (2004) 3958–3965.
[14] J.A. Engelman, K. Zejnullahu, C.M. Gale, E. Lifshits, A.J. Gonzales, T. Shimamura, F. Zhao, P.W. Vincent, G.N. Naumov, J.E. Bradner, I.W. Althaus, L. Gandhi, G.I. Shapiro, J.M. Nelson, J.V. Heymach, M. Meyerson, K.K. Wong, P.A. Janne, PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib, Cancer Research 67 (2007) 11924–11932.
[15] T. Kosaka, E. Yamaki, A. Mogi, H. Kuwano, Mechanisms of resistance to EGFR TKIs and development of a new generation of drugs in non-small-cell lung cancer, Journal of Biomedicine and Biotechnology 2011 (2011) 165214.
[16] L.V. Sequist, B. Besse, T.J. Lynch, V.A. Miller, K.K. Wong, B. Gitlitz, K. Eaton, C. Zacharchuk, A. Freyman, C. Powell, R. Ananthakrishnan, S. Quinn, J.C. Soria,

Neratinib, an irreversible pan-ErbB receptor tyrosine kinase inhibitor: results of a phase II trial in patients with advanced non-small-cell lung cancer, Journal of Clinical Oncology 28 (2010) 3076–3083.
[17] 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. Janne, Novel mutant-selective EGFR kinase inhibitors against EGFR T790M, Nature 462 (2009) 1070–1074.
[18] T.R. Fennell, S.C. Sumner, R.W. Snyder, J. Burgess, R. Spicer, W.E. Bridson, M.A. Friedman, Metabolism and hemoglobin adduct formation of acrylamide in humans, Toxicological Sciences 85 (2005) 447–459.
[19] T. Barf, A. Kaptein, Irreversible protein kinase inhibitors: balancing the benefits and risks, Journal of Medicinal Chemistry 55 (2012) 6243–6262.
[20] C. Carmi, E. Galvani, F. Vacondio, S. Rivara, A. Lodola, S. Russo, S. Aiello, F. Bordi, G. Costantino, A. Cavazzoni, R.R. Alfieri, A. Ardizzoni, P.G. Petronini, M. Mor, Irreversible inhibition of epidermal growth factor receptor activity by 3-aminopropanamides, Journal of Medicinal Chemistry 55 (2012) 2251–2264.
[21] N.I. Wenzel, N. Chavain, Y. Wang, W. Friebolin, L. Maes, B. Pradines, M. Lanzer, V. Yardley, R. Brun, C. Herold-Mende, C. Biot, K. Toth, E. Davioud-Charvet, Antimalarial versus cytotoxic properties of dual drugs derived from 4-aminoquinolines and mannich bases: interaction with DNA, Journal of Medicinal Chemistry 53 (2010) 3214–3226.
[22] D. Fukumura, S. Kashiwagi, R.K. Jain, The role of nitric oxide in tumour progression, Nature Reviews Cancer 6 (2006) 521–534.
[23] B. Bonavida, S. Baritaki, S. Huerta-Yepez, M.I. Vega, D. Chatterjee, K. Yeung, Novel therapeutic applications of nitric oxide donors in cancer: roles in chemo- and immunosensitization to apoptosis and inhibition of metastases, Nitric Oxide 19 (2008) 152–157.
[24] C. Han, Z. Huang, C. Zheng, L. Wan, L. Zhang, S. Peng, K. Ding, H. Ji, J. Tian, Y. Zhang, Novel hybrids of (phenylsulfonyl)furoxan and anilinopyrimidine as potent and selective epidermal growth factor receptor inhibitors for intervention of non-small-cell lung cancer, Journal of Medicinal Chemistry 56 (2013) 4738–4748.
[25] C. Han, Z. Huang, C. Zheng, L. Wan, Y. Lai, S. Peng, K. Ding, H. Ji, Y. Zhang, Nitric oxide donating anilinopyrimidines: synthesis and biological evaluation as EGFR inhibitors, European Journal of Medicinal Chemistry 66 (2013) 82–90.
[26] S. Chang, L. Zhang, S. Xu, J. Luo, X. Lu, Z. Zhang, T. Xu, Y. Liu, Z. Tu, Y. Xu, X. Ren, M. Geng, J. Ding, D. Pei, K. Ding, Design, synthesis, and biological evaluation of novel conformationally constrained inhibitors targeting epidermal growth factor receptor threonine^{790} –> methionine^{790} mutant, Journal of Medicinal Chemistry 55 (2012) 2711–2723.
[27] E.A. Jares-Erijman, T.M. Jovin, FRET imaging, Nature Biotechnology 21 (2003) 1387–1395.
[28] L. Chen, Y. Zhang, X. Kong, E. Lan, Z. Huang, S. Peng, D.L. Kaufman, J. Tian, Design, synthesis, and antihepatocellular carcinoma activity of nitric oxide releasing derivatives of oleanolic acid, Journal of Medicinal Chemistry 51 (2008) 4834–4838.
[29] Z. Huang, Y. Zhang, L. Zhao, Y. Jing, Y. Lai, L. Zhang, Q. Guo, S. Yuan, J. Zhang, L. Chen, S. Peng, J. Tian, Synthesis and anti-human hepatocellular carcinoma activity of new nitric oxide-releasing glycosyl derivatives of oleanolic acid, Organic & Biomolecular Chemistry 8 (2010) 632–639.
[30] Y. Ling, X. Ye, H. Ji, Y. Zhang, Y. Lai, S. Peng, J. Tian, Synthesis and evaluation of nitric oxide-releasing derivatives of farnesylthiosalicylic acid as anti-tumor agents, Bioorganic & Medicinal Chemistry 18 (2010) 3448–3456.
[31] Y. Ling, X. Ye, Z. Zhang, Y. Zhang, Y. Lai, H. Ji, S. Peng, J. Tian, Novel nitric oxide-releasing derivatives of farnesylthiosalicylic acid: synthesis and evaluation of antihepatocellular carcinoma activity, Journal of Medicinal Chemistry 54 (2011) 3251–3259.
[32] P.N. Coneski, M.H. Schoenfisch, Nitric oxide release: part III. Measurement and reporting, Chemical Society Reviews 41 (2012) 3753–3758.