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Synthesis and Biological Evaluation of Pyrimidine-Based Dual Inhibitors of Human Epidermal Growth Factor Receptor 1 (HER-1) and HER-2 Tyrosine Kinases

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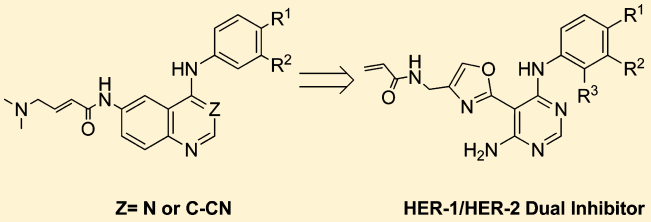
S *Supporting Information*

ABSTRACT: A novel series of N^4-(3-chlorophenyl)-5-(oxazol-2-yl)pyrimidine-4,6-diamines were synthesized and evaluated as dual inhibitors of HER-1/HER-2 tyrosine kinases. In contrast to the currently approved HER-2-targeted agent (lapatinib, **1**), our irreversible HER-1/HER-2 inhibitors have the potential to overcome the clinically relevant and mutation-induced drug resistance. The selected compound (**19a**) showed excellent inhibitory activity toward HER-1/HER-2 tyrosine kinases with selectivity over 20 other kinases and inhibited the proliferation of both cancer cell types: lapatinib-sensitive cell lines (SK-Br3, MDA-MB-175, and N87) and lapatinib-resistant cell lines (MDA-MB-453, H1781, and H1975). The excellent pharmacokinetic profiles of **19a** in mice and rats led us to further investigation of a novel therapeutic agent for HER-2-targeting treatment of solid tumors, especially HER-2-positive breast/gastric cancer and HER-2-mutated lung cancer.



INTRODUCTION

HER-2 is a transmembrane oncoprotein encoded by the *Her-2/neu* gene and is a member of HER/ErbB/EGFR tyrosine kinases family, which is composed of HER-1 (ErbB-1, EGFR), HER-2 (neu, ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4). The signaling cascade of EGFRs plays a crucial role in regulation of cell proliferation and differentiation in many tissue types, and the dysregulation of EGFR signaling pathway may contribute to malignant transformation.[1−3] In particular, HER-2 is overexpressed in 20−25% of breast or gastric cancer, which is associated with poor prognosis.[4,5] Therefore, HER-2 has been recognized as a therapeutic target with good clinical outcomes.[6] For example, trastuzumab[7,8] (a humanized monoclonal antibody targeting the extracellular domain of HER-2) was approved for HER-2-positive metastatic breast cancer and gastric cancer by U.S. Food and Drug Administration (FDA). Lapatinib[9,10] (reversible small-molecule tyrosine kinase inhibitor of HER-1 and HER-2) was also approved for HER-2-positive metastatic breast cancer. Even though these therapeutic agents are effective for patients with HER-2-positive breast or gastric cancer, trastuzumab has potential cardiotoxicity when combined with chemotherapy (especially with anthracyclines) and lapatinib did not show the overall survival advantage in metastatic breast cancer.[11,12] In addition, the intrinsic or acquired resistance against trastuzumab or lapatinib has been an important issue in HER-2-targeted cancer therapy.[13−15]

As shown in Figure 1, several small-molecule inhibitors of HER-2 tyrosine kinases have been developed.[16,17] The first

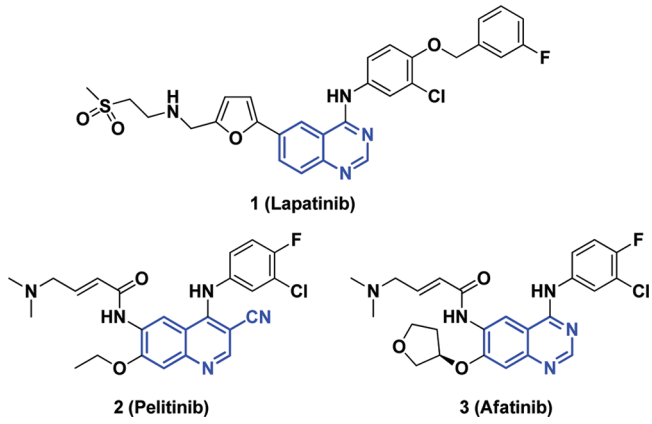


Figure 1. Representative inhibitors of HER-2 tyrosine kinase.

HER-2-targeting small-molecule agent is lapatinib (**1**), the quinazoline-based HER-1/HER-2 dual inhibitor. Since HER-2 tyrosine kinase binds either HER-1 or HER-2 tyrosine kinases to form a hetero- or homodimer for the signal transduction of

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EGFR signal cascade, HER-1/HER-2 dual inhibition can be more effective than just HER-1 or HER-2 inhibition.[11] The next class includes the irreversible EGFR inhibitors cyanoquinoline-based pelitinib (**2**) and quinazoline-based afatinib (**3**).[18−20] Among these HER-2 inhibitors, we focused on irreversible EGFR inhibitors because of their interesting mode of action; both HER-1 and HER-2 tyrosine kinases were inhibited by the irreversible modification of a Cys residue (Cys773 of HER-1 and Cys805 of HER-2) at their active sites. Their strong inhibitory activity toward both HER-1 and HER-2 and the unique mode of action can provide the potential to overcome the limited efficacy of current HER-2-targeted therapy. Therefore, we designed new HER-2-targeting small-molecule inhibitors with a distinct scaffold for improved antitumor efficacy to address the limited activity profile of the currently approved HER-2-targeting small molecular therapy, lapatinib.

RESULTS AND DISCUSSION

Chemical Design. To overcome the drug resistance and the limited activity of the current anti-HER-2 therapy, we pursued the discovery of novel HER-1/HER-2-targeting therapeutic agents with enhanced potency. We selected pyrimidine as a novel core skeleton, inspired by quinazoline- or cyanoquinoline-based irreversible EGFR inhibitors (**2**, **3**, and their general molecular framework **4** shown in Figure 2).

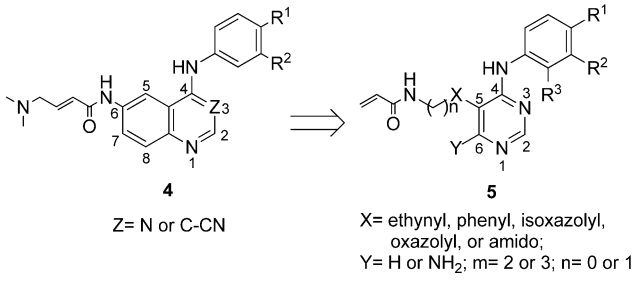
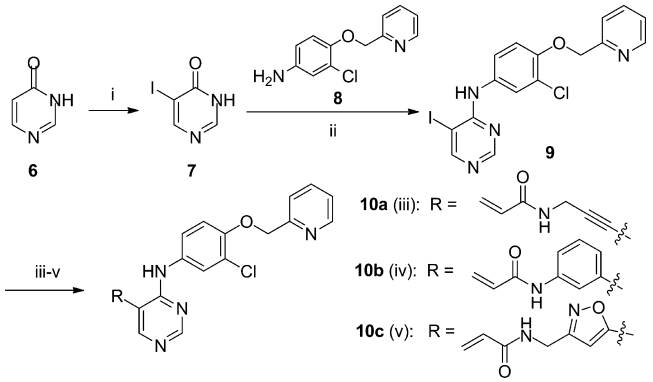


Figure 2. Design of novel HER-1/HER-2 dual inhibitors.

According to previous reports,[21] N1 and N3 (if Z = N; quinazoline) or 3-carbonitrile (if Z = C−CN; cyanoquinoline) in the molecular framework **4** is essential for the critical hydrogen bonding interaction with Met769 and Thr766 (or Thr830) of EGFR, respectively. In addition, the introduction of substituents at the C8 position of compound **4** resulted in the loss of EGFR inhibitory activity.[21] In contrast, the aniline moiety at the C4 position of compound **4** is essential to achieve bioactive conformation. Bulky substituents such as pyridin-2-ylmethoxy or substituted benzyloxy moiety, as shown in **1**, at the R^1 position increase HER-2 inhibition, and small electron-withdrawing lipophilic groups such as chloride or fluoride at the R^2 position increase HER-1 and HER-2 inhibitory activity. On the basis of this structure−activity relationship (SAR) information, we designed pyrimidine-based analogues **5** with an *N*-acryloyl moiety at the C5 position and bulky aniline moiety at the C4 position to secure irreversible HER-2 inhibitory activity. Herein, we report the identification of N^4-(3-chlorophenyl)-5-(oxazol-2-yl)pyrimidine-4,6-diamines with *N*-acryloyl moiety as potential candidates and compound **19a** as a novel HER-1/HER-2 dual inhibitor for HER-2-targeting anticancer therapy.

Selection of Active Scaffold. The modification at the X position of compound **5** provides the important element that can influence the orientation of *N*-acryloyl group as a Michael acceptor for the alkylation to desired cysteine residues. Therefore, we synthesized a series of *N*-4-((3-chloro-4-(pyridin-2-ylmethoxy)phenyl)amino)pyrimidin-5-linked acrylamide analogues **10a−d** to introduce various moieties at the X position of pyrimidine scaffold as an irreversible HER-1/HER-2 dual inhibitor. As shown in Scheme 1, the synthesis of

Scheme 1[a]



[a]Reagents and conditions: (i) *N*-iodosuccinimide, AcOH, 100 °C; (ii) (a) $POCl_3$, reflux, (b) **8**, 2-propanol, reflux; (iii) (a) *N*-Boc-propargylamine, $PdCl_2(PPh_3)_2$, CuI, TEA, THF, rt; (b) TFA, DCM, rt; (c) acryloyl chloride, $NaHCO_3$, aq THF (THF/H_2O = 5:1), 0 °C; (iv) (a) 3-aminophenylboronic acid, $Pd(OAc)_2$, K_2CO_3, MeOH, 70 °C; (b) acryloyl chloride, $NaHCO_3$, aq THF (THF/H_2O = 5:1), 0 °C; (v) (a) trimethylsilyl acetylene, $PdCl_2(PPh_3)_2$, CuI, TEA, THF, rt; (b) TBAF, THF, 0 °C; (c) 2-(1,3-dioxoisoindolin-2-yl)-*N*-hydroxyacetimidoyl chloride, TEA, THF, reflux; (d) hydrazine monohydrate, EtOH, 65 °C; (e) acryloyl chloride, $NaHCO_3$, aq THF (THF/H_2O = 5:1), 0 °C.

analogues **10a−c** was initiated using 4(3*H*)-pyrimidinone **6** treated with *N*-iodosuccinimide at 100 °C in acetic acid to yield 5-iodopyrimidin-4(3*H*)-one **7**. The resulting compound **7** was chlorinated with phosphorus oxychloride under reflux conditions and subjected to substitution with aniline **8** in refluxing 2-propanol to yield *N*-(3-chloro-4-(pyridin-2-ylmethoxy)-phenyl)-5-iodopyrimidin-4-amine **9** as a key intermediate. This intermediate **9** was reacted with *N*-Boc-propargylamine under Sonogashira coupling conditions in the presence of bis(triphenylphosphine)palladium(II) dichloride and copper(I) iodide. Subsequently, the Boc group was removed upon treatment with 30% trifluoroacetic acid in dichloromethane, followed by amide coupling with acryloyl chloride in the presence of sodium bicarbonate at 0 °C to yield propargyl analogue **10a**. Phenyl analogue **10b** was synthesized through Suzuki coupling reaction of the key intermediate **9** with 3-aminophenylboronic acid in the presence of palladium(II) acetate, followed by N-acryloylation. For the preparation of isoxazolylmethyl analogue **10c**, the key intermediate **9** was treated with trimethylsilyl (TMS) acetylene under the identical Sonogashira coupling conditions used above. The desired analogue **10c** was synthesized through a series of reactions including TMS deprotection using tetrabutylammonium fluoride (TBAF), 1,3-dipolar cycloaddition with 2-(1,3-dioxoisoindolin-2-yl)-*N*-hydroxyacetimidoyl chloride, phthalimide deprotection using hydrazine, and subsequent N-acryloylation.

For the synthesis of oxazolylmethyl analogue **10d**, ethyl 4-((3-chloro-4-(pyridin-2-ylmethoxy)phenyl)amino)pyrimidine-

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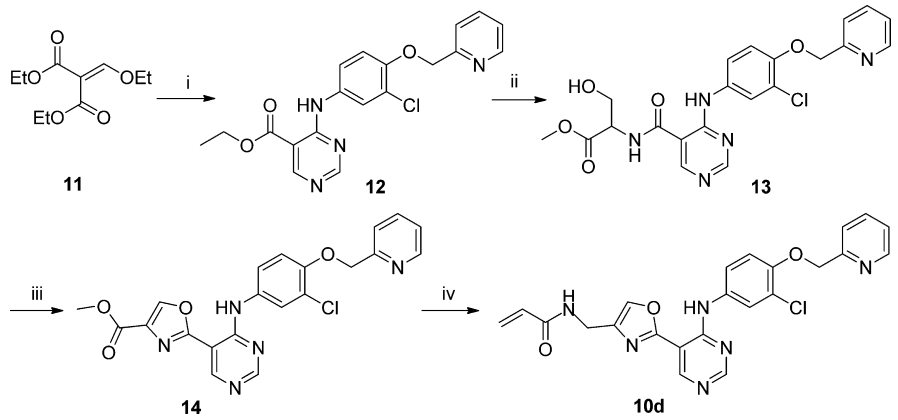
Scheme 2[a]



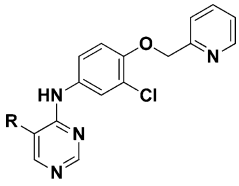
[a]Reagents and conditions: (i) (a) formamidine hydrochloride, 160 °C; (b) $POCl_3$, $SOCl_2$, DIPEA, reflux; (c) **8**, 2-propanol, reflux; (ii) (a) 2 N NaOH aq solution, EtOH, 80 °C; (b) DL-serine methyl ester hydrochloride, EDCI, HOBt, DIPEA, DMF, rt; (iii) (a) DAST, DCM, −78 to 0 °C; (b) DBU, $CBrCl_3$, DCM, −40 °C to rt; (ix) (a) LAH, THF, 0 °C; (b) NaN_3, PPh_3, DMF/CCl_4 (4:1), 90 °C; (c) PPh_3, H_2O, THF, 60 °C; (d) acryloyl chloride, $NaHCO_3$, aq THF (THF/H_2O = 5:1), 0 °C.

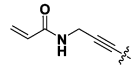
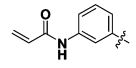
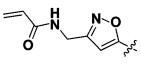
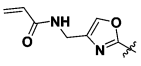
5-carboxylate (**12**) was prepared by condensation of diethyl ethoxymethylenemalonate (**11**) with formamidine, chlorination using thionyl chloride and phosphorus oxychloride in the presence of diisopropylethylamine, and substitution with aniline **8** in the refluxing 2-propanol (Scheme 2). Then the resulting ethyl carboxylate **12** was hydrolyzed in 2 N sodium hydroxide aqueous solution and coupled with DL-serine methyl ester using EDCI and HOBt to provide *β*-hydroxyamide **13**, which was converted to oxazole **14** via the mild cyclodehydration reaction using diethylaminosulfur trifluoride (DAST) and oxidation using bromotrichloromethane and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). Finally, oxazolylmethyl analogue **10d** was prepared by the reduction of methyl ester of oxazole **14**, substitution of hydroxyl group to azide, Staudinger reduction of azide to amine, and subsequent N-acryloylation.

All synthesized analogues **10a−d** were initially tested for their ability to inhibit cellular proliferation in two representative HER-1 and HER-2-positive cell lines: A431 (HER-1-overexpressing human vaginal epidermoid cancer cell line)[22] and SK-Br3 (HER-2-overexpressing human breast cancer cell line).[23] As shown in Table 1, analogues containing *N*-acryloyl moiety with phenyl group (**10b**, IC_{50} = 6.1 nM) or oxazolylmethyl group (**10d**, IC_{50} = 9.7 nM) at the C5 position of pyrimidine showed stronger growth inhibition potency in SK-Br3 cell-based proliferation assay, compared to analogues with propargyl group (**10a**, IC_{50} > 1000 nM) or isoxazolylmethyl group (**10c**, IC_{50} = 122 nM). In addition, the oxazolylmethyl derivative **10d** (IC_{50} = 105 nM) showed better potency compared to the phenyl derivative **10b** (IC_{50} = 593 nM) in A431 cell lines. It was quite exciting to see that the growth inhibitory activity of analogue **10d** is comparable to that of **1**, which is an approved HER-1/HER-2 dual inhibitor for the treatment of patients with HER-2-positive metastatic breast cancer.

Optimization of Active Analogue 10d. On the basis of the results of cell-based proliferation assay against **10a−d**, the *N*-acryloyl moiety with oxazolylmethyl group was selected for position X of pyrimidine. For the further optimization of active analogue **10d**, we performed a series of docking studies using the EGFR crystal structure (PDB code 1XKK) cocrystallized with **1**. As shown in Figure 3, the modification at the Y position of compound **5** with amine group allows the additional

Table 1. In Vitro Cellular Activity[a] of R-Modified Analogues



Entry	Compd	R	Cell assay, IC_{50} (nM) A431[b]	SK-Br3[c]
1	**10a**		>1,000	>1,000
2	**10b**		593	6.1
3	**10c**		2.2	122
4	**10d**		105	9.7
	1[d]	-	104	29

[a]All biological data are mean values of three independent experiments performed in duplicate. [b]A431 is HER-1-overexpressing human vaginal epidermoid cancer cell line. [c]SK-Br3 is HER-2-overexpressing human breast cancer cell line. [d]**1** was synthesized using the synthetic protocol reported in WO99/35146.

hydrogen bonding interaction with Gly796 (Grid score, which is not considered for H-bond: −53.65 for **10d** vs −57.04 for amine (Y) containing analogue of **10d**). Therefore, we aimed to introduce the amine moiety at the Y position of pyrimidine **5** to enhance its binding affinity at the active site of EGFR. The synthesis of the amine-containing analogue of **10d** at the Y position is outlined in Scheme 3. 4,6-Dichloropyrimidine-5-carbaldehyde **15** was chlorinated under radical conditions, followed by amide coupling with DL-serine methyl ester to give *β*-hydroxyamide **16**. The C6-amine was introduced to **16** using ammonia gas in 50% ethanolic toluene in a sealed tube, followed by oxazoline formation using DAST. The C4-chloride of oxazoline **17** was substituted with various anilines (R-NH_2) under acidic conditions and oxidized to oxazole using

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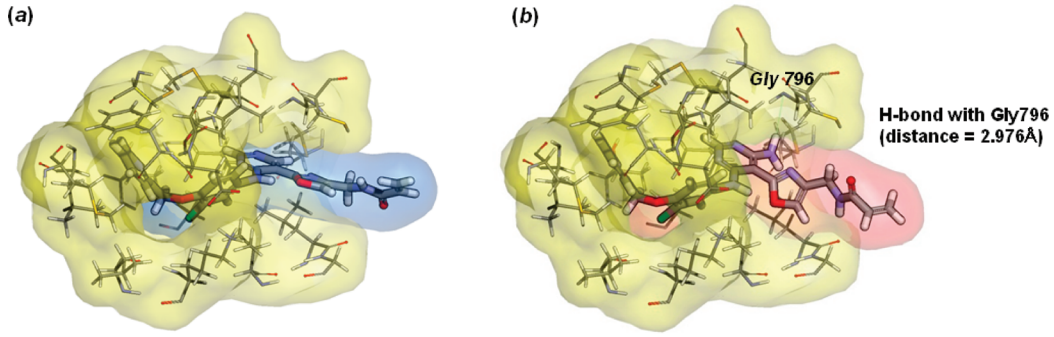
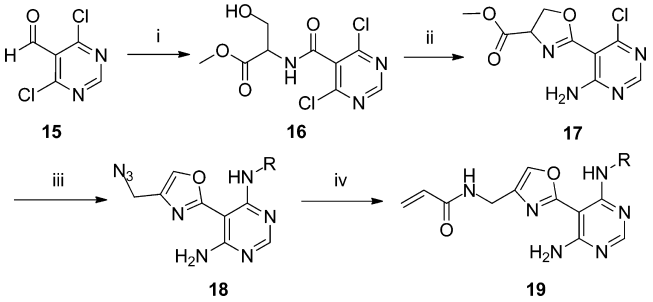


Figure 3. Binding mode of **10d** (a) and its analogue with amine at the Y position (b) to the active site of EGFR crystal structure cocrystallized with **1** (PDB entry 1XKK).

Scheme 3[a]



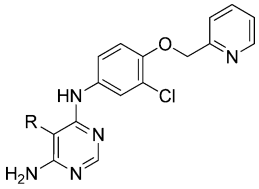
[a]Reagents and conditions: (i) (a) $SOCl_2$, AIBN, CCl_4, 80 °C; (b) DL-serine methyl ester hydrochloride, DIPEA, THF, 0 °C to rt; (ii) (a) NH_3 (gas), toluene/EtOH (1:1), sealed tube, 60 °C; (b) DAST, DCM, −78 to 0 °C; (iii) (a) R-NH_2, 4 N HCl in 1,4-dioxane, *t*-BuOH, 60 °C; (b) DBU, $CBrCl_3$, DCM, −40 °C to rt; (c) LAH, THF, 0 °C; (d) NaN_3, PPh_3, DMF/CCl_4 (4:1), 90 °C; (iv) (a) PPh_3, H_2O, THF, 60 °C; (b) acryloyl chloride, $NaHCO_3$, aq THF (THF/H_2O = 5:1), 0 °C.

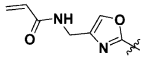
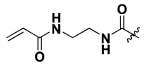
bromotrichloromethane and DBU. After the reduction of methyl ester in **17** and the substitution of alcohol to azide, the resulting azide moiety in azidomethyloxazole **18** was reduced to amine in the presence of PPh_3 and subsequently N-acryloylated to obtain the desired analogue **19**.

Compound **19a**, an analogue of **10d** with an amine moiety at the C6 position of pyrimidine, showed 10-fold and 14-fold enhancement in its antiproliferative activity compared to **10d** in A431 (IC_{50} of 10.8 vs 105 nM) and SK-Br3 (IC_{50} of 0.7 vs 9.7 nM) cell lines, respectively (Table 2). Compound **21** with an amide moiety as a bioisostere of oxazole was also prepared to ensure the significance of oxazole's conformation (see Scheme S1). As shown in Table 2, amide analogue **21** showed no antiproliferative activity in both cancer cell lines (entry 2, IC_{50} > 1000 nM).

After significant enhancement of its antiproliferative activity via the introduction of an amine moiety at the C6 position of the pyrimidine scaffold, we turned our attention to the structure−activity relationship study of substituents (R^1, R^2, and R^3 moieties) on the aryl ring of compound **19**. As stated earlier, the introduction of small electron-withdrawing lipophilic groups such as chloride or fluoride at the R^2 position increases HER-1/HER-2 inhibitory activity and the introduction of bulkier moieties such as pyridin-2-ylmethoxy or substituted benzyloxy at the R^1 position increases HER-2 inhibitory activity. In addition, we aimed to develop an orally available anticancer agent with respect to the patients'

Table 2. In Vitro Cellular Activity[a] **of Analogues 19a and 21**



Entry	Compd	R	Cell assay, IC_{50} (nM) A431[b]	SK-Br3[c]
1	**19a**		10.8	0.7
2	**21**		>1,000	>1,000
	1[d]	-	104	29

[a]All biological data are mean values of three independent experiments performed in duplicate. [b]A431 is HER-1-overexpressing human vaginal epidermoid cancer cell line. [c]SK-Br3 is HER-2-overexpressing human breast cancer cell line. [d]**1** was synthesized using the synthetic protocol of WO99/35146.

convenience. Because the first-pass metabolism by liver is one of the major causes of poor oral bioavailability and short in vivo half-life, the in vitro metabolic stability using liver microsome is one of the efficient in vitro guidelines for the evaluation of oral bioavailability and used in a prospective manner to choose the potential candidates for further development with desirable pharmacokinetic properties including long half-life time and low oral clearance.[24,25] Therefore, we focused on the improvement of HER-1/HER-2 potency and liver microsomal stability through the substituent modification at the R^1, R^2, and R^3 positions of compound **19**. Analogues **19b−r** were synthesized in a similar manner as **19a** for the systematic introduction of various substituents such as halogen, alkyl, pyridylmethyloxy, benzyloxy, phenyloxy, or heteroaryloxy on phenyl moiety and subjected to the cell-based growth inhibition assay using the HER-1-positive A431 cell line and the HER-2-positive SK-Br3 cell line, along with the microsomal stability.

As shown in Table 3, the HER-1/HER-2 cellular inhibitory activity was decreased by the replacement of 3-chloro (**19a**, IC_{50} = 11 nM for A431 and IC_{50} = 0.7 nM for SK-Br3) with 3-fluoro (**19b**, IC_{50} = 63 nM for A431 and IC_{50} = 6.1 nM for SK-Br3) at the R^2 position or the replacement of 4-(2-pyridine) (**19a**) with 4-(3-fluorophenyl) (**19c**, IC_{50} = 63 nM for A431 and IC_{50} = 6.1 nM for SK-Br3) at the R^1 position, which shares the same structure−activity relationship pattern of phenyl

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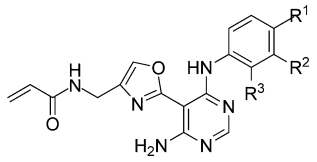
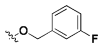
Table 3. In Vitro Cellular Activity[a] and Microsomal Stability of Analogues 19



Entry	Compd	R^1	R^2	R^3	Cell assay IC_{50} (nM)		Microsomal stability[d] (min)			
					A431[b]	SK-Br3[c]	Mouse	Rat	Dog	Human
1	**19a**		Cl	-	11	0.7	17	23	46	25
2	**19b**		F	-	63	6.1	9	16	44	27
3	**19c**		Cl	-	59	3.9	NT[e]	29	*[f]	45
4	**19d**		Cl	-	66	4.0	39	25	51	*
5	**19e**		Cl	-	65	3.3	*	*	*	*
6	**19f**		Cl	-	60	3.1	48	59	*	*
7	**19g**		Cl	-	184	1.9	57	51	*	96
8	**19h**		Cl	-	105	6.5	NT	NT	NT	NT
9	**19i**		Cl	-	113	18.2	NT	NT	NT	NT
10	**19j**		Cl	-	29	4.7	49	44	65	45
11	**19k**		Cl	-	75	0.5	*	54	*	*
12	**19l**		Cl	-	39	6.0	*	66	*	*
13	**19m**		Cl	-	36	1.7	15	16	24	39
14	**19n**		Cl	-	68	2.4	18	9	39	15
15	**19o**		Cl	-	68	6.1	NT	NT	NT	NT
16	**19p**		Me	-	64	1.1	43	17	41	39
17	**19q**	F	Cl	-	52	34.1	9	24	9	16
18	**19r**	Cl	Cl	F	282	92.6	74	*	*	*

[a]All biological data are mean values of three independent experiments performed in duplicate. [b]A431 is HER-1-overexpressing human vaginal epidermoid cancer cell line. [c]SK-Br3 is HER-2-overexpressing human breast cancer cell line. [d]Half-life (HL) of metabolism by incubation of parent molecule with liver microsomes of mouse, rat, dog, and human (duplicate). [e]NT means "not tested". [f]The asterisk (*) means very stable with a HL of >100 min.

substituents in the case of **1**. The reduced anticancer activities were observed in a series of analogues **19d–p** substituted with 4-phenyloxy or 4-heteroaryloxy at the R^1 position. Analogue **19e** containing the 3-trifluoromethyl-1*H*-pyrazol-5-yloxy moiety had excellent microsomal stability, but this compound also showed extremely high affinity to plasma proteins (PPB = 100%, data not shown). Compound **19k** showed relatively low growth inhibitory activity in A431 cells, but it showed comparable SK-Br3 cellular activity with improved microsomal stability. Analogues **19q–r** with small anilines containing only fluoro or chloro substituents at the R^1 position showed relatively low cellular HER-2 inhibitory activity (for SK-Br3: IC_{50}(**19q**) = 34.1 nM and IC_{50}(**19r**) = 92.6 nM vs IC_{50}(**19a**) = 0.7 nM). Among these analogues, **19a** showed the highest potency in growth inhibition of A431 and SK-Br3 cells with acceptable microsomal stability.

Pharmacokinetic Studies of 19a. Prior to further evaluation, our leading compound **19a** was subjected to pharmacokinetic studies in ICR (imprinting control region) mice and SD (Sprague–Dawley) rats. As shown in Table 4, pharmacokinetic parameters of **19a** were determined after a single oral administration (10 mg/kg for mouse and 5 mg/kg for rat) and intravenous injection (2 mg/kg for mouse and 1 mg/kg for rat). Compound **19a** demonstrated fair oral bioavailability with >20% (21.6% for mouse and 21.9% for rat) and desirable exposure levels (C_{max} and AUC) in mice and rats.

Enzymatic Activity and EGFR Irreversible Inhibition of 19a. We examined the specificity of **19a** conducting in vitro

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Table 4. Pharmacokinetic Profile of 19a[a] in Mouse and Rat

parameters (po)	mouse[b]	rat[c]
dose (mg/kg)	10	5
AUC_{last} (ng·h/mL)	454.8	439.7
C_{max} (ng/mL)	282.5	189.0
$T_{1/2}$ (h)	27.7	3.23
F (%)	21.6	21.9

[a]**19a** was treated as HCl salt in a vehicle as a solution of 30% PEG400 and 5% ethanol in distilled water. [b]The pharmacokinetic parameters were determined after a single oral administration (10 mg/kg, n = 3) and intravenous injection (2 mg/kg, n = 3) in ICR mice. [c]The pharmacokinetic parameters were determined after a single oral administration (5 mg/kg, n = 3) and intravenous injection (1 mg/kg, n = 3) in SD rats.

assays against purified tyrosine kinases of the EGFR family (Table 5) and against a panel of other protein kinases (Table 6). **19a** effectively inhibited EGFR family kinases with IC_{50} values of 3.3, 3.2, 22.4, and 4.0 nM against $EGFR^{WT}$, $EGFR^{T790M}$, HER-2, and HER-4, respectively. In particular, **19a** showed excellent inhibitory activity against mutated EGFR ($EGFR^{T790M}$) and HER-4, both of which have been resistant to **1**. **19a** also showed excellent selectivity over other 20 kinases confirmed by in vitro enzymatic assays (IC_{50} > 3 μM except for Blk).

Table 5. In Vitro Enzymatic Activity[a] of 19a

	enzyme assay, IC_{50} (nM)			
	EGFR			
compd	WT	T790M	HER-2	HER-4
19a	3.3	3.2	22.4	4.0
1[b]	52.2	>1000	36.3	>1000

[a]All biological data are mean values of three independent experiments performed in duplicate. [b]**1** was synthesized using the synthetic protocol of WO99/35146.

Table 6. Selectivity Profile of 19a against 20 Kinases[a]

kinase	% inhibition at 3 μM
Abl	15
AMPKα1	0
Aurora-A	42
Blk	77
CDK1/cyclinB	0
cKIT	0
FAK	0
FGFR1	0
Flt1	15
Flt3	0
IGF-1R	0
IR	0
JAK2	0
LKB1	0
MEK1	14
Met	13
PDGFRα	0
PDGFRβ	0
Pim-1	2
Syk	15

[a]This test was conducted by Millipore Kinase Profiling Service.

19a is an oxazolylpyrimidine-based HER-1/HER-2 dual inhibitor containing a functional α,β-unsaturated amide as Michael acceptor. This moiety is responsible for the irreversible inhibition of **19a** via the covalent modifications at the active site (Cys773 of EGFR and Cys805 of HER-2) of EGFR tyrosine kinase domains in a manner similar to that with other irreversible EGFR inhibitors, such as **2** and **3**. To validate this hypothesis, the phosphorylation levels (% relative to positive control) of EGFR tyrosine kinases (EGFR and HER-2) in EGFR-overexpressing A431 and HER-2-overexpressing SK-Br3 cells were measured immediately or in 8 h after the removal of **19a** from the medium. We observed the prolonged inhibitory effect on the phosphorylation of EGFR (97.5% inhibition at 0 h and 87.5% at 8 h after medium washing) and HER-2 (87.2% inhibition at 0 h and 68.2% at 8 h after medium washing) upon treatment of **19a** in both A431 and SK-Br3 cells, which might be caused by the irreversible modification of **19a** to EGFR and HER-2 (see Figure S1).

Cellular Activity of 19a. On the basis of the results of the cellular growth inhibition assays in A431 and SK-Br3 cell lines along with in vitro/in vivo pharmacokinetic study, we selected compound **19a** for further biological evaluation as a potential HER-1/HER-2-targeting therapeutic agent. The effects of **19a** were evaluated using a panel of cancer cell lines, which express various levels of HER-2 and other genetic information with sensitivity or resistance to **1**. **19a** showed excellent growth inhibitory activity in lapatinib-sensitive breast (MDA-MB-175, IC_{50} = 1.6 nM) and gastric (NCI-N87, IC_{50} = 3.0 nM) cancer cell lines with 12- to 27-fold superiority to **1**.

HER-2-directed therapy including **1** is an important treatment for breast cancer. However, some tumors do not respond or develop resistance to this agent.[26] Although the overexpression of HER-2 correlates with sensitivity to the growth inhibition by **1**, some cancer cells that overexpress HER-2 do not respond to **1**.[27] Even though the factors that confer primary or acquired resistance to **1** are not well characterized, several hypotheses including low PTEN expression, *PIK3CA* mutations, or ER (estrogen receptor) activation are reported to be associated with resistance in HER-2-positive breast cancer. Analogue **19a** showed excellent growth inhibitory activity with IC_{50} of 5.0 nM in lapatinib-resistant MDA-MB-453 cell line (IC_{50}(**1**) = 555 nM) (Table 7), which is conferred by low PTEN expression and *PIK3CA* mutations with high expression of HER-2. But **19a** did not induce the growth inhibitory effect in JIMT-1 cells with low HER-2 expression level (IC_{50}(**19a**) = 1880 nM and IC_{50}(**1**) > 10000 nM) and MDA-MB-361 cells with ER activation (IC_{50}(**19a**) = 562 nM and IC_{50}(**1**) = 1029 nM). In addition, analogue **19a** induced the growth inhibition in HER-2-mutated (G776insV_G/C, NCI-H1781, IC_{50} = 22 nM) and EGFR-mutated (L858R/T790M, NCI-H1975, IC_{50} = 40 nM) NSCLC cell lines with resistance to **1** (IC_{50}(NCI-H1781) = 3047 nM and IC_{50}(NCI-H1975) > 1000 nM).

SUMMARY

HER-2 is overexpressed in 20−25% of metastatic breast or gastric cancers and is associated with an aggressive tumor type and reduced survival rate. Therefore, HER-2-targeted therapy such as trastuzumab or lapatinib is beneficial for cancer patients with high HER-2 expression level. However, most cancers that initially respond to HER-2-targeted therapy begin to progress

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Table 7. In Vitro Cellular Activities[a] of 19a

tissue	cell line	character	IC_{50} (nM) 19a	IC_{50} (nM) 1[b]
breast cancer	MDA-MB-175	Src++, Ret+++, low HER-2	1.6	44.4
	MDA-MB-361	Her-2++, Src+++, *PIK3CA* mutation, ER activation	562.0	1029
	MDA-MB-453	HER-2++, PTEN deficiency, *PIK3CA* mutation	5.0	555.0
	JIMT-1	low HER-2	1,880	>10000
gastric cancer	N87	HER-2+++	3.0	36
NSCLC[c]	H1781	HER-2 G776insV_G/C	22.0	3047
	H1975	EGFR L858R/T790M	40.0	>1000

[a]All biological data are mean values of three independent experiments performed in duplicate. [b]**1** was synthesized using the synthetic protocol of WO99/35146. [c]NSCLC: non-small-cell lung cancer.

again within 1 year. The several mechanisms by HER-2 therapy contribute to address this resistance. Herein, we have described the synthesis of a novel series of N^4-(3-chlorophenyl)-5-(oxazol-2-yl)pyrimidine-4,6-diamines which are explored as HER-1/HER-2 dual inhibitors for the treatment of resistant patients to the current HER-2-targeted therapy, lapatinib. The selected compound (**19a**) showed excellent HER-1 and HER-2 inhibition activity and also showed excellent selectivity in an enzyme-based inhibition assay with a panel of 20 kinases. We confirmed that our leading HER-1/HER-2 dual inhibitor (**19a**) has an irreversible binding mode to HER-1 and HER-2 confirmed by a cell washout test in A431 and SK-Br3 cell lines. In contrast to the currently approved HER-2-targeted agent, our selected compound (**19a**) is an irreversible small-molecule inhibitor of HER-1 and HER-2 tyrosine kinases with the potential to overcome clinically relevant, mutation-induced drug resistance. **19a** showed excellent inhibitory activity toward cellular growth of lapatinib-sensitive or resistant cancer cells. The excellent pharmacokinetic profiles of **19a** in rats and mice clearly demonstrate its merit for further investigation as a novel therapeutic agent for HER-2-targeting treatment of solid tumors, especially HER-2-positive breast or gastric cancer.

EXPERIMENTAL SECTION

Chemistry. General Information. All commercial chemicals and solvents were reagent grade and were used without further purification unless otherwise specified. All reported yields are isolated yields after flash column chromatography or crystallization. ^{1}H spectra were recorded on a Bruker DRX-300 [Bruker Biospin, Germany]. Chemical shifts are reported in ppm relative to the residual solvent peak ($CDCl_3$, TMS: 0.00). Multiplicity was indicated as follows: s (singlet); d (doublet); t (triplet); q (quartet); m (multiplet); dd (doublet of doublet); dt (triplet of doublet); td (doublet of triplet); br s (broad singlet); etc. Products were purified by flash column chromatography on silica gel (230−400 mesh). The eluent used for purification is reported in parentheses. The purity of all synthesized compounds (>95% area) was analyzed by high performance liquid chromatography using a Shimadzu HPLC instrument equipped with a reverse phase column (XDB C18, 5 μm, 4.6 mm × 150 mm) and a UV detector (254 nm). HPLC column was equilibrated with 5% acetonitrile (CAN) in H_2O (0.1% trifluoroacetic acid, TFA) for 5 min. After the injection of 10 μL of individual samples, the eluent was changed from 5% ACN in H_2O (0.1% TFA) to 100% ACN (0.1% TFA) over 45 min with a flow rate of 1.0 mL/min. Thin-layer chromatography (TLC) was performed on precoated glass-backed plates (silica gel 60 F_{254}, 0.25 mm), and the components were visualized under UV light (254 and 365 nm) or by treating the plates with anisaldehyde, $KMnO_4$, and phosphomolybdic acid, followed by heating. Distilled water was treated using ion exchange and filtration.

***N*-(3-(4-((3-Chloro-4-(pyridin-2-ylmethoxy)phenyl)amino)-pyrimidin-5-yl)prop-2-yn-1-yl)acrylamide (10a).** ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.95 (s, 1H), 8.83 (s, 1H), 8.62−8.60 (m, 1H), 7.79−7.76 (m, 1H), 7.68−7.65 (m, 1H), 7.47−7.46 (m, 1H), 7.17−7.14 (m, 1H), 6.62 (s, 1H), 6.31−6.25 (m, 1H), 6.11−6.07 (m, 1H), 5.75 (m, 1H), 5.71−5.68 (m, 1H), 5.36 (s, 2H), 4.59−4.57 (d, 2H). MS (ESI^+): m/z = 420.7 $[M + H]^+$.

***N*-(3-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)phenylamino)-pyrimidin-5-yl)phenyl)acrylamide (10b).** *Step 1. Preparation of 5-(3-Aminophenyl)-N-(3-chloro-4-(pyridin-2-ylmethoxy)phenyl)-pyrimidin-4-amine.* 300 mg of compound **9** and 142 mg of 3-aminophenylboronic acid hydrochloride were dissolved in 6 mL of methanol, and 7.7 mg of palladium acetate and 284 mg of potassium carbonate were added thereto. The mixture was stirred at 70 °C for 2 h. After the reaction was completed, the reaction mixture was condensed under a reduced pressure, and the resulting residue was subjected to flash column chromatography ($CHCl_3/CH_3OH$ = 15: 1) to obtain the title compound (120 mg, yield 44%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.70 (s, 1H), 8.60 (d, 1H), 8.21 (s, 1H), 7.76 (t, 2H), 7.69 (d, 1H), 7.65 (d, 1H), 7.34 (m, 2H), 7.24 (d, 1H), 6.97 (d, 1H), 6.80 (m, 1H), 6.73 (s, 1H), 5.28 (s, 2H), 3.88 (s, 2H).

*Step 2. Preparation of N-(3-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)-phenylamino)pyrimidin-5-yl)phenyl)acrylamide (**10b**).* 51 mg of the compound obtained in step 1 and 32 mg of sodium bicarbonate were dissolved in a mixture of 2 mL of tetrahydrofuran (THF) and 0.5 mL of distilled water at 0 °C. Then 10 μL of acryloyl chloride was added to the mixture and stirred for 30 min. After the reaction was completed, the resulting mixture was added to a saturated aqueous solution of sodium bicarbonate and extracted twice with chloroform. The separated organic layer was washed with water and saturated saline, dried over anhydrous sodium sulfate, filtered, and distilled under a reduced pressure. The resulting residue was subjected to flash column chromatography ($CHCl_3/CH_3OH$ = 15: 1) to obtain the title compound (12 mg, yield 21%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.68 (s, 1H), 8.59 (d, 1H), 8.19 (s, 1H), 8.13 (s, 1H), 7.74 (m, 3H), 7.65 (t, 2H), 7.50 (t, 1H), 7.35 (m, 1H), 7.22 (m, 2H), 6.95 (s, 1H), 6.94 (d, 1H), 6.48 (d, 1H), 6.33 (m, 1H), 5.82 (d, 2H), 5.26 (s, 2H). MS (ESI^+): m/z = 458.1 $[M + H]^+$.

***N*-((5-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)phenylamino)-pyrimidin-5-yl)isooxazol-3-yl)methyl)acrylamide (10c).** *Step 1. Preparation of N-(3-Chloro-4-(pyridin-2-ylmethoxy)phenyl)-5-((trimethylsilyl)ethinyl)pyrimidin-4-amine.* 5.67 g of the compound **9**, 181 mg of dichlorobis(triphenylphosphine)palladium-(II), and 98 mg of copper(I) iodide were dissolved in 60 mL of THF, and 7.2 mL of triethylamine and 2.15 mL of trimethylsilylacetylene were added to the mixture. The mixture was stirred at room temperature for 24 h. After the reaction was completed, a saturated aqueous solution of sodium bicarbonate was added thereto, and the resulting mixture was extracted twice with ethyl acetate. The separated organic layer was washed with water and saturated saline, dried over anhydrous sodium sulfate, filtered, and distilled under a reduced pressure. The resulting residue was subjected to column chromatography (ethyl acetate/hexanes = 1:10) to obtain the title compound (1.8 g, yield 34%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.62 (s, 1H), 8.60 (d, 1H), 8.41 (s, 1H), 7.80 (d, 1H), 7.75 (m, 1H), 7.65 (d,

1H), 7.37 (m, 1H), 7.23 (m, 2H), 7.00 (d, 1H), 5.29 (s, 2H), 1.77 (s, 1H), 0.32 (s, 9H).

Step 2. Preparation of N-(3-Chloro-4-(pyridin-2-ylmethoxy)-phenyl)-5-ethinyl pyrimidin-4-amine. 1.8 g of the compound obtained in step 1 was dissolved in 30 mL of THF, and 8.8 mL of 1.0 M tetrabutylammonium fluoride solution in THF was added thereto. The mixture was stirred at room temperature for 1 h. After the reaction completion monitored by TLC, the reaction was quenched by the addition of saturated aqueous solution of sodium bicarbonate. The reaction mixture was extracted twice with ethyl acetate, and the combined organic layer was dried over anhydrous sodium sulfate, filtered, and distilled under a reduced pressure. The resulting residue was subjected to flash column chromatography (ethyl acetate/hexanes = 1:1) to obtain the title compound (1.4 g, yield 94%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.65 (s, 1H), 8.60 (m, 1H), 8.45 (s, 1H), 7.76 (m, 2H), 7.64 (d, 1H), 7.41 (m, 1H), 7.24 (m, 2H), 6.99 (d, 1H), 5.29 (s, 2H), 3.67 (s, 1H).

Step 3. Preparation of 2-((5-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)phenylamino)pyrimidin-5-yl)isooxazol-3-yl)methyl)-isoindoline-1,3-dione. 6.8 mL of oxalylchloride was dissolved in 250 mL of CH_2Cl_2, and the mixture was cooled to −78 °C. Then 8.9 mL of dimethyl sulfoxide was added to the mixture. The mixture was stirred for 10 min. 10 g of *N*-(2-hydroxyethyl)phthalimide was gradually added to the mixture, and the mixture was stirred for 30 min, and 36.5 mL of triethylamine was added thereto. The resulting mixture was heated to 0 °C and stirred for 1 h. After the reaction was completed, the resulting mixture was added to a saturated aqueous solution of NH_4Cl and extracted twice with CH_2Cl_2. The combined organic layer was dried over anhydrous sodium sulfate, filtered, and condensed under a reduced pressure. 5.5 g of the solid thus obtained was dissolved in 50 mL of pyridine, and 6.1 g of hydroxylamine hydrochloride was added thereto, followed by stirring at 65 °C for 5 h. After the reaction was completed, the reaction mixture was condensed under a reduced pressure, ethyl acetate was added thereto, and the mixture was washed three times with water. The combined organic layer was dried over anhydrous sodium sulfate, filtered, and condensed under a reduced pressure. The resulting residue was crystallized in diethyl ether. The obtained solid was filtered, washed with diethyl ether, and dried under a reduced pressure. 588 mg of the solid was dissolved in 10 mL of THF, and 595 mg of *N*-chlorosuccinimide and 36 μL of pyridine were added thereto, followed by stirring at 70 °C for 2 h. 500 mg of the compound obtained in step 2 and 621 μL of triethylamine were added to the mixture, and the mixture was stirred at 70 °C for 12 h. After the reaction completion monitored by TLC, the reaction mixture was condensed under a reduced pressure and the resulting residue was subjected to flash column chromatography (ethyl acetate/hexanes = 1:1) to obtain the desired compound (450 mg, yield 56%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.84 (s, 1H), 8.61 (s, 1H), 8.59 (s, 1H), 8.56 (d, 1H), 7.90 (m, 5H), 7.67 (d, 1H), 7.54 (d, 1H), 7.40 (m, 1H), 7.35 (m, 1H), 7.18 (d, 1H), 7.05 (s, 1H), 5.25 (d, 2H), 4.92 (d, 2H).

Step 4. Preparation of 5-(3-(Aminomethyl)isooxazol-5-yl)-N-(3-chloro-4-(pyridin-2-ylmethoxy)phenyl)pyrimidin-4-amine. 450 mg of the compound obtained in step 3 was dissolved in 10 mL of ethanol, and 203 μL of hydrazine hydrate was added thereto, followed by stirring at 70 °C for 1 h. After the reaction completion monitored by TLC, the reaction mixture was cooled to 0 °C and the solid thus obtained was filtered and washed with chloroform. The filtrate therefrom was condensed under a reduced pressure. The resulting residue was subjected to flash column chromatography ($CHCl_3$/CH_3OH = 15:1) to obtain the title compound (200 mg, yield 59%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.79 (m, 1H), 8.61 (s, 1H), 8.57 (d, 1H), 7.86 (t, 1H), 7.71 (d, 1H), 7.55 (d, 1H), 7.45 (m, 1H), 7.35 (t, 1H), 7.20 (d, 1H), 7.04 (s, 1H), 5.26 (d, 2H), 3.79 (s, 2H).

*Step 5. Preparation of N-((5-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)phenylamino)pyrimidin-5-yl)isooxazol-3-yl)methyl)-acrylamide (**10c**).* 42 mg of acrylic acid was dissolved in 10 mL of CH_2Cl_2, and 140 mg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, 20 mg of *N*-hydroxybenzotriazole, and 255 μL of *N*,*N*-diisopropylethylamine were added thereto, followed by stirring at room temperature for 10 min. 200 mg of the compound obtained in step 4 was added to the mixture, and the mixture was stirred at room temperature for 3 h. After the reaction completion monitored by TLC, the resulting mixture was added to a saturated aqueous solution of sodium bicarbonate and extracted twice with chloroform. The combined organic layer was dried over anhydrous sodium sulfate, filtered, and condensed under a reduced pressure. The resulting residue was subjected to silica gel flash column chromatography ($CHCl_3$/CH_3OH = 50:1) to obtain the title compound (86 mg, yield 38%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.67 (s, 1H), 8.59 (s, 1H), 8.57 (s, 1H), 7.74 (m, 2H), 7.67 (d, 1H), 7.38 (m, 1H), 7.27 (m, 1H), 7.01 (m, 2H), 6.71 (s, 1H), 6.37 (m, 1H), 6.20 (m, 1H), 5.74 (m, 1H), 5.28 (s, 2H), 4.64 (d, 2H). MS (ESI$^+$): m/z = 463.1 [M + H]$^+$.

***N*-((2-(4-(3-Chloro-4-(pyridin-2-ylmethoxy)phenylamino)-pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (10d).** ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.56 (s, 1H), 8.95 (s, 1H), 8.72 (s, 1H), 8.59 (d, 1H), 7.86 (s, 1H), 7.76−7.67 (m, 3H), 7.53 (dd, 1H), 7.00 (d, 1H), 6.40 (s, 1H), 6.19 (s, 1H), 5.73 (d, 1H), 5.30 (s, 2H), 4.56 (d, 2H). MS (ESI$^+$): m/z = 463.3 [M + H]$^+$.

Methyl 2-(4,6-Dichloropyrimidin-5-carboxamido)-3-hydroxypropanoate (16). 4,6-Dichloropyrimidin-5-carbaldehyde **15** (10 g) was dissolved in 100 mL of carbon tetrachloride, and 7.8 mL of sulfuryl chloride and 0.51 g of 2,2-azobis(2-methylpropionitrile) were added thereto, followed by stirring under reflux conditions at 80 °C for 1.5 h. After the reaction completion monitored by TLC, the reaction mixture was cooled to room temperature and filtered under a reduced pressure. The filtrate was condensed under a reduced pressure to obtain 4,6-dichloropyrimidin-5-carbonyl chloride without further purification (11.4 g, yield 95%). The resulting 4,6-dichloropyrimidin-5-carbonyl chloride (8.6 g) and DL-serine methyl ester (12.6 g) were dissolved in 100 mL of THF, and 14.2 mL of *N*,*N*-diisopropylethylamine was added thereto dropwise at 0 °C. Then the reaction mixture was heated to room temperature, followed by stirring for 30 min. After the reaction completion monitored by TLC, the reaction mixture was added to water and extracted twice with chloroform. The combined organic layer was washed with water and saturated saline and dried over anhydrous sodium sulfate, followed by filtration and condensation under a reduced pressure. The obtained residue was subjected to silica gel flash column chromatography (ethyl acetate/CH_2Cl_2/CH_3OH = 30:30:1) to obtain the title compound (10.2 g, yield 85%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.31 (d, 1H), 8.96 (s, 1H), 5.15 (s, 1H), 4.58 (m, 1H), 3.85 (m, 1H), 3.68 (s, 3H), 3.67 (m, 1H).

Methyl 2-(4-Amino-6-chloropyrimidin-5-yl)-4,5-dihydrooxazol-4-carboxylate (17). Compound **16** (7.0 g) was dissolved in 50 mL of toluene and 50 mL of ethanol in a sealed tube, and ammonia gas was bubbled through the solution, followed by stirring at 60 °C for 2 h. Ammonia gas was further bubbled into the solution until the reaction was substantially completed, and the reaction mixture continued to be stirred at the same temperature for an additional 1 h. After the reaction completion monitored by TLC, the reaction mixture was condensed under a reduced pressure to obtain methyl 2-(4-amino-6-chloropyrimidin-5-carboxamido)-3-hydroxypropanoate (6.54 g). The resulting compound (5.0 g) was dissolved in 50 mL of CH_2Cl_2, and 2.65 mL of (diethylamino)sulfur trifluoride was added thereto dropwise at −78 °C. The reaction mixture was slowly warmed up to 0 °C and stirred for 2.5 h. After the reaction was completed, the reaction mixture was basificated (pH 8) with a saturated aqueous solution of sodium bicarbonate and extracted twice with CH_2Cl_2. The combined organic layer was dried over anhydrous sodium sulfate, filtered, and condensed under a reduced pressure. The obtained residue was crystallized with a mixed solvent of ethyl acetate and diethyl ether (v/v, 3:1), followed by filtering and drying under a reduced pressure to obtain the desired compound (2.38 g, yield 51%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.70 (s, 1H), 8.30 (s, 1H), 6.10 (s, 1H), 4.97 (m, 1H), 4.63 (m, 2H), 3.82 (s, 3H).

***N*-((2-(4-Amino-6-(3-chloro-4-(pyridin-2-ylmethoxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19a).** *Step 1. Preparation of Methyl 2-(4-Amino-6-(3-chloro-4-(pyridin-2-ylmethoxy)phenylamino)pyrimidin-5-yl)-4,5-dihydrooxazol-4-carboxylate.* Compound **17** (1.5 g) and 3-chloro-4-(pyridin-2-ylmethoxy)benzenamine **8** (1.37 g) were dissolved

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in 25 mL of *tert*-butanol, and 0.73 mL of 4 N hydrochloric acid solution in 1,4-dioxane was added thereto dropwise. The mixture was heated to 70 °C and stirred for 1.5 h, followed by condensation under a reduced pressure when the reaction was completed. After removal of solvents, 25 mL of 2-propanol was added thereto, followed by stirring at 0 °C for 2 h. The resulting solid was washed with 2-propanol, followed by filtering and drying under a reduced pressure to obtain the title compound (2.10 g, yield 79%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.24 (s, 1H), 8.63 (d, 1H), 8.26 (s, 1H), 8.10 (m, 1H), 7.95 (m, 1H), 7.75 (d, 1H), 7.61 (d, 1H), 7.43 (m, 1H), 7.32 (m, 1H), 7.25 (d, 1H), 5.31 (s, 2H), 5.05 (m, 1H), 4.63 (m, 2H), 3.71 (s, 3H).

Step 2. Preparation of Methyl 2-(4-Amino-6-(3-chloro-4-(pyridin-2-ylmethoxy)phenylamino)pyrimidin-5-yl)oxazol-4-carboxylate. 2.0 g of the compound obtained in step 1 was dissolved in 40 mL of dichloromethane, and 1.9 mL of 1,8-diazabicyclo[5.4.0]undec-7-ene was added thereto at −40 °C, followed by adding thereto 1.3 mL of bromotrichloromethane dropwise for 20 min. The mixture was warmed to room temperature and stirred for 2 h. After the reaction completion monitored by TLC, the reaction mixture was condensed under a reduced pressure and then crystallized with ethyl acetate. The resulting solid was washed with ethyl acetate, followed by filtering and drying under a reduced pressure to obtain the desired compound (0.4 g, yield 20%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.16 (s, 1H), 8.88 (s, 1H), 8.58 (m, 1H), 8.11 (m, 1H), 7.85 (m, 2H), 7.54 (m, 3H), 7.37 (m, 2H), 7.22 (d, 1H), 5.26 (s, 2H), 3.87 (s, 3H).

Step 3. Preparation of (2-(4-Amino-6-(3-chloro-4-(pyridin-2-ylmethoxy)phenylamino)pyrimidin-5-yl)oxazol-4-yl)methanol. 0.35 g of the compound obtained in step 2 was dissolved in 10 mL of dichloromethane at 0 °C, and 1.6 mL of 1.0 M lithium aluminum hydride ether solution was added dropwise thereto. The reaction mixture was stirred at the same temperature for 1 h. After the reaction completion monitored by TLC, an aqueous solution of Rochelle salt (potassium sodium tartrate, $NaKC_4H_4O_6$) was added thereto. The resulting mixture was stirred for 1 h and extracted twice with a mixed solvent of chloroform and 2-propanol (v/v, 3:1). The combined organic layer was washed with distilled water and saturated saline and dried over anhydrous sodium sulfate, followed by filtering and distilling under a reduced pressure. The obtained residue was subjected to silica gel flash column chromatography (ethyl acetate/CH_2Cl_2/CH_3OH = 10:10:1) to obtain the title compound (0.27 g, yield 82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.70 (s, 1H), 8.58 (d, 1H), 8.15 (s, 1H), 7.72 (m, 3H), 7.64 (d, 1H), 7.33 (m, 1H), 7.26 (m, 1H), 6.92 (d, 1H), 5.25 (s, 2H), 4.72 (s, 2H).

Step 4. Preparation of (5-(4-(Aminomethyl)oxazol-2-yl)-N4-(3-chloro-4-(pyridin-2-ylmethoxy)phenyl)pyrimidin-4,6-diamine. 0.27 g of the compound obtained in step 3 was dissolved in 10 mL of *N*,*N*-dimethylformamide and 2 mL of carbon tetrachloride, and 50 mg of sodium azide and 400 mg of triphenylphosphine were added thereto. The reaction mixture was heated to 90 °C and stirred for 30 min. Subsequently, the mixture was condensed under a reduced pressure to remove carbon tetrachloride, and distilled water was added thereto, followed by the extraction twice with ethyl acetate. The combined organic layer was washed with water and saturated saline and dried over anhydrous sodium sulfate, followed by filtering and condensing under a reduced pressure. The obtained residue was dried under a reduced pressure, dissolved in 10 mL of THF, and 437 mg of triphenylphosphine and 0.1 mL of distilled water were added thereto, followed by stirring at 60 °C for 2 h. After the reaction completion monitored by TLC, the reaction mixture was condensed under a reduced pressure and then subjected to silica gel flash column chromatography ($CHCl_3$/CH_3OH = 1:1) to obtain the desired compound (50 mg, yield 19%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.71 (s, 1H), 8.58 (d, 1H), 8.19 (s, 1H), 7.72 (m, 2H), 7.64 (d, 2H), 7.41 (m, 1H), 7.24 (m, 1H), 6.98 (d, 1H), 5.28 (s, 2H), 3.90 (s, 2H). MS (ESI$^+$): m/z = 424.3 [M + H]$^+$.

Step 5. Preparation of N-((2-(4-Amino-6-(3-chloro-4-(pyridin-2-ylmethoxy)phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)-acrylamide (19a). 45 mg of the compound obtained in step 4 and 27 mg of sodium bicarbonate were dissolved in 2 mL of THF and 0.5 mL of distilled water at 0 °C, and 8.6 μL of acryloyl chloride was added thereto, followed by stirring at the same temperature for 10 min. After the reaction was completed, a saturated aqueous solution of sodium bicarbonate was added thereto and the mixture was extracted twice with chloroform. The isolated layer was washed with water and saturated saline, dried over anhydrous sodium sulfate, followed by filtering and condensing under a reduced pressure. The resulting residue was subjected to silica gel flash column chromatography ($CHCl_3$/CH_3OH = 15:1) to obtain the desired compound **19a** (20 mg, yield 39%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.65 (s, 1H), 8.59 (d, 1H), 8.18 (s, 1H), 7.72 (m, 3H), 7.65 (d, 1H), 7.41 (m, 1H), 7.24 (m, 1H), 6.96 (d, 1H), 6.35 (m, 1H), 6.13 (m, 2H), 5.70 (m, 2H), 5.27 (s, 2H), 4.53 (d, 2H). MS (ESI$^+$): m/z = 478.2 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-fluoro-4-(pyridin-2-ylmethoxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19b).** The similar procedure of **19a** was repeated to obtain the compound **19b** (40 mg, final yield 51%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.69 (s, 1H), 8.59 (d, 1H), 8.20 (s, 1H), 7.74 (m, 1H), 7.71 (s, 1H), 7.64 (m, 2H), 7.22 (m, 2H), 6.99 (t, 1H), 6.36 (m, 1H), 6.14 (m, 1H), 6.00 (m, 1H), 5.71 (m, 1H), 5.27 (s, 2H), 4.54 (m, 2H). MS (ESI$^+$): m/z = 462.2 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(3-fluorobenzyloxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19c).** The similar procedure of **19a** was repeated to obtain the compound **19c** (9 mg, final yield 47%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.04 (s, 1H), 7.88 (m, 1H), 7.80−7.79 (d, 1H), 7.64−7.03 (m, 6H), 6.30 (s, 1H), 6.28−6.27 (d, 1H), 5.70−5.66 (m, 1H), 5.18 (s, 2H), 4.48 (s, 2H). MS (ESI$^+$): m/z = 495.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(1-methyl-1*H*-pyrazol-5-yloxy)phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)-acrylamide (19d).** The similar procedure of **19a** was repeated to obtain the compound **19d** (68 mg, final yield 31%). ^{1}H NMR (300 MHz, $CDCl_3$ + CD_3OD) δ 10.96 (bs, 1H), 8.21 (s, 1H), 7.93−7.91 (m, 1H), 7.74 (s, 1H), 7.57−7.53 (m, 1H), 7.14−7.11 (d, 1H), 6.50 (m, 1H), 6.40−6.33 (m, 1H), 6.20−6.11 (m, 1H), 5.73−5.69 (m, 1H), 5.52 (m, 1H), 4.54 (d, 2H), 3.80 (s, 3H). MS (ESI$^+$): m/z = 467.2 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(1-methyl-3-(trifluoromethyl)-1*H*-pyrazol-5-yloxy)phenylamino)pyrimidin-5-yl)oxazol-4-yl)-methyl)acrylamide (19e).** The similar procedure of **19a** was repeated to obtain the compound **19e** (25 mg, final yield 67%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.12 (s, 1H), 8.64 (t, 1H), 8.19 (d, 1H), 8.16 (s, 1H), 8.05 (s, 1H), 7.72 (m, 1H), 7.49 (s, 2H), 7.41 (d, 1H), 6.25 (m, 2H), 6.02 (s, 1H), 5.62 (m, 1H), 4.39 (d, 2H), 3.84 (s, 3H). MS (ESI$^+$): m/z = 535.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-phenoxyphenylamino)-pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19f).** The similar procedure of **19a** was repeated to obtain the compound **19f** (29 mg, final yield 67%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.86 (bs, 1H), 8.25 (s, 1H), 7.92 (d, 1H), 7.74 (s, 1H), 7.55−7.52 (m, 1H), 7.37−7.31 (m, 2H), 7.10−6.97 (m, 4H), 6.41−6.35 (m, 1H), 6.19 (m, 1H), 5.73 (m, 1H), 4.57 (d, 2H). MS (ESI$^+$): m/z = 463.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(2,5-dichlorophenoxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19g).** The similar procedure of **19a** was repeated to obtain the compound **19g** (14 mg, final yield 40%). ^{1}H NMR (300 MHz, $CDCl_3$ + CD_3OD) δ 8.37 (s, 1H), 7.97 (m, 1H), 7.75 (s, 1H), 7.50 (m, 1H), 7.40−7.37 (m, 1H), 7.05−6.75 (m, 2H), 6.60 (m, 1H), 6.17 (m, 1H), 5.73 (m, 1H), 4.55 (m, 2H). MS (ESI$^+$): m/z = 531.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(2,4-dichlorophenoxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19h).** The similar procedure of **19a** was repeated to obtain the compound **19h** (14 mg, final yield 41%). ^{1}H NMR (300 MHz, $CDCl_3$ + CD_3OD) δ 8.24 (s, 1H), 7.96 (m, 1H), 7.75 (s, 1H), 7.55 (m, 1H), 7.49 (m, 1H), 7.18−7.14 (m, 1H), 6.98−6.95 (d, 1H), 6.78−6.75 (d, 1H), 6.41−6.35 (m, 1H), 6.20−6.17 (m, 1H), 5.75−5.71 (m, 1H), 4.58−4.56 (m, 2H). MS (ESI$^+$): m/z = 531.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(2,3-dichlorophenoxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19i).** The similar procedure of **19a** was repeated to obtain the

compound **19i** (11 mg, final yield 39%). ^{1}H NMR (300 MHz, $CDCl_3$+CD_3OD) δ 8.20 (s, 1H), 7.94−7.93 (m, 1H), 7.40 (m, 1H), 7.20 (m, 1H), 7.10 (m, 1H), 6.98 (m, 1H), 6.60 (m, 1H), 6.34 (m, 1H), 6.10 (m, 1H), 5.80 (m, 1H), 4.56−4.54 (m, 2H). MS (ESI$^+$): m/z = 531.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(2-fluorophenoxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19j).** The similar procedure of **19a** was repeated to obtain the compound **19j** (7 mg, final yield 21%). ^{1}H NMR (300 MHz, $CDCl_3$ + CD_3OD) δ 8.20 (s, 1H), 7.87 (m, 1H), 7.73 (s, 1H), 7.49 (m, 1H), 7.19 (m, 1H), 7.07 (m, 2H), 6.94 (m, 2H), 6.36 (m, 2H), 6.16 (m, 1H), 5.70 (m, 1H), 4.53 (d, 2H). MS (ESI$^+$): m/z = 481.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(3-fluorophenoxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19k).** The similar procedure of **19a** was repeated to obtain the compound **19k** (14 mg, final yield 38%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (s, 1H), 7.95 (m, 1H), 7.72 (s, 1H), 7.59−7.56 (m, 1H), 7.25 (m, 1H), 7.09−7.06 (m, 1H), 6.77−6.71 (m, 2H), 6.67−6.64 (m, 1H), 6.63 (m, 1H), 6.19−6.15 (m, 1H), 5.73−5.69 (m, 1H), 4.56 (s, 2H). MS (ESI$^+$): m/z = 481.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(3-fluorophenoxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19l).** The similar procedure of **19a** was repeated to obtain the compound **19l** (21 mg, final yield 44%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (s, 1H), 7.90 (m, 1H), 7.73 (s, 1H), 7.51 (m, 1H), 7.02−6.93 (m, 5H), 6.40−6.34 (m, 1H), 6.19−6.10 (m, 1H), 5.74−5.70 (m, 1H). MS (ESI$^+$): m/z = 481.2 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(pyridin-2-yloxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19m).** The similar procedure of **19a** was repeated to obtain the compound **19m** (7 mg, final yield 30%). ^{1}H NMR (300 MHz, $CDCl_3$ + CD_3OD) δ 10.93 (s, 1H), 8.20 (s, 1H), 7.90 (s, 1H), 7.73−7.71 (m, 2H), 7.54 (dd, 1H), 7.18 (d, 2H), 7.01−6.96 (m, 2H), 6.37 (d, 1H), 6.18 (t, 1H), 5.68 (d, 1H), 4.53 (d, 2H). MS (ESI$^+$): m/z = 464.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(pyridin-3-yloxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19n).** The similar procedure of **19a** was repeated to obtain the compound **19n** (20 mg, final yield 44%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.10 (s, 1H), 8.64 (t, 1H), 8.33 (m, 2H), 8.17 (m, 2H), 8.05 (s, 1H), 7.70 (m, 1H), 7.49 (s, 1H), 7.39 (m, 1H), 7.25 (m, 2H), 6.20 (m, 2H), 5.62 (m, 2H), 4.39 (d, 2H). MS (ESI$^+$): m/z = 464.3 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-(6-methylpyridin-3-yloxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19o).** The similar procedure of **19a** was repeated to obtain the compound **19o** (1 mg, final yield 2%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.23 (d, 1H), 8.21 (s, 1H), 7.92 (d, 1H), 7.75 (s, 1H), 7.55 (m, 1H), 7.16 (m, 2H), 7.02 (m, 2H), 6.50 (m, 1H), 6.37 (m, 1H), 6.15 (m, 1H), 5.71 (m, 1H), 4.55 (d, 2H), 2.54 (s, 3H). MS (ESI$^+$): m/z = 478.2 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-methyl-4-(6-methylpyridin-3-yloxy)-phenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19p).** The similar procedure of **19a** was repeated to obtain the compound **19p** (34 mg, final yield 76%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.65 (bs, 1H), 8.28−8.27 (d, 1H), 8.23 (s, 1H), 7.74 (s, 1H), 7.53 (d, 1H), 7.48−7.44 (m, 1H), 7.10 (m, 2H), 6.92−6.89 (d, 1H), 6.40−6.34 (m, 1H), 6.20−6.11 (m,1H), 5.74−5.70 (m, 1H), 4.57 (d, 2H), 2.54 (s, 3H), 2.28 (s, 3H). MS (ESI$^+$): m/z = 458.4 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3-chloro-4-fluorophenylamino)pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19q).** The similar procedure of **19a** was repeated to obtain the compound **19q** (19 mg, final yield 16%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (s, 1H), 7.75 (dd, 1H), 7.69 (s, 1H), 7.42−7.37 (m, 1H), 7.06 (t, 1H), 6.28 (d, 1H), 6.20 (d, 1H), 5.63 (d, 1H), 4.42 (s, 2H). MS (ESI$^+$): m/z = 389.2 [M + H]$^+$.

***N*-((2-(4-Amino-6-(3,4-dichloro-2-fluorophenylamino)-pyrimidin-5-yl)oxazol-4-yl)methyl)acrylamide (19r).** The similar procedure of **19a** was repeated to obtain the compound **19r** (26 mg, final yield 45%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.38−8.30 (m, 1H), 8.13 (s, 1H), 8.04 (s, 1H), 7.61−7.48 (m, 3H), 6.32−6.23 (m, 1H), 6.16−6.09 (m, 1H), 4.35 (s, 2H). MS (ESI$^+$): m/z = 423.2 [M + H]$^+$.

***N*-(2-Acrylamidoethyl)-4-amino-6-((3-chloro-4-(pyridin-2-ylmethoxy)phenyl)amino)pyrimidine-5-carboxamide (21).** Compound **21** was synthesized in 14.4% yield over three steps from **20**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (s, 1H), 8.56 (s, 1H), 8.08−7.87 (m, 1H), 7.75−7.69 (m, 2H), 7.38−7.32 (m, 2H), 7.02 (d, 1H), 6.23 (dd, 2H), 5.68 (d, 1H), 5.28 (s, 2H), 3.43 (t, 2H), 3.36 (t, 2H). MS (ESI$^+$): m/z = 468.8 [M + H]$^+$.

General Procedures for the Cell Growth Inhibitory Assay. A human vaginal epidermoid cancer cell line A431 (ATCC, CRL-1555) and a human breast cancer cell line SK-Br3 (ATCC, HTB-30) were used to measure the inhibitory activities of synthesized compounds toward cancer cell growth. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and 1.5 g/L sodium bicarbonate and were supplemented with 10% fetal bovine serum (FBS). In addition, MDA-MB-175, MDA-MB-361, MDA-MB-453, NCI-N87, NCI-H1781, and NCI-H1975 cells were purchased from the American Type Culture Collection (ATCC). JIMT-1 cell line was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH) and was incubated in the same conditions as above. MDA-MB-175, MDA-MB-361, and MDA-MB-453 cells were incubated in L-15 medium, and NCI-N87, NCI-H1781, and NCI-H1975 cells were incubated in RPMI medium containing 1 mM sodium pyruvate. All culture media were supplemented with 1% penicillin−streptomycin and 10% fetal bovine serum (FBS). Cells were incubated in a humidified atmosphere under 5% CO_2 except for MDA-MB-175, MDA-MB-361, and MDA-MB-453 cells (CO_2 free) at 37 °C. Cell growth inhibition assays and GI_{50} determinations were performed as previously described.[28]

General Procedures for the Microsomal Stability Assay. All assays were conducted in duplicate. The incubation mixtures were prepared in E-tube and were contained with 5 μM synthesized analogue, 1 mg/mL liver microsomes (from mouse, rat, dog, and human), and NADPH regeneration solution (1.3 mM $NADP^+$, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, and 0.4 U/mL glucose 6-phosphate dehydrogenase) in 100 mM potassium phosphate buffer solution. Reactions were initiated by the addition of NADPH and kept in a shaking water bath at 37 °C. At the each sampling time, aliquots were removed and added to termination solvent (acetonitrile). The samples were centrifuged for 4−5 min at 13 000 rpm, and the supernatant was subjected to HPLC analysis (HPLC/Agilent 1200 series). In the determination of the in vitro $t_{1/2}$ (half-life, HL), the analyte peak areas were converted to percentage of drug remaining, using the T = 0 peak area values as 100%. The slope of the linear regression from log percentage remaining versus incubation time relationships ($-k$) was used in the conversion to the in vitro $t_{1/2}$, values by the in vitro $t_{1/2}$= $-0.693/k$. The percent remaining of test compound is calculated compared to the initial quantity at 0 time.

Pharmacokinetic Studies. Male mice (imprinting control region mice, body-weight range of 27 ± 5 g, iv, n = 3, po, n = 3) and male rats (Sprague−Dawley rats, body-weight range of 250 ± 10 g, iv, n = 3, po, n = 3) were administered analogue **19a** intravenously via the tail vein at 2 and 1 mg/kg, respectively, or orally at 10 and 5 mg/kg, respectively, by gavage in a solution of 30% PEG400 and 5% ethanol in distilled water. For the in vivo study, **19a** was prepared as HCl salt. At predetermined times 24 h or more after dosing, 0.3 mL blood was collected from the jugular vein using a tube containing anticoagulant (1000 IU/mL, heparin, 3 μL). The plasma was separated by centrifugation (12 000 rpm, 2 min, Eppendorf). The concentrations of the compound were measured in the plasma using LC/MS/MS after protein precipitation with acetonitrile. The relevant estimated pharmacokinetic parameters for plasma were derived using WinNonlin, version 5.2 (Pharsight).

General Procedures for the EGFR Enzyme Assay. An amount of 10 μL of EGFR enzyme ($EGFR^{WT}$, $EGFR^{T790M}$, HER-2, or HER-4 kinase, Upstate) was added to each well of a 96-well microplate. As an EGFR inhibitor, 10 μL of serially diluted solution of synthesized compounds was added to the individual wells, and the plate was incubated at room temperature for 10 min. Then 10 μL of Poly (Glu,

Tyr 4:1, Sigma) and 10 μL of ATP were successively added to initiate a kinase reaction, and the resulting mixture was incubated at room temperature for 1 h. 10 μL of 100 mM EDTA was added to each well, and the mixture was stirred for 5 min to terminate the kinase reaction. Then 10 μL of 10× anti-phosphotyrosine antibody (Pan Vera), 10 μL of 10× protein tyrosine kinase (PTK) green tracer (Pan Vera), and 30 μL of fluorescence polarization (FP)-diluted buffer were added to the reacted mixture, followed by incubation in the dark at room temperature for 30 min. The FP value of each well was determined using a VICTORIII fluorescence meter (Perkin-Elmer) at 488 nm. The IC_{50}, i.e., the concentration at which 50% inhibition was observed, was determined by setting the maximum value (0% inhibition) to the polarized light value for a well untreated with EGFR inhibitor and the minimum value (100% inhibition). IC_{50} calculations and analysis were carried out using Microsoft Excel.

Prolongation of Phosphorylation Inhibition. Cells were plated at a density of 5×10^5/well in six-well plates under normal culture conditions (10% FBS and 1% penicillin−streptomycin). After 24 h, the medium was changed to 0.1% FBS medium and cells were incubated for 16 h. Cells were then treated with 1 μM analogue **19a** for 4 h. Each set was washed 4 times with warmed compound-free medium and incubated for 0 and 8 h. Each set was stimulated with EGF (100 ng/mL) for 5 min. The phosphorylation rates of EGFR or HER-2 were measured by Western blotting.

■ ASSOCIATED CONTENT

Supporting Information

Scheme showing the synthesis of compound **21** and figure showing prolonged inhibitory results on the phosphorylation of EGFR for compound **19a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

HER, human epidermal growth factor receptor; EGFR, epidermal growth factor receptor; IC_{50}, half-maximal inhibitory concentration; po, per os; AUC_{last}, area under the curve from 0 h to last time; C_{max}, maximum plasma concentration; $T_{1/2}$, drug half-life; *F*, oral bioavailability

■ REFERENCES

(1) Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **2000**, *103*, 211−225.

(2) Hynes, N. E.; Lane, H. A. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer* **2005**, *5*, 341−354.

(3) Yarden, Y.; Sliwkowski, M. X. Untangling the ErbB signaling network. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 127−137.

(4) Slamon, D. J.; Clark, G. M.; Wong, S. G.; Levin, W. J.; Ullrich, A.; McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **1987**, *235*, 177−182.

(5) Slamon, D. J.; Godolphin, W.; Jones, L. A.; Holt, J. A.; Wong, S. G.; Keith, D. E.; Levin, W. J.; Stuart, S. G.; Udove, J.; Ullrich, A.; Press, M. F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **1989**, *244*, 707−712.

(6) Nahta, R.; Shabaya, S.; Ozbay, T.; Rowe, D. L. Personalizing HER2-targeted therapy in metastatic breast cancer beyond HER2 status: what we have learned from clinical specimens. *Curr. Pharm. Pharmacogenomics Pers. Med.* **2009**, *7*, 263−274.

(7) Vogel, C. L.; Cobleigh, M. A.; Tripathy, D.; Gutheil, J. C.; Harris, L. N.; Fehrenbacher, L.; Slamon, D. J.; Murphy, M.; Novotny, W. F.; Burchmore, M.; Shak, S.; Stewart, S. J.; Press, M. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J. Clin. Oncol.* **2002**, *20*, 719−726.

(8) Slamon, D. J.; Leyland-Jones, B.; Shak, S.; Fuchs, H.; Paton, V.; Bajamonde, A.; Fleming, T.; Eiermann, W.; Wolter, J.; Pegram, M.; Baselga, J.; Norton, L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **2001**, *344*, 783−792.

(9) Wenle, X.; Mullin, R. J.; Keith, B. R.; Liu, L. H.; Ma, H.; Rusnak, D. W.; Owens, G.; Alligood, K. J.; Spector, N. L. Anti-tumor activity of GW-572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* **2002**, *21*, 6255−6263.

(10) Johnston, S. R. D.; Leary, A. Lapatinib: a novel EGFR/HER2 tyrosine kinase inhibitor for cancer. *Drugs Today* **2006**, *42*, 441−453.

(11) Speyer, J. Cardiac dysfunction in the trastuzumab clinical experience. *J. Clin.Oncol.* **2002**, *20*, 1156−1157.

(12) Abraham, J. Dual HER1/HER2 inhibitor lapatinib in advanced/metastatic breast cancer. *Commun. Oncol.* **2007**, *4*, 73−78.

(13) Nahta, R.; Esteva, F. J. Herceptin: mechanisms of action and resistance. *Cancer Lett.* **2006**, *232*, 123−138.

(14) O'Brien, N. A.; Browne, B. C.; Chow, L.; Wang, Y.; Ginther, C.; Arboleda, J.; Duffy, M. J.; Crown, J.; O'Donovan, N.; Slamon, D. J. Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. *Mol. Cancer Ther.* **2010**, *9*, 1489−1502.

(15) Köninki, K.; Barok, M.; Tanner, M.; Staff, S.; Pitkänen, J.; Hemmilä, P.; Ilvesaro, J.; Isola, J. Multiple molecular mechanisms underlying trastuzumab and lapatinib resistance in JIMT-1 breast cancer cells. *Cancer Lett.* **2010**, *294*, 211−219.

(16) Adjei, A. A. Epidermal growth factor receptor tyrosine kinase inhibitors in cancer therapy. *Drugs Future* **2001**, *26*, 1087−1092.

(17) Renhowe, P. A. Inhibitors of growth factor receptor kinase-dependent signaling pathways in anticancer chemotherapy-clinical progress. *Curr. Opin. Drug Discovery Dev.* **2002**, *5*, 214−224.

(18) Wissner, A.; Overbeek, E.; Reich, M. F.; Floyd, M. B.; Johnson, B. D.; Mamuya, N.; Rosfjord, E. C.; Discafani, C.; Davis, R.; Shi, X.; Rabindran, S. K.; Gruber, B. C.; Ye, F.; Hallett, W. A.; Nilakantan, R.; Shen, R.; Wang, Y.; Greenberger, L. M.; Tsou, H. Synthesis and structure−activity relationships of 6,7-disubstituted 4-anilinoquinoline-3-carbonitriles: the design of an orally active, irreversible inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor-2 (HER-2). *J. Med. Chem.* **2003**, *46*, 49−63.

(19) Tsou, H. R.; Overbeek-Klumpers, E. G.; Hallett, W. A.; Reich, M. F.; Floyd, M. B.; Johnson, B. D.; Michalak, R. S.; Shen, R.; Shi, X.; Wang, Y. F.; Upeslacis, J.; Wissner, A. Optimization of 6,7-disubstituted-4-(arylamino)quinoline-3-carbonitriles as orally active, irreversible inhibitors of human epidermal growth factor receptor-2 kinase activity. *J. Med. Chem.* **2005**, *48*, 1107−1131.

(20) Li, D.; Ambrogio, L.; Shimamura, T.; Kubo, S.; Takahashi, M.; Chirieac, L. R.; Padera, R. F.; Shapiro, G. I.; Baum, A.; Himmelsbach, F.; Rettig, W. J.; Meyerson, M.; Solca, F.; Greulich, H.; Wong, K. K. BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene* **2008**, *27*, 4702−4711.

(21) Kamath, S.; Buolamwini, J. K. Targeting EGFR and HER-2 receptor tyrosine kinases for cancer drug discovery and development. *Med. Res. Rev.* **2006**, 1−26.

(22) Shintani, S.; Matsuo, K.; Crohin, C. C. Intragenic mutation analysis of the human epidermal growth factor receptor (EGFR) gene in malignant human oral keratinocytes. *Cancer Res.* **1999**, *59*, 4142−4147.

(23) Wildenhain, Y.; Pawson, T.; Blackstein, M. E.; Andrulis, I. L. p185neu is phosphorylated on tyrosine in human primary breast tumors which overexpress neu/erbB-2. *Oncogene* **1990**, *5*, 879−883.

(24) Di, L.; Kerns, E. H.; Hong, Y.; Kleintop, T. A.; McConnell, O. J.; Huryn, D. M. Optimization of a higher throughput microsomal stability screening assay for profiling drug discovery candidates. *J. Biomol. Screening* **2003**, *8*, 453−462.
(25) Obach, R. S. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab. Dispos.* **1999**, *27* (11), 1350−1359.
(26) Liu, L.; Greger, J.; Shi, H.; Liu, Y.; Greshock, J.; Annan, R.; Halsey, W.; Sathe, G. M.; Martin, A.; Gilmer, T. M. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. *Cancer Res.* **2009**, *69*, 6871−6878.
(27) Rusnak, D. W.; Alligood, K. J.; Mullin, R. J.; Spehar, G. M.; Arenas-Elliott, C.; Martin, A. M.; Degenhardt, Y.; Rudolph, S. K.; Haws, T. F. Jr.; Hudson-Curtis, B. L.; Gilmer, T. M. Assessment of epidermal growth factor receptor (EGFR, ErbB1) and HER2 (ErbB2) protein expression levels and response to lapatinib (Tykerb, GW572016) in an expanded panel of human normal and tumour cell lines. *Cell Proliferation* **2007**, *40*, 580−594.
(28) Cha, M. Y.; Lee, K.-O.; Kim, J. W.; Lee, C. G.; Song, J. Y.; Kim, Y. H.; Lee, G. S.; Park, S. B.; Kim, M. S. Discovery of a novel Her-1/Her-2 dual tyrosine kinase inhibitor for the treatment of Her-1 selective inhibitor-resistant non-small cell lung cancer. *J. Med. Chem.* **2009**, *52*, 6880−6888.