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Design and Synthesis of Tetrahydropyridothieno[2,3-*d*]pyrimidine Scaffold Based Epidermal Growth Factor Receptor (EGFR) Kinase Inhibitors: The Role of Side Chain Chirality and Michael Acceptor Group for Maximal Potency

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HTS hit **7** was modified through hybrid design strategy to introduce a chiral side chain followed by introduction of Michael acceptor group to obtain potent EGFR kinase inhibitors **11** and **19**. Both **11** and **19** showed over 3 orders of magnitude enhanced HCC827 antiproliferative activity compared to HTS hit **7** and also inhibited gefitinib-resistant double mutant (DM, T790M/L858R) EGFR kinase at nanomolar concentration. Moreover, treatment with **19** shrinked tumor in nude mice xenograft model.

Introduction

Lung cancer is the most common human cancer, with 85% being nonsmall cell lung cancer (NSCLC[a]). In ∼50% of NSCLC patients, epidermal growth factor receptor (EGFR) is overexpressed with concomitant dysregulation in their downstream signaling pathway, and this overexpression is correlated with poor prognosis.[1] EGFR is a transmembrane protein with an external ligand binding receptor domain and an intracellular tyrosine kinase activity domain. Once a native ligand (eg., EGF) binds to the extracellular domain, it dimerizes with another EGFR family member (eg, EGFR, Her2), resulting in activation of the intracellular tyrosine kinase (TK) activity. This results in autoposphorylation of EGFR and further activation of downstream signaling pathway affects cell survival and proliferation.[2]

Gefitinib (**1**) and erlotinib (**2**) were approved by US FDA in 2002 and 2004, respectively, for the treatment of NSCLC (Figure 1).[2] Gefitinib or erlotinib competitively binds to the adenosine triphosphate (ATP) binding pocket of intracellular EGFR TK domain and inhibit its activity, thereby blocking the aberrant EGFR downstream signaling essential for tumor survival and proliferation. Although the two drugs showed high response rates in specific subsets of NSCLC patients, for most patients, who respond initially to gefitinib or erlotinib,

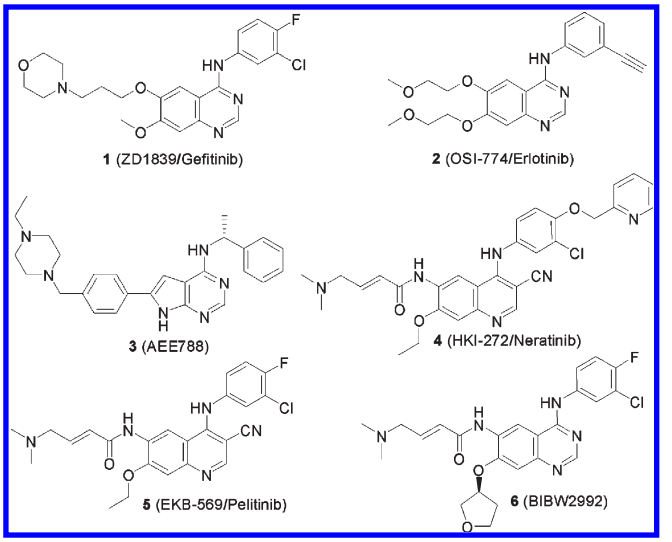

Figure 1. Reversible and irreversible EGFR tyrosine kinase inhibitors.

drug resistance often occurs as a result of a secondary mutation such as the $Thr^{790} \rightarrow Met^{790}$ (T790M) mutation.[1,3,4] Therefore, identification of second-generation EGFR tyrosine kinase inhibitors that overcome the acquired T790M mutation (such as the $EGFR^{L858R/T790M}$ double mutation) can be beneficial for the treatment of gefinitib- or erlotinib-resistant NSCLC.

We have recently used an EGFR-transfected 32D cell-based high-throughput screening (HTS) assay to identify compounds that inhibit EGFR activation and/or EGFR-mediated downstream signaling pathway.[5] This cell-based

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[a]Abbreviations: ATP, adenosine triphosphate; DM, double mutant (L858R/T790M); EGFR, epidermal growth factor receptor; HTS, high-throughput screening; LCMS, liquid chromatography coupled mass spectrometry; NSCLC, nonsmall cell lung cancer; PK, pharmacokinetic; TK, tyrosine kinase; WT, wild type.

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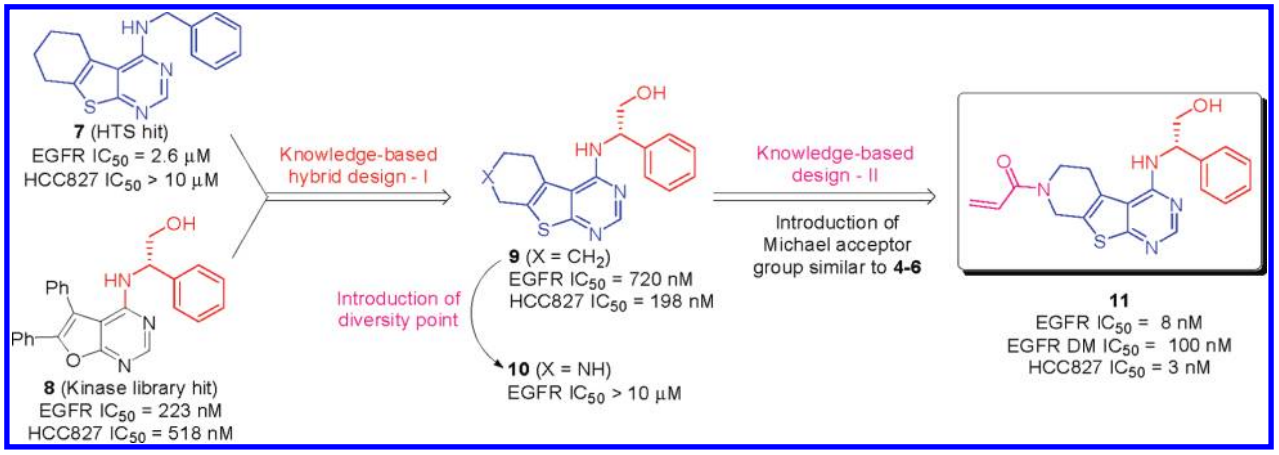


Figure 2. Design of novel and potent tetrahydropyridothieno[2,3-*d*]pyrimidine scaffold EGFR tyrosine kinase inhibitors, through knowledge-based design strategy.

screening protocol allowed us to identify several leads which disrupt cell proliferation mediated by aberrant EGFR signaling, among which **7**, a novel tricyclic tetrahydrobenzothieno-[2,3-*d*]pyrimidine core compound, was identified as an initial hit with an enzyme inhibition $IC_{50} = 2.6\ \mu M$ (Figure 2). In this brief communication, we describe the development path of hit **7**, a weak EGFR kinase inhibitor, to that of potent EGFR kinase inhibitor **11** ($IC_{50} = 8$ nM) and **19** ($IC_{50} = 6$ nM), which can also inhibit gefitinib resistant $EGFR^{L858R/T790M}$ double mutant kinase. Further, in vivo activity for **19** is demonstrated in HCC827 (NSCLC cell line with Del722-726 mutation) xenograft nude mice model, proving the potential of **19** for cancer treatment.

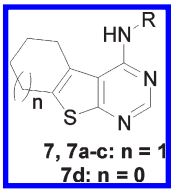
Results and Discussion

Initial attempts to improve the activity of **7** through increasing the side chain length (**7a**), substitution in the phenyl ring (**7b**,**c**), or converting the fused cyclohexyl ring to cyclopentyl ring (**7d**) led to decreased EGFR kinase activity (Table 1). Meanwhile, through a concurrent program for the development of aurora kinase inhibitors, we have synthesized a set of kinase-targeted compound library based on furanopyrimidine scaffold. This library was counter-screened for EGFR kinase inhibition, leading to the identification of an EGFR kinase selective furanopyrimidine inhibitor **8** (Figure 2) with an EGFR kinase $IC_{50} = 223$ nM (aurora A kinase $IC_{50} > 10\ \mu M$).[6]

Scrutinizing both the structures of HTS hit **7** and kinase library hit **8** revealed that even though both have different core structures (scaffolds), they have very similar side chain. The kinase library hit **8** possessed a chiral *S*-2-phenyl-2-aminoethanol side chain, which is very similar to that of the HTS hit **7** except for the presence of methanol branching with an *S*-configuration. This novel chiral phenylaminoethanol side chain is not present in any of the EGFR kinase inhibitors reported so far. As a majority of EGFR kinase inhibitors in market or in development bear anilino type side chains linked to quinazoline/quinoline cores (**1**, **2**, **4**−**6**) (Figure 1), we contemplated the development of new EGFR kinase inhibitors with this novel chiral phenylaminoethanol side chain, both to explore the importance of chirality for EGFR inhibition and to develop novel proprietary molecules. It should also be noted that a closely related *R*-1-phenylethylamino side chain has been reported a few times in EGFR kinase inhibitors,[7,8] and compound **3** (AEE788)[9] bearing *R*-1-phenylethylamino side chain is reported to be in clinical testing; however, the importance of chirality for EGFR inhibition has not been explored in much detail through structure−activity relationship (SAR) studies in those reports.

With this agenda, we synthesized the hybrid compound **9** (Figure 2) possessing the tricyclic core of hit **7** and the chiral

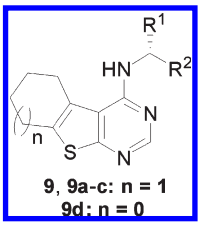
Table 1. Inhibition of EGFR WT Kinase by **7** and **7a**−**d** Analogues[a]



compd	*n*	R	EGFR-WT kinase % inhibition at 20 μM
7	1	$-CH_2Ph$	76.9
7a	1	$-CH_2CH_2Ph$	44.3
7b	1	$-CH_2(4\text{-}OCH_3\text{-}Ph)$	1.1
7c	1	$-CH_2(4\text{-}F\text{-}Ph)$	52.7
7d	0	$-CH_2Ph$	69.1

[a] WT: wild type kinase.

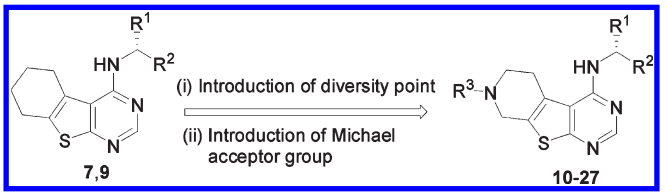
Table 2. Inhibition of EGFR-WT Kinase and HCC827 Cell Line by **9** and **9a**−**d** Analogues[a]

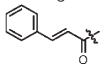
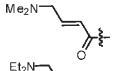
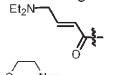
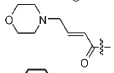
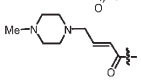
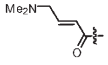
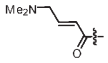
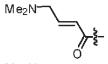
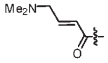
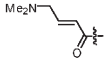


compd	R^1	R^2	EGFR-WT kinase inhibition IC_{50}[b] (μM)	HCC827 IC_{50} (μM)[b]
9	CH_2OH	Ph	0.720	0.198
9a	CH_3	Ph	3.240	
9b	CH_2OH	H	>20[c]	
9c	Ph	CH_2OH	>20[c]	
9d	CH_2OH	Ph	1.090	0.324

[a] WT: wild type kinase. [b] Values are expressed as the mean of at least two independent experiments and are mostly within 15% error margins. [c] < 50% enzyme inhibition at 20 μM.

Table 3. Inhibition of EGFR Kinase and HCC827 Cell Line by **7**, **9**−**27**[d]



Compd	R^1/R^2	R^3	EGFR kinase inhibition (μM)		HCC827 IC_{50} (μM)[a]
			WT IC_{50}[a]	DM IC_{50}[a]	
7	H/Ph	-	2.600	> 10[c]	> 10
***S*-9**	CH_2OH/Ph	-	0.720	> 10[c]	0.198
***S*-10/33a**	CH_2OH/Ph	H	> 20[b]	> 10[c]	-
***S*-11**	CH_2OH/Ph		0.008	0.100	0.003
***S*-12**	CH_2OH/Ph		> 20[b]	> 10[c]	-
***S*-13**	CH_2OH/Ph		1.270	> 10[c]	-
***S*-14**	CH_2OH/Ph		0.091	2.480	0.273
***R*-15**	Ph/CH_2OH		3.057	>10[c]	-
***R*-16**	CH_3/Ph		0.020	0.739	0.019
***R*-17**	CH_2CH_3/Ph		0.046	2.200	0.024
***S*-18**	CH_2OH/Ph		3.503	> 10[c]	-
***S*-19**	CH_2OH/Ph		0.007	0.305	0.002
***S*-20**	CH_2OH/Ph		0.009	0.930	0.008
***S*-21**	CH_2OH/Ph		0.021	1.399	0.004
***S*-22**	CH_2OH/Ph		0.089	> 10[c]	0.038
***R*-23**	Ph/CH_2OH		> 20[b]	> 10[c]	-
***R*-24**	CH_3/Ph		0.012	0.596	0.002
***R*-25**	CH_2CH_3/Ph		0.012	5.750	0.007
***S*-26**	CH_2OH/ CH_2Ph		0.149	4.107	0.032
27	CH_2OH/H		4.884	> 10[c]	-
Gefitinib (**1**)	-	-	0.02	2.815	0.013

[a] Values are expressed as the mean of at least two independent experiments and are mostly within 15% error margins. [b] < 50% enzyme inhibition at 20 μM. [c] < 50% enzyme inhibition at 10 μM. [d] WT: wild−type EGFR kinase. DM: double mutant EGFR kinase.

phenylaminoethanol side chain of hit **8**. Subsequently, the hybrid compound **9** showed submicromolar EGFR kinase inhibition (IC_{50} = 720 nM) and further exhibited potent antiproliferative activity in HCC827 cell lines (IC_{50} = 198 nM), which is over 50-fold better than that of the original HTS hit **7**. On the basis of these findings, new lead **9** was selected for further structural modification to deeply investigate SAR in order to optimize both the kinase and cell-based activity.

It was found that either removal of the secondary hydroxyl group (**9a**) or phenyl group (**9b**) from the side chain of **9** led to decreased EGFR kinase inhibition, particularly removal of the phenyl group led to complete loss of activity, showing the indispensible nature of the phenyl ring in maintaining activity (Table 2). Moreover, the orientation of the phenyl ring is critical, as the *R*-enantiomer (**9c**) of **9** was found to be completely inactive. Also, converting the six-membered carbocyclic ring fused to the thienopyrimidine to five-membered ring (**9d**) did not improve the activity. Although compounds **9** and **9d** showed nanomolar level inhibition in wild-type enzyme and cell-based assay, both were not capable of

inhibiting double mutant EGFR kinase (< 50% inhibition at 10 μM), which could overcome gefitinib resistance.

Recent research reveals that a possible way to overcome gefitinib-resistant T790M mutant EGFR kinase is through irreversible inhibition of the enzyme by covalent modification of Cys733 residue in the ATP binding domain of the kinase.[1,4,10,11] This has been achieved with three clinical trial compounds **4** (HKI-272),[12,13] **5** (EKB-569),[14,15] and **6** (BIBW2992)[16] for the treatment of gefitinib-resistant NSCLC. These clinical trial compounds possess a Michael acceptor group in common, which plays a crucial role in inhibiting gefitinib resistant T790M mutant kinase irreversibly through covalent modification of cysteine residue.[11,16] This knowledge stimulated us to introduce a Michael acceptor group in our series of compounds to incorporate activity toward gefitinib-resistant T790M mutant kinase. One possibility to introduce a Michael acceptor group into the tricyclic core of **9** is through the introduction of additional diversity point by replacing one methylene unit with a nitrogen in the six-membered carbocyclic ring to form **10**. Then the newly designed tricyclic tetrahydropyridothieno[2,3-*d*]pyrimidine core was used to construct **11** incorporating an acrylamide Michael acceptor group at the diversity point (Figure 2).

First, the hybrid compound **11** with the acrylamide Michael acceptor group was examined for both wild-type and double mutant EGFR kinase inhibition (Table 3). It not only displayed an excellent wild-type enzyme inhibition but also displayed double mutant kinase inhibition at nanomolar level. Compared to **9**, **11** displayed 90-fold enhanced EGFR wild-type kinase inhibition. Most interestingly, **11** displayed significantly enhanced double mutant kinase inhibition, which is over 25-fold better than gefitinib. In addition to showing potent EGFR kinase inhibition, **11** also showed potent antiproliferative activity in HCC827 cell line, with growth inhibition in the single-digit nanomolar range (IC_{50} = 3 nM). It should be noted that compound **10**, a close analogue of **11** lacking the Michael acceptor acrylamide function shows complete loss of activity. Thus incorporation of acrylamide Michael acceptor group had resulted in both enhancing the wild-type enzyme inhibition as well as imparting potent activity toward gefitinib-resistant double mutant kinase. Further, the importance of the acrylamide Michael acceptor group in imparting EGFR activity was shown by complete inactivity of **12**, wherein the terminal double bond of **11** is reduced to give a simple propanamide functional group. Next, we attempted to replace the acrylamide group with butynamide (**13**) or with vinyl sulfonamide (**14**) as different Michael acceptor groups, both of which led to loss of activity. The butynamide analogue **13** showed poor activity in both wild and double mutant kinase, while the vinyl sulfonamide analogue **14** inhibited the double mutant kinase in a range very similar to that of gefitinib, suggesting the superiority of acrylamide **11** as an EGFR inhibitor.

Second, the full potential of **11** was probed by evaluating its ability to interfere with EGFR signaling inside the cell by carrying out Western blotting analysis. For this purpose, HCC827 cells were treated with **11** in dose-dependent manner and then analyzed for EGFR autophosphorylation (pEGFR at residue Tyr1068) (Figure 3). When compared to gefitinib, **11** showed better inhibition of EGFR autoposphorylation, further demonstrating the potential of **11** as an EGFR kinase inhibitor.

Third, we carried out in vivo pharmacokinetic (PK) study in rat for **11**, which showed that the compound could not be detected in appreciable amounts in plasma within 10 min of IV injection (data not shown). The poor PK properties precluded testing of **11** for in vivo efficacy, and hence further efforts were aimed at identifying potent compounds with improved PK profile compared to that of **11**. For this purpose, we first turned our attention to the chiral phenylaminoethanol side chain linked to the pyrimidine core. When the *S*-enatiomer **11** was converted to *R*-enatiomer **15**, the compound lost EGFR wild and double mutant kinase activity over 200-fold compared to that of **11**. However, either the removal of hydroxyl group (**16**) or converting it to methyl group (**17**) led to loss of 3−6-fold potency for wild-type and 8−22-fold loss of potency for double mutant kinase compared to that of **11**. These results suggest that presence of hydroxyl group plays an important role in maintaining potent activity for both wild-type and double mutant EGFR kinase. Hence, by retaining the chiral phenylaminoethanol side chain linked to the pyrimidine core, we attempted modification in the Michael acceptor region of **11** in order to identify potent compounds.

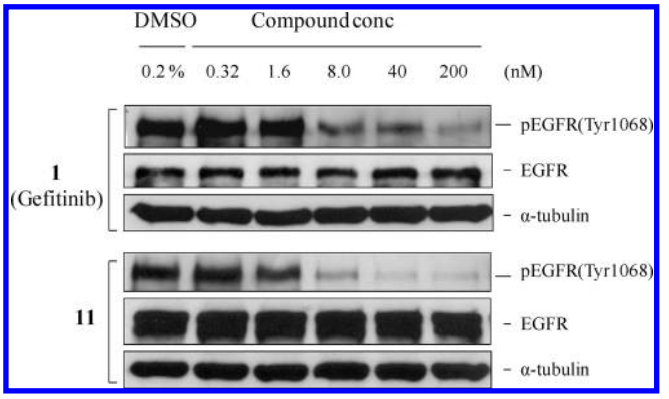


Figure 3. Western blot analysis using anti-phospho-EGFR Y1068 and anti-EGFR immunoblots of HCC827 cell lysates treated with **1** or **11** for 2 h. A representative anti-tubulin immunoblot is shown as a loading control.

Introduction of a hydrophobic phenyl ring at the terminal double bond (**18**) led to complete loss of potency for both wild-type and double mutant kinase. On the basis of this observation, a hydrophilic *N*,*N*-dimethyl group was introduced through a methylene linker to the terminal double bond of **11** to get **19**, which retained the wild-type kinase activity and led to only a 3-fold loss of activity for double mutant kinase compared to that of **11**. When the size of the hydrophilic moiety was increased by changing the *N*,*N*-dimethyl group (**19**) to *N*,*N*-diethyl (**20**), morpholine (**21**), or *N*-methyl piperazine (**22**), both the wild-type as well as double mutant kinase activity decreased; the loss of potency was more prominent in the case of double mutant kinase activity, particularly *N*-methyl piperazine analogue **22** was completely inactive.

As compound **19** is found to be better than related analogues **20**−**22** by retaining the *N*,*N*-dimethyl side chain on the Michael acceptor side of **19**, we further attempted to explore the SAR in the chiral phenylaminoethanol side chain linked to the pyrimidine core. It was found that the *R*-enatiomer (**23**) again lost the activity for both wild and double mutant kinase completely. Removal of hydroxyl group (**24**) only led to 2-fold loss of wild-type and double mutant kinase activity, while converting it to methyl group (**25**) led to loss of double mutant kinase activity (IC_{50} > 5 μM) without much affecting the wild-type kinase activity compared to that of **19**. These findings in

the chiral phenylaminoethanol side chain are very similar to the SAR observation in **11** containing the acrylamide Michael acceptor group. This demonstrates that apart from the presence of Michael acceptor functionality, the presence of the hydroxyl group and the orientation of the phenyl group in the side chain has played a critical role determining the activity in this series, in particular for double mutant kinase activity. To further explore the role of the phenyl group, it was moved one carbon away from the chiral center or completely removed from the side chain, which resulted in **26** and **27**, respectively. Both moving the phenyl group through chain extension or removing the phenyl group had resulted in drastic loss of activity for both wild-type and double mutant kinase, with compound **27** being completely inactive toward both enzymes and **26** still maintaining nanomolar level wild-type kinase inhibition. These studies further demonstrate that the presence as well as orientation of the phenyl group in the side chain is essential for potent EGFR kinase activity in this series of compounds.

Table 4. Intravenous (iv) Pharmacokinetics Profile of **19** in Rat

parameter	unit	value (SE)
N		3
dose	mg/kg	5
$t_{1/2}$	hr	1.4 (0.1)
clearance	mL/min/kg	248.7 (7.6)
V_{ss}	L/kg	27.3 (3.7)
AUC (0−∞)	ng/mL·h	337 (7)

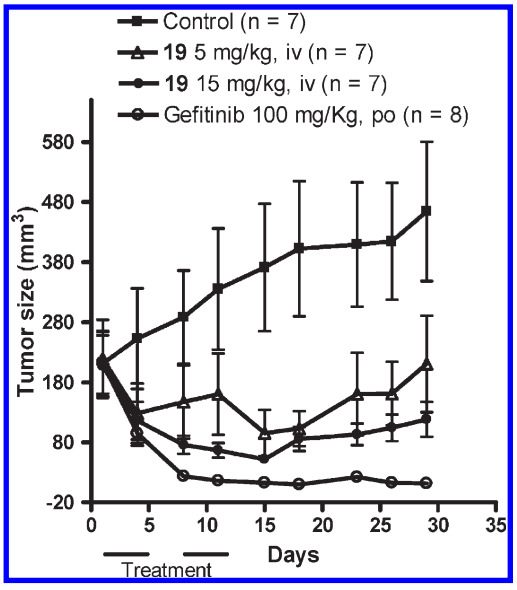


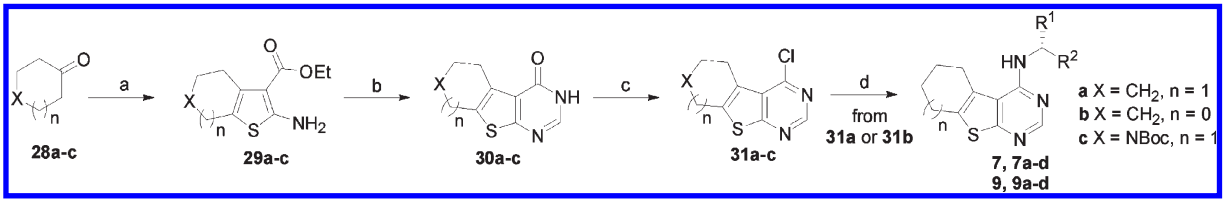
Figure 4. In vivo antitumor effect of **19** in human nonsmall cell lung cancer (HCC827) xenograft nude mice model. The growth of HCC827 tumor xenograft is inhibited by **19** (5 mg/kg, iv or 15 mg/kg, iv) and gefitinib (100 mg/kg, po) with $p < 0.05$. Drug treatment on days 1−5 and 8−12.

Having identified **19**, which has similar EGFR kinase inhibition profile as that of **11**, we carried out PK evaluation of **19** in rats and found that **19** showed better IV PK profile (Table 4) compared to that of **11**, suggesting that **19** is a more suitable candidate for in vivo efficacy evaluation. The in vivo efficacy of **19** was evaluated in nude mice bearing HCC827 tumor xenografts. Treatment mice received **19** as IV injection through the tail veins for 5 days/week for 2 consecutive weeks (days 1−5 and 8−12). At the dosage of 5 and 15 mg/kg, **19** shrank tumor growth significantly ($p < 0.05$), indicating potent in vivo anticancer activity for **19** (Figure 4). Administration of reference compound gefitinib (**1**) 100 mg/kg by perioral route using the same dosing schedule also shrinks the tumor significantly ($p < 0.05$).

Synthesis. Compounds **7**, **7a**−**d**, **9**, and **9a**−**d** were synthesized as shown in Scheme 1. Appropriate cyclic ketones **28a**,**b** were condensed with ethyl cyanoacetate under basic condition and then cyclized in the presence of sulfur to construct the thiophene cores **29a**,**b** through Gewald reaction.[17] The thieno[2,3-*d*]pyrimidine ring system was then constructed using a modified Niementowski quinazoline synthesis by condensation of **29a**,**b** with formamide to afford **30a**,**b**. Finally, chlorination of pyrimidone **30a**,**b** with phosphrous oxychloride gave the intermediate **31a**,**b**, which underwent nucleophilic reaction with appropriate amines to give the desired compounds **7**, **7a**−**d**, **9**, and **9a**−**d**.

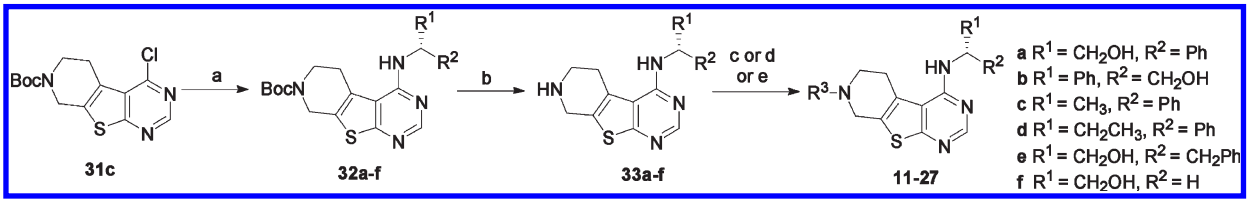
For the preparation of thieonpyrimidines **11**−**27** with the nitrogen bridge in the alicyclic ring, the key intermediate **31c** was constructed starting from 4-oxo-piperidine-1-carboxylic acid *tert*-butyl ester (**28c**) using a similar sequence of reaction

Scheme 1[a]



[a] Reagents and conditions: (a) $NCCH_2CO_2Et$, S_8, EtOH, Et_3N, reflux or rt, 16 h; (b) formamide, reflux, 2 h or formamidine acetate, DMF, 100 °C, 16 h; (c) $POCl_3$, 0 → 60 °C, 3 h; (d) primary amine ($R^1R^2CHNH_2$), *n*-BuOH, reflux, 8 h.

Scheme 2[a]



[a] Reagents and conditions: (a) amine ($R^1R^2CHNH_2$), EtOH, reflux, 8 h; (b) TFA, CH_2Cl_2, 0 °C → rt, 2 h; (c) acid (RCO_2H), EDCI, CH_2Cl_2, rt, 2 h (d) $Cl(CH_2)_2SO_2Cl$, CH_2Cl_2, rt, 4 h; (e) (i) (*E*)-$BrCH_2CH{=}CHCO_2H$, EDCI, CH_2Cl_2, rt, 2 h; (ii) secondary amine (R_2NH), THF, 0 °C, 2 h.

as that for obtaining **31a**,**b** (Scheme 1). Nucleophilic reaction of **31c** with appropriate primary amines gave **32a**−**f**, which were then subjected to Boc deprotection using TFA resulting in the intermediates **33a**−**f**. The desired compounds **11**−**13** and **15**−**18** were obtained by amide coupling reaction of appropriate secondary amine (**33**) and the acid (RCO_2H) using EDCI as a coupling agent. For the preparation of compounds **19**−**27**, a two-step reaction sequence was utilized to get the final products; an initial amide coupling of 4-bromo crotonoic acid with appropriate intermediate **33**, followed by S_N2 reaction with different secondary amines (R_2NH), was carried out. For the sulphonamide derivative **14**, acylation of **33a** with sulphonyl chloride was carried out (Scheme 2).

Conclusion

In summary, HTS hit **7** identified from cell-based screening strategy was modified through a knowledge-based design approach to obtain potent and novel tetrahydropyridothieno-[2,3-*d*]pyrimidine scaffold compounds as EGFR kinase inhibitors. Designing-in an unusual *S*-chiral 2-phenyl-2-aminoethanol side chain identified from counter-screening approach, and then introduction of Michael acceptor group into the tricyclic core through a diversity point "NH" in HTS hit **7**, led to the development of **11** and **19**. Both **11** and **19** showed > 300-fold enhanced EGFR wild-type kinase inhibition and > 3000-fold enhanced HCC827 antiproliferative activity compared to HTS hit **7** and also inhibited gefitinib-resistant double mutant (DM, T790M/L858R) EGFR kinase at nanomolar concentration. Detailed SAR study in this series reveals that, in the presence of the side chain, the nature of chirality and the presence of the phenyl group are very critical for maintaining activity, while the presence of secondary hydroxyl function provides maximum potency. The presence of *S*-chiral 2-phenyl-2-aminoethanol side chain along with the acrylamide-type Michael acceptor group imparts maximal activity toward gefitinib-resistant double mutant EGFR kinase. Six of the compounds (**11**, **19**−**21**, **24**, **25**) possess excellent antiproliferative activity against HCC827 lung cancer cell line in single-digit nanomolar range, with compound **19** showing in vivo efficacy in nude mice bearing HCC827 tumor xenografts. Further testing could identify the full potential of this novel series for the treatment of cancers.

Experimental Section

General Methods. All commercial chemicals and solvents are reagent grade and were used without further treatment unless otherwise noted. All reactions were carried out under an atmosphere of dry nitrogen. Reactions were monitored by TLC using Merck 60 F_{254} silica gel glass backed plates (5 cm × 10 cm); zones were detected visually under ultraviolet irradiation (254 nm) or by spraying with phosphomolybdic acid reagent (Aldrich), followed by heating at 80 °C. Flash column chromatography was done using silica gel (Merck Kieselgel 60, no. 9385, 230−400 mesh ASTM). ^{1}H NMR spectra were obtained with a Varian Mercury-300 spectrometer operating at 300 MHz. Chemical shifts were recorded in parts per million (ppm, δ) and were reported relative to the solvent peak or TMS. High-resolution mass spectra (HRMS) were measured with a Finnigan (MAT-95XL) electron impact (EI) or by using Finnigan/Thermo Quest MAT 95XL FAB mass spectrometer. LCMS data were measured on an Agilent MSD-1100 ESI-MS/MS System. Purity of the final compounds were determined with an Hitachi 2000 series HPLC system using C-18 column (Agilent ZORBAX Eclipse XDB-C18 5 μm. 4.6 mm × 150 mm) using mobile phase A-acetonitrile and mobile phase B-water containing 0.1% formic acid + 10 mmol NH_4OAc. Elution condition, at 0 min phase A 10% + phase B 90%; at 45 min phase A 90% + phase B 10%; at 50 min phase A 10% + phase B 90%; at 60 min phase A 10% + phase B 90%. The flow-rate was 0.5 mL/min, and the injection volume was 5 μL. The system operated at 25 °C. Peaks were detected at 210 nm. Purity of all the tested compounds were found to be ≥95% unless otherwise stated.

Ethyl 2-Amino-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate (29a). To a mixture of cyclohexanone (**28a**) (9.80 g, 100.0 mmol), ethyl cyanoacetate (11.32 g, 100.0 mmol), and sulfur (3.20 g, 100.0 mmol) in absolute ethanol (200 mL) was added triethylamine (20 mL) and refluxed for 16 h; the reaction mixture was concentrated and the residue was partitioned between water and ethyl acetate. The organic layer was separated, dried over $MgSO_4$, and concentrated, and the crude product was purified by silica gel column chromatography using a mixture of hexanes: ethyl acetate (4:1), to give **29a** (18.6 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31 (t, 3H, J = 7.2 Hz), 1.68−1.77 (m, 4H), 2.45−2.49 (m, 2H), 2.66−2.69 (m, 2H), 4.23 (q, 2H, J = 7.2 Hz), 5.90 (brs, 2H). LCMS (ESI): m/z 226.0 $[M + H]^+$.

Ethyl 2-Amino-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate (29b). Compound **29b** was synthesized in 78% yield from cyclopentanone (**28b**), in a manner similar to **29a**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.30 (t, 3H, J = 7.2 Hz), 2.28 (dddd, 2H, J = 7.2, 7.2, 7.2, 7.2 Hz), 2.66−2.71 (m, 2H), 2.77−2.81 (m, 2H), 4.21 (q, 2H, 7.2 Hz), 5.59 (brs, 2H). LCMS (ESI): m/z 212.0 $[M + H]^+$.

6-(*tert*-Butyl) 3-Ethyl 2-Amino-4,5,6,7-tetrahydrothieno[2,3-*c*]-pyridine-3,6-dicarboxylate (29c). To a mixture of 4-oxo-piperidine-1-carboxylic acid *tert*-butyl ester (**28c**) (12.90 g, 64.7 mmol), ethyl cyanoacetate (7.31 g, 64.7 mmol), and sulfur (2.07 g, 64.7 mmol) in absolute ethanol (50 mL) was added triethylamine (9 mL). After stirring for 16 h at room temperature, the precipitate was collected by filtration and washed with ethanol to give **29c** (18.7 g, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.30 (t, 3H, J = 7.2 Hz), 1.44 (s, 9H), 2.72−2.80 (brs, 2H), 3.58 (t, 2H, J = 6.0 Hz), 4.23 (q, 2H, J = 7.2 Hz), 4.31 (s, 2H), 6.02 (s, 2H). LCMS (ESI): m/z 327.0 $[M + H]^+$.

3,4,5,6,7,8-Hexahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-one (30a). A mixture of **29a** (0.90 g, 4.0 mmol) and formamide (10 mL) was refluxed for 2 h. The reaction mixture was cooled, water was added, and the precipitate formed was collected by filtration, and washed thoroughly with water to give **30a** (0.8 g, 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.83−2.02 (m, 4H), 2.76−2.79 (m, 2H), 2.99−3.02 (m, 2H), 7.91 (s, 1H). LCMS (ESI): m/z 207.0 $[M + H]^+$.

3,5,6,7-Tetrahydro-4*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidin-4-one (30b). Compound **30b** was synthesized in 83% yield from **29b**, in a manner similar to **30a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.47 (dddd, 2H, J = 7.2, 7.2, 7.2, 7.2 Hz), 2.95−2.98 (m, 2H), 3.05−3.09 (m, 2H), 8.03 (s, 1H). LCMS (ESI): m/z 193.0 $[M + H]^+$.

***tert*-Butyl 4-Oxo-3,4,5,6,7,8-hexahydropyrido[4′,3′:4,5]thieno-[2,3-*d*]pyrimidine-7-carboxylate (30c).** A mixture of **29c** (18.50 g, 56.7 mmol) and formamidine acetate (8.85 g, 85.0 mmol) in DMF (100 mL) were heated at 100 °C for 16 h. The reaction mixture was cooled, DMF removed under vacuum, and the solid obtained was washed thoroughly with water to give **30c** (15.8 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 9H), 3.02−3.08 (brs, 2H), 3.62−3.70 (brs, 2H), 4.56−4.62 (brs, 2H), 7.88 (s, 1H). LCMS (ESI): m/z 308.1 $[M + H]^+$.

4-Chloro-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidine (31a). A mixture of **30a** (12.0 g, 58.2 mmol) and $POCl_3$ (10 mL) was heated at 55−65 °C for 3 h. The reaction mixture was cooled in an ice bath and then carefully neutralized by the addition of aqueous sodium bicarbonate solution. The resulting mixture was extracted with ethyl acetate, the organic layer was separated, dried over $MgSO_4$, and concentrated, and the crude product was purified by silica gel column chromatography using a mixture of hexanes:ethyl acetate (20:1), to give **31a** (9.6 g, 73%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.89−1.92 (m, 4H), 2.88−2.86 (m, 2H), 3.10−3.07 (m, 2H), 8.69 (s, 1H). LCMS (ESI): m/z 225.3 $[M + H]^+$.

4-Chloro-6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidine (31b). Compound **31b** was synthesized in 88% yield from **30b** in a manner similar to **31a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.48−2.56 (m, 2H), 3.04−3.08 (m, 2H), 3.13−3.17 (m, 2H), 8.70 (s, 1H). LCMS (ESI): m/z 210.9 $[M + H]^+$.

***tert*-Butyl 4-Chloro-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno-[2,3-*d*]pyrimidine-7-carboxylate (31c).** To a mixture of $POCl_3$ (3 mL) and triethylamine (3 mL) at 0 °C was added **30c** (2.3 g, 7.5 mmol). The reaction mixture was heated at 55−60 °C for 3 h, then the reaction mixture was cooled, excess of $POCl_3$ removed under vacuum, and the remaining residue neutralized carefully by adding saturated aqueous sodium bicarbonate solution. The resulting mixture was extracted with dichloromethane, the organic layer separated, dried over $MgSO_4$, concentrated, and the crude product obtained was purified by silica gel column chromatography using a mixture of hexane:ethyl acetate (10:1) to give **31c** (1.85 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46 (s, 9H), 3.14−3.18 (brs, 2H), 3.74 (t, 2H, J = 5.6 Hz), 4.69 (s, 2H), 8.70 (s, 1H). LCMS (ESI): m/z 326.0 $[M + H]^+$.

***N*-Benzyl-*N*-(5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-yl)amine (7).** A mixture of **31a** (45 mg, 0.2 mmol) and benzylamine (86 mg, 4.0 equiv, 0.8 mmol) in *n*-butanol (2 mL) was refluxed for 8 h. The reaction mixture was concentrated, and the residue was partitioned between water and dichloromethane. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The crude product was purified by silica gel column chromatography using a mixture of hexane:ethyl acetate (6:1) to give **7** (42 mg, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.84−1.88 (m, 4H), 2.75−2.78 (m, 2H), 2.82−2.84 (m, 2H), 4.77 (d, 2H, J = 5.6 Hz), 5.54 (brs, 1H), 7.24−7.34 (m, 5H), 8.38 (s, 1H). HRMS (EI): calcd for $C_{17}H_{17}N_3S$ (M^+) 295.1143, found 295.1129.

***N*-Phenethyl-*N*-(5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-yl)amine (7a).** Compound **7a** was synthesized in 52% yield from **31a** and phenylethylamine, in a manner similar to **7**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.75−1.80 (m, 4H), 2.49−2.52 (m, 2H), 2.70−2.72 (m, 2H), 2.94 (t, 2H, J = 6.4 Hz), 3.80 (td, 2H, 6.4, 6.4 Hz), 5.19 (m, 1H), 7.19−7.23 (m, 3H), 7.28−7.31 (m, 2H), 8.35 (s, 1H). LCMS (ESI): m/z 310.0 $[M + H]^+$. HPLC purity 90.63%.

***N*-(4-Methoxybenzyl)-*N*-(5,6,7,8-tetrahydrobenzo[4,5]thieno-[2,3-*d*]pyrimidin-4-yl)amine (7b).** Compound **7b** was synthesized in 65% yield from **31a** and 4-methoxybenzylamine, in a manner similar to **7**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.80−1.85 (m, 4H), 2.75−2.78 (m, 2H), 2.80−2.82 (m, 2H), 3.78 (s, 3H), 4.68 (d, 2H, J = 5.2 Hz), 5.45 (m, 1H), 6.85−6.88 (m, 2H), 7.26−7.28 (m, 2H), 8.39 (s, 1H). LCMS (ESI): m/z 326.0 $[M + H]^+$.

***N*-(4-Fluorobenzyl)-*N*-(5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-yl)amine (7c).** Compound **7c** was synthesized in 76% yield from **31a** and 4-fluorobenzylamine, in a manner similar to **7**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.86−1.91 (m, 4H), 2.80−2.82 (m, 2H), 2.85−2.88 (m, 2H), 4.77 (d, 2H, J = 6.0 Hz), 5.53 (m, 1H), 7.01−7.06 (m, 2H), 7.32−7.35 (m, 2H), 8.41 (s, 1H). LCMS (ESI): m/z 314.0 $[M + H]^+$.

***N*-Benzyl-*N*-(6,7-dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidin-4-yl)amine (7d).** Compound **7d** was synthesized in 79% yield from **31b** and benzylamine in a manner similar to **7**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.45−2.53 (m, 2H), 2.94−2.98 (m, 4H), 4.80 (d, 2H, J = 5.6 Hz), 5.37 (m, 1H), 7.24−7.35 (m, 5H), 8.40 (s, 1H). HRMS (EI): calcd for $C_{16}H_{15}N_3S$ (M^+) 281.0987, found 281.1009.

(2*S*)-2-Phenyl-2-(5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]-pyrimidin-4-ylamino)ethan-1-ol (9). A mixture of **31a** (0.05 g, 0.2 mmol) and *S*-(+)-2-amino-2-phenyl-ethanol (0.04 g, 1.5 equiv, 0.3 mmol) in *n*-butanol (2 mL) was refluxed for 8 h. The reaction mixture was concentrated, and the residue was partitioned between water and dichloromethane. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The crude product was purified by silica gel column chromatography using a mixture of dichloromethane:methanol (30:1), to give **9** (0.048 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.83−1.88 (m, 4H), 2.74 (m, 2H), 2.88−2.93 (m, 2H), 3.95−3.99 (m, 2H), 5.36 (dd, 1H, J = 5.6, 9.6 Hz), 6.14 (d, 1H, J = 6.0 Hz), 7.26−7.34 (m, 5H), 8.20 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.3 (CH_2), 22.4 (CH_2), 25.3 (CH_2), 26.1 (CH_2), 57.0 (CH), 67.1 (CH_2), 116.2 (C), 125.3 (C), 126.4 (CH), 127.7 (CH). 128.8 (CH), 133.6 (C), 139.7 (C), 152.4 (CH), 156.8 (C), 165.0 (C). HRMS (EI): calcd for $C_{18}H_{19}N_3OS$ (M^+) 325.1249, found 325.1247.

***N*-[(1*R*)-1-Phenylethyl]-*N*-(5,6,7,8-tetrahydrobenzo[4,5]thieno-[2,3-*d*]pyrimidin-4-yl)amine (9a).** Compound **9a** was synthesized in 94% yield from **31a** and *R*-(+)-methylbenzylamine in a manner similar to **9**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.60 (d, 3H, J = 6.6 Hz), 1.85−1.92 (m, 4H), 2.76−2.81 (m, 2H), 2.89−2.91 (m, 2H), 5.51−5.52 (m, 1H), 7.25−7.39 (m, 5H), 8.33 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.4 (CH_2), 22.5 (CH_2), 22.6 (CH_3), 25.4 (CH_2), 26.3 (CH_2), 49.7 (CH), 115.9 (C), 125.2 (C), 126.0 (CH), 127.3 (CH), 128.3 (CH), 133.2 (C), 143.7 (C), 153.0 (CH), 156.5 (C), 165.3 (C). HRMS (EI): calcd for $C_{18}H_{19}N_3S$ (M^+) 309.1300, found 309.1296.

2-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-*d*]pyrimidin-4-ylamino)-1-ethanol (9b). Compound **9b** was synthesized in 88% yield from **31a** and ethanolamine in a manner similar to **9**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.78−1.84 (m, 4H), 2.70−2.72 (m, 2H), 2.82−2.84 (m, 2H), 3.66 (dt, 2H, J = 5.2, 5.2 Hz), 3.82 (t, 2H, J = 5.2 Hz), 5.80 (t, 1H, J = 5.2 Hz), 8.24 (s,1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.3 (CH_2), 22.3 (CH_2), 25.2 (CH_2), 26.1 (CH_2), 43.9 (CH_2), 62.0 (CH_2), 116.1 (C), 125.5 (C), 133.3 (C), 152.2 (CH), 157.4 (C), 164.7 (C). HRMS (EI): calcd for $C_{12}H_{15}N_3OS$ (M^+) 249.0936, found 249.0958.

(2*R*)-2-Phenyl-2-(5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-*d*]-pyrimidin-4-ylamino)ethan-1-ol (9c). Compound **9c** was synthesized in 59% yield from **31a** and *R*-(−)-2-amino-2-phenyl-ethanol in a manner similar to **9**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.82−1.88 (m, 4H), 2.73−2.76 (m, 2 H), 2.88−2.93 (m, 2 H), 3.93−3.99 (m, 2 H), 5.35−5.38 (m, 1 H), 6.11 (d, 1 H, J = 6.4 Hz), 7.25−7.34 (m, 5 H), 8.20 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.3 (CH_2), 22.4 (CH_2), 25.3 (CH_2), 26.1 (CH_2). 57.0 (CH), 67.1 (CH_2), 116.2 (C), 125.3 (C), 126.4 (CH), 127.7 (CH), 128.8 (CH), 133.6 (C), 139.7 (C), 152.4 (CH), 156.8 (C), 165.0 (C). HRMS (EI): calcd for $C_{18}H_{19}N_3OS$ (M^+) 325.1249, found 325.1254.

(2*S*)-2-(6,7-Dihydro-5*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidin-4-ylamino)-2-phenylethan-1-ol (9d). Compound **9d** was synthesized in 94% yield from **31b** and *S*-(+)-2-amino-2-phenyl-ethanol in a manner similar to **9**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.48−2.55 (m, 2H), 2.93−3.06 (m, 4H), 3.83 (brs, 1H), 4.00 (dd, 2H, J = 5.2, 5.2 Hz), 5.34 (ddd, 1H, J = 5.2, 5.2, 5.2 Hz), 5.80 (d, 1H, J = 6.0 Hz), 7.29−7.40 (m, 5H), 8.31 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.9 (CH_2), 28.9 (CH_2), 29.4 (CH_2), 57.1 (CH), 67.2 (CH_2), 113.4 (C), 126.4 (CH), 127.8 (CH), 128.9 (CH), 134.3 (C), 139.1 (C), 139.6 (C), 152.5 (CH), 156.3 (C), 165.0 (C). HRMS (EI): calcd for $C_{17}H_{17}N_3OS$ (M^+) 311.1092, found 311.1096.

***tert*-Butyl 4-[(1*S*)-2-Hydroxy-1-phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidine-7-carboxylate (32a).** A mixture of **31c** (0.25 g, 0.7 mmol) and *S*-(+)-2-amino-2-phenyl-ethanol (0.13 g, 1.1 mmol) in ethanol (1 mL) was refluxed for 8 h. The reaction mixture was concentrated, and the residue was partitioned between water and dichloromethane; the organic layer separated, dried over $MgSO_4$, and concentrated, and the crude product was purified by silica gel column chromatography using a mixture of dichloromethane:methanol (20:1) to give **32a** (0.31 g, 95%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H), 2.90−3.08 (m, 2H), 3.72−3.81 (m, 2H), 3.97−4.02 (m, 2H), 4.62 (s, 2H), 5.36−5.43 (m, 1H), 6.04 (d, 1H, J = 6.6 Hz), 7.26−7.38 (m, 5H), 8.25 (s, 1H). LCMS (ESI): m/z 427.0 $[M + H]^+$.

***tert*-Butyl 4-[(1*R*)-2-Hydroxy-1-phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidine-7-carboxylate (32b).** Compound **32b** was synthesized in 99% yield from **31c** and *R*-(−)-2-amino-2-phenyl-ethanol in a manner similar to **32a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.46 (s, 9H), 2.98−3.02 (m, 2H), 3.73−3.76 (m, 2H), 3.96−3.99 (m, 2H), 4.61 (s, 2H), 5.37−5.38

(m, 1H), 6.04 (d, 1H, J = 6.0 Hz), 7.26−7.34 (m, 5H), 8.23 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.8 (CH_2), 27.7 (CH_2), 28.1 (CH_3), 39.8 (CH_2), 40.9 (CH_2), 42.6 (CH_2), 43.4 (CH_2), 56.0 (CH), 65.7 (CH_2), 80.3 (C), 115.3 (C), 124.4 (C), 126.3 (CH), 127.2 (CH), 128.4 (CH), 129.2 (C), 139.6 (C), 152.7 (CH), 154.0 (C), 156.5 (C), 164.9 (C). LCMS (ESI): m/z 427.1 $[M + H]^+$.

***tert*-Butyl 4-[(1*R*)-1-Phenylethyl]amino-5,6,7,8-tetrahydropyrido-[4′,3′:4,5]thieno[2,3-*d*]pyrimidine-7-carboxylate (32c).** Compound **32c** was synthesized in 90% yield from **31c** and *R*-(+)-methylbenzylamine in a manner similar to **32a**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.47 (s, 9H), 1.57 (d, 3H, J = 9.6 Hz), 2.95−3.03 (m, 2H), 3.73−3.79 (m, 2H), 4.62 (brs, 2H), 5.35−5.37 (m, 1H), 5.49−5.54 (m, 1H), 7.24−7.38 (m, 5H), 8.37 (s, 1H). LCMS (ESI): m/z 411.2 $[M + H]^+$.

***tert*-Butyl 4-[(1*R*)-1-phenylpropyl]amino-5,6,7,8-tetrahydropyrido-[4′,3′:4,5]thieno[2,3-*d*]pyrimidine-7-carboxylate (32d).** Compound **32d** was synthesized in 95% yield from **31c** and *R*-(+)-1-phenylpropylamine in a manner similar to **32a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.93 (t, 3H, J = 7.2 Hz), 1.42 (s, 9H), 1.87−2.00 (m, 2H), 2.98−3.00 (m, 2H), 3.77−3.78 (m, 2H), 4.62 (s, 2H), 5.29 (td, 1H, J = 7.2, 7.2 Hz), 5.41 (d, 1H, J = 7.2 Hz), 7.22−7.33 (m, 5H), 8.33 (s, 1H). LCMS (ESI): m/z 425.1 $[M + H]^+$.

***tert*-Butyl 4-[(1*S*)-1-Benzyl-2-hydroxyethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidine-7-carboxylate (32e).** Compound **32e** was synthesized in 87% yield from **31c** and *S*-(−)-2-amino-3-phenyl-1-propanol in a manner similar to **32a**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (s, 9H), 2.37−2.44 (m, 1H), 2.66−2.71 (m, 1H), 2.91 (dd, 1H, J = 8.4, 13.8 Hz), 3.06 (dd, 1H, J = 6.6, 13.8 Hz), 3.60 (m, 2H), 3.72 (dd, 1H, J = 5.4, 10.8 Hz), 3.84 (dd, 1H, J = 2.4, 10.8 Hz), 4.43−4.48 (m, 1H), 4.56 (s, 2H), 5.37 (d, 1H, J = 5.7 Hz), 7.20−7.32 (m, 5H), 8.32 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.8 (CH_2), 28.3 (CH_3), 37.2 (CH_2), 39.9 (CH_2), 41.0 (CH_2), 42.9 (CH_2), 43.6 (CH_2), 54.7 (CH), 65.1 (CH_2), 80.6 (C), 115.5 (C), 124.5 (C), 127.0 (CH), 128.8 (CH), 129.3 (CH), 137.2 (C), 152.7 (CH), 154.2 (C), 157.0 (C), 165.8 (C). LCMS (ESI): m/z 441.1 $[M + H]^+$.

***tert*-Butyl 4-[(2-Hydroxyethyl)amino]-5,6,7,8-tetrahydropyrido-[4′,3′:4,5]thieno[2,3-*d*]pyrimidine-7-carboxylate (32f).** Compound **32f** was synthesized in 91% yield from **31c** and ethanolamine in a manner similar to **32a**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (s, 9H), 2.97−3.00 (m, 2H), 3.71−3.77 (m, 4H), 3.84−3.88 (m, 2H), 4.62 (s, 2H), 5.81−5.83 (m, 1H), 8.33 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.9 (CH_2), 28.4 (CH_2), 39.9 (CH_2), 41.0 (CH_2), 43.5 (CH_2), 61.3 (CH_2), 80.6 (C), 115.3 (C), 124.5 (C), 129.3 (C), 152.6 (CH), 154.3 (C), 157.3 (C), 165.0 (C). LCMS (ESI): m/z 351.0 $[M + H]^+$.

(2*S*)-2-Phenyl-2-(5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]-pyrimidin-4-ylamino)ethan-1-ol (33a/10). To a mixture of **32a** (0.6 g, 1.4 mmol) in dichloromethane (2 mL) at 0 °C was added trifluoroacetic acid (TFA) (1 mL) and then warmed to room temperature. The reaction mixture was stirred for 2 h, removed the solvent under vacuum, and neutralized the residue by slow addition of sodium bicarbonate solution. The precipitate formed was collected by filtration and washed with water to give **33a**/**10** (0.4 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.84−3.04 (m, 2H), 3.20 (t, 2H, J = 4.4 Hz), 3.99 (d, 2H, J = 3.2 Hz), 4.01 (d, 2H, J = 7.6 Hz), 5.38 (d, 1H, J = 4.8 Hz), 6.04 (d, 1H, J = 6.4 Hz), 7.26−7.38 (m, 5H), 8.28 (s, 1H). LCMS (ESI): m/z 327.1 $[M + H]^+$.

(2*R*)-2-Phenyl-2-(5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]-pyrimidin-4-ylamino)ethan-1-ol (33b). Compound **33b** was synthesized in 97% yield from **32b** in a manner similar to **33a**. ^{1}H NMR (400 MHz, CD_3OD): δ 3.14−3.17 (m, 2H), 3.21−3.24 (m, 2H), 3.89−3.97 (m, 2H), 4.03−4.04 (m, 2H), 5.38 (dd, 1H, J = 5.6, 5.6 Hz), 7.21−7.25 (m, 1H), 7.29−7.33 (m, 2H), 7.38−7.41 (m, 2H), 8.15 (s, 1H). LCMS (ESI): m/z 327.0 $[M + H]^+$.

***N*-[(1*R*)-1-Phenylethyl]-*N*-(5,6,7,8-tetrahydropyrido[4′,3′:4,5]-thieno[2,3-*d*]pyrimidin-4-yl)amine (33c).** Compound **33c** was synthesized in 90% yield from **32c** in a manner similar to **33a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.60 (d, 3H, J = 6.8 Hz), 2.89−2.95 (m, 2H), 3.21−3.44 (m, 2H), 4.04 (brs, 2H), 5.26−5.41 (m, 1H), 5.49−5.53 (m, 1H), 7.24−7.38 (m, 5H), 8.35 (s, 1H). LCMS (ESI): m/z 311.1 $[M + H]^+$.

***N*-[(1*R*)-1-Phenylpropyl]-*N*-(5,6,7,8-tetrahydropyrido[4′,3′:4,5]-thieno[2,3-*d*]pyrimidin-4-yl)amine (33d).** Compound **33d** was synthesized in 82% yield from **32d** in a manner similar to **33a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.93 (t, 3H, J = 7.6 Hz), 1.89−1.97 (m, 2H), 2.90−3.00 (m, 2H), 3.24 (t, 2H, 5.6 Hz), 4.04 (s, 2H), 5.30 (td, 1H, 7.2, 7.2 Hz), 5.46 (d, 1H, 7.2 Hz), 7.22−7.33 (m, 5H), 8.32 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 10.6 (CH_3), 27.2 (CH_2), 29.6 (CH_2), 42.9 (CH_2), 45.0 (CH_2), 55.6 (CH), 115.7 (C), 123.9 (C), 126.4 (CH), 127.2 (CH), 128.6 (CH), 132.2 (C), 142.4 (C), 153.3 (CH), 156.9 (C), 165.4 (C). LCMS (ESI): m/z 325.0 $[M + H]^+$.

(2*S*)-3-Phenyl-2-(5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]-pyrimidin-4-ylamino)propan-1-ol (33e). Compound **33e** was synthesized in 96% yield from **32e** in a manner similar to **33a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.38−2.42 (m, 1H), 2.67−2.71 (m, 1H), 2.89−2.95 (m, 1H), 3.03−3.19 (m, 3H), 3.73 (dd, 1H, J = 5.6, 11.2 Hz), 3.76 (dd, 1H, J = 2.8, 11.2 Hz), 4.05 (s, 2H), 4.44−4.47 (m, 1H), 5.37 (d, 1H, J = 6.0 Hz), 7.22−7.31 (m, 5H), 8.32 (s, 1H). LCMS (ESI): m/z 341.0 $[M + H]^+$.

2-(5,6,7,8-Tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-4-ylamino)-1-ethanol (33f). Compound **33f** was synthesized in 49% yield from **32f**, in a manner similar to **33a**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.96 (t, 2H, J = 5.6 Hz), 3.18 (t, 2H, J = 5.6 Hz), 3.65 (t, 2H, J = 5.6 Hz), 3.76 (t, 2H, J = 5.6 Hz), 4.00 (s, 2H), 8.18 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 26.6 (CH_2), 43.1 (CH_2), 44.1 (CH_2), 44.9 (CH_2), 61.3 (CH_2), 116.9 (C), 126.4 (C), 131.1 (C), 153.8 (CH), 158.6 (C), 165.6 (C). LCMS (ESI): m/z 251.0 $[M + H]^+$.

1-(4-(((1*S*)-2-Hydroxy-1-phenylethyl)amino)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-2-propen-1-one (11). A mixture of acrylic acid (12 mg, 0.16 mmol) and EDCI (32 mg, 0.16 mmol) in anhydrous dicloromethane was stirred for 2 h and **33a**/**10** (50 mg, 0.15 mmol) was then added. The resulting mixture was stirred for 15 min, then partitioned between water and ethyl acetate; the organic layer was dried over $MgSO_4$ and concentrated and the residue was purified by silica gel column chromatography using a mixture of dichloromethane:methanol (20:1) to give **11** (35 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.06−3.12 (m, 2H), 3.94−3.99 (m, 4H), 4.76−4.83 (m, 2H), 5.39 (m, 1H), 5.75 (dd, 1H, J = 10.4, 0.8 Hz), 5.96−6.11 (m, 1H), 6.27−6.39 (m, 1H), 6.52−6.64 (m,1H), 7.25−7.36 (m, 5H), 8.26 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.8 (CH_2), 27.0 (CH_2), 39.3 (CH_2), 42.0 (CH_2), 42.9 (CH_2), 45.3 (CH_2), 56.6 (CH), 66.8 (CH_2), 115.4 (C), 123.6 (C), 125.4 (C), 126.4 (CH), 126.9 (CH), 127.3 (CH), 127.8 (CH), 128.5 (C), 128.9 (CH), 130.0 (C), 139.4 (C), 153.2 (CH), 156.8 (C), 165.7 (C). HRMS (EI) calcd for $C_{20}H_{20}N_4O_2S$ (M^+) 380.1307, found 380.1310.

1-(4-[(1*S*)-2-Hydroxy-1-phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-1-propanone (12). Compound **12** was synthesized in 63% yield from **33a** and propionic acid in a manner similar to **11**. ^{1}H NMR (400 MHz, CD_3OD): δ 1.12 (t, 1.2H, J = 7.6 Hz), 1.17 (t, 1.8 H, J = 7.6 Hz), 2.48 (q, 0.8 H, J = 7.6 Hz), 2.55 (q, 1.2H, J = 7.6 Hz), 3.14−3.26 (m, 2H), 3.87−3.98 (m, 4H), 4.77−4.79 (m, 2H), 5.38−5.40 (m, 1H), 7.20−7.23 (m, 1H), 7.24−7.33 (m, 2H), 7.38−7.42 (m, 2H), 8.15 (s, 1H). HRMS (EI): calcd for $C_{20}H_{21}N_4O_2S$ (M^+) 382.1463, found 382.1466.

1-(4-[(1*S*)-2-Hydroxy-1-phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-2-butyn-1-one (13). Compound **13** was synthesized in 68% yield from **33a** and 2-butynoic acid in a manner similar to **11**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.97−2.03 (m, 3H), 2.96−3.24 (m, 2H), 3.84−4.15 (m, 4H), 4.67−4.82 (m, 1H), 4.92 (s, 1H), 5.35−5.45 (m, 1H), 6.13−6.16 (m, 1H), 7.23−7.36 (m, 5H), 8.22 (s, 0.5H), 8.24 (s, 0.5H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 4.1 (CH_3), 25.7 (CH_2), 26.7 (CH_2), 29.6 (C), 38.4 (CH_2), 41.0 (CH_2), 43.7 (CH_2), 46.2 (CH_2), 56.5 (CH), 56.7 (CH), 66.4 (CH_2), 66.5 (CH_2), 72.4 (C), 72.5 (C), 90.1 (C), 91.3 (C), 115.3 (C), 115.4 (C), 124.0 (C), 124.9 (C), 126.4

(CH), 126.5 (C), 127.7 (C), 127.8 (CH), 128.6 (CH), 128.8 (C), 128.8 (C), 129.0 (C), 139.3 (C), 139.4 (C), 152.8 (C), 153.2 (CH), 153.3 (CH), 156.7 (C), 156.9 (C), 164.9 (C), 165.1 (C). HRMS (EI): calcd for $C_{21}H_{20}N_4O_2S$ (M^+) 392.1307, found 392.1311.

(2*S*)-2-Phenyl-2-[7-(vinylsulfonyl)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-4-yl]aminoethan-1-ol (14). A mixture of **33a** (88 mg, 0.27 mmol), Et_3N (0.2 mL, 5 equiv), and 2-chloroethanesulfonyl chloride (175 mg, 1.08 mmol) in dichloromethane (2 mL) was stirred at room temperature for 4 h. The reaction mixture was concentrated, and the residue was partitioned between water and dichloromethane; the organic layer was washed with brine, dried over $MgSO_4$, and concentrated under vacuo. The crude product was purified by silica gel column chromatography using a mixture of dichloromethane:methanol (40:1), to give **14** (66 mg, 59%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.04−3.18 (m, 2H), 3.63 (t, 2H, J = 4.8 Hz), 3.96−4.23 (m, 2H), 4.47 (s, 2H), 5.41 (m, 1H), 5.99 (d, 1H, J = 6.4 Hz), 6.02 (d, 1H, J = 9.6 Hz), 6.32 (d, 1H, J = 16.4 Hz), 6.44 (dd, 1H, J = 9.6, 16.4 Hz), 7.28−7.39 (m, 5H), 8.32 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.2 (CH_2), 42.5 (CH_2), 44.6 (CH_2), 56.9 (CH), 66.9 (CH_2), 115.3 (C), 124.0 (C), 126.4 (CH), 128.0 (CH), 128.3 (C), 128.9 (CH), 129.0 (CH_2), 133.2 (CH), 139.3 (C), 153.2 (CH), 156.9 (C), 165.7 (C). HRMS (EI): calcd for $C_{19}H_{20}N_4O_3S_2$ (M^+) 416.0977, found 416.0977. HPLC purity 92.63%.

1-(4-[(1*R*)-2-Hydroxy-1-phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno [2,3-*d*]pyrimidin-7-yl)-2-propen-1-one (15). Compound **15** was synthesized in 68% yield from **33b** and acrylic acid in a manner similar to **11**. ^{1}H NMR (400 MHz, CD_3OD): δ 3.25−3.27 (m, 2H), 3.89 (dd, 2H, J = 6.0, 11.2 Hz), 3.94−4.04 (m, 2H), 4.90 (m, 2H), 5.39 (dd, 1H, J = 5.2, 5.2 Hz), 5.79−5.84 (m, 1H), 6.25−6.30 (m, 1H), 6.80−6.93 (m, 1H), 7.20−7.24 (m, 1H), 7.29−7.33 (m, 2H), 7.39−7.41 (m, 2H), 8.16 (s, 1H). LCMS (ESI): m/z 381.1 $[M + H]^+$. HPLC purity 93.46%.

1-(4-[(1*R*)-1-Phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-2-propen-1-one (16). Compound **16** was synthesized in 74% yield from **33c** and acrylic acid in a manner similar to **11**. ^{1}H NMR (300 MHz, CD_3OD): δ 1.61 (d, 3H, J = 7.2 Hz), 3.01−3.22 (m, 2H), 3.91−4.05 (m, 2H), 4.78−5.01 (m, 2H), 5.48 (dd, 1H, J = 6.9, 13.8 Hz), 5.81 (d, 1H, J = 10.8 Hz), 6.26 (d, 1H, J = 16.5 Hz), 6.75−6.93 (m, 1H), 7.18−7.42 (m, 5H), 8.20 (s, 1H). LCMS (ESI): m/z 365.1 $[M + H]^+$.

1-(4-[(1*R*)-1-Phenylpropyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-2-propen-1-one (17). Compound **17** was synthesized in 60% yield from **33d** and acrylic acid in a manner similar to **11**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (t, 3H, J = 7.6 Hz), 1.81−2.01 (m, 2H), 2.97−3.11 (m, 2H), 3.81−4.10 (m, 2H), 4.74−4.95 (m, 2H), 5.26−5.43 (m, 2H), 5.74−5.78 (m, 1H), 6.24−6.36 (m, 1H), 6.52−6.67 (m, 1H), 7.21−7.35 (m, 5H), 8.33 (s, 1H). LCMS (ESI): m/z 379.1 $[M + H]^+$. HPLC purity 92.06%.

(*E*)-1-(4-[(1*S*)-2-Hydroxy-1-phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-3-phenyl-2-propen-1-one (18). Compound **18** was synthesized in 73% yield from **33a** and *trans*-cinnamic acid in a manner similar to **11**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.08 (br, 2H), 3.92−4.04 (m, 4H), 4.75−4.92 (m, 2H), 5.41 (s, 1H), 6.16 (s, 1H), 6.91 (d, 1H, J = 15.6 Hz), 7.24−7.27 (m, 8H), 7.47−7.52 (m, 2H), 7.67 (d, 1H, J = 15.6 Hz), 8.23 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.8 (CH_2), 27.1 (CH_2), 39.4 (CH_2), 42.2 (CH_2), 42.9 (CH_2), 45.2 (CH_2), 56.7 (CH), 66.4 (CH_2), 115.4 (C), 116.2 (CH), 116.6 (CH), 123.9 (C), 125.5 (C), 126.5 (CH), 127.8 (CH), 128.8 (CH), 128.8 (CH), 129.0 (C), 129.9 (CH), 134.7 (C), 139.4 (C), 143.8 (CH), 152.7 (CH), 156.8 (C), 165.0 (C), 165.8 (C). HRMS (EI): calcd for $C_{26}H_{24}N_4O_2S$ (M^+) 456.1620, found 456.1616. HPLC purity 92.65%.

(*E*)-4-(Dimethylamino)-1-(4-[(1*S*)-2-hydroxy-1-phenylethyl]-amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-2-buten-1-one (19). A mixture of 4-bromocrotonoic acid (49 mg, 0.33 mmol) and EDCI (63 mg, 0.33 mmol) in anhydrous dichloromethane (3 mL) was stirred for 15 min, and **33a** (0.100 g, 0.30 mmol) was then added. The resulting mixture was stirred for 2 h, and then the solvent was removed under vacuum. The residue was reconstituted in THF (3 mL), *N*,*N*-dimethylamine (0.5 mL, 28% aqueous solution) was added, and stirring was continued for another 2 h. At the end of the reaction, the resulting mixture was partitioned between water and ethyl acetate; the organic layer was separated, dried over $MgSO_4$, and concentrated, and the residue was purified by silica gel column chromatography using a mixture of dichloromethane:methanol (5:1) to give **19** (88 mg, 67%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.24 (s, 6 H), 3.01−3.20 (m, 4H), 3.90−3.99 (m, 2H), 4.00−4.12 (m, 2H), 4.78 (s, 1H), 4.86 (s, 1H), 5.39 (s, 1H), 6.03 (d, 1H, J = 13.6 Hz), 6.38−6.57 (m, 1H), 6.80−6.98 (brs, 1H), 7.28−7.40 (m, 5H), 8.30 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.6 (CH_2), 26.9 (CH_2), 39.2 (CH_2), 41.9 (CH_2), 42.7 (CH_2), 45.1 (CH_2), 45.1 (CH_3), 56.0 (CH), 60.2 (CH_2), 65.8 (CH_2), 115.2 (C), 121.8 (CH), 123.8 (C), 125.4 (C), 126.3 (CH), 127.3 (CH), 128.0 (C), 128.5 (CH), 129.5 (C), 139.8 (C), 142.8 (CH), 142.3 (CH), 153.1 (CH), 153.2 (CH), 156.6 (C), 156.7 (C), 165.1 (C), 165.4 (C). HRMS (EI): calcd for $C_{23}H_{27}N_5O_2S$ (M^+) 437.1885, found 437.1869.

(*E*)-4-(Diethylamino)-1-(4-(((1*R*)-2-hydroxy-1-phenylethyl)-amino)-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-2-buten-1-one (20). Compound **20** was synthesized in 59% yield from **33a**, 4-bromocrotonoic acid, and *N*,*N*-diethylamine in a manner similar to **19**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.96−1.06 (m, 6H), 2.55−2.58 (m, 4H), 3.11−3.28 (m, 4H), 3.93−4.02 (m, 2H), 4.08−4.13 (m, 2H), 4.80−4.85 (m, 1H), 4.85−4.92 (m, 1H), 5.39−5.44 (m, 1H), 6.08 (s, 1H), 6.52 (dd, 1H, J = 14.7, 15.6 Hz), 6.90−6.97 (m, 1H), 7.27−7.41 (m, 5H), 8.32 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.6 (CH_3), 25.7 (CH_2), 27.0 (CH_2), 39.2 (CH_2), 42.0 (CH_2), 42.8 (CH_2), 45.1 (CH_2), 47.0 (CH_2), 54.1 (CH_2), 56.3 (CH), 66.3 (CH_2), 115.3 (C), 121.4 (CH), 122.2 (CH_2), 123.7 (CH_2), 126.4 (CH), 127.6 (CH), 128.2 (CH_2), 128.7 (CH), 129.8 (CH_2), 139.7 (C), 143.0 (CH), 144.5 (CH), 153.2 (CH), 153.3 (CH), 156.7 (C), 165.5 (C), 165.6 (C). HRMS (EI): calcd for $C_{25}H_{31}N_5O_2S$ (M^+) 465.2198, found 465.2195.

(*E*)-1-(4-[(1*R*)-2-Hydroxy-1-phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-4-morpholino-2-buten-1-one (21). Compound **21** was synthesized in 61% yield from **33a**, 4-bromocrotonoic acid, and morpholine in a manner similar to **19**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.42 (m, 4H), 3.10−3.11 (m, 4H), 3.60−3.67 (m, 4H), 3.88−3.97 (m, 4H), 4.79 (m, 1H), 4.74 (m, 1H), 4.81 (m, 1H), 5.36−5.38 (m, 1H), 6.04−6.09 (m, 1H), 6.53 (t, 1H, J = 16.2 Hz), 6.84−6.88 (m, 1H), 7.22−7.33 (m, 5H), 8.23 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.8 (CH_2), 27.0 (CH_2), 39.4 (CH_2), 42.0 (CH_2), 42.9 (CH_2), 45.2 (CH_2), 53.6 (CH_2), 56.5 (CH), 59.9 (CH_2), 66.6 (CH_2), 66.7 (CH_2), 115.3 (C), 121.9 (CH), 122.2 (CH), 123.6 (C), 125.5 (C), 126.4 (CH), 127.7 (CH), 128.3 (C), 128.8 (CH), 130.0 (C), 139.5 (C), 142.9 (CH), 153.2 (CH), 153.3 (CH), 156.7 (C), 165.2 (C), 165.8 (C). HRMS (EI): calcd for $C_{25}H_{29}N_5O_3S$ (M^+) 479.1991, found 479.1991.

(*E*)-1-(4-[(1*S*)-2-Hydroxy-1-phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-4-(4-methylpiperazino)-2-buten-1-one (22). Compound **22** was synthesized in 55% yield from **33a**, 4-bromocrotonoic acid, and *N*-methylpiperazine in a manner similar to **19**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.28 (s, 3H), 2.60 (m, 8H), 3.13−3.16 (m, 4H), 3.89−4.03 (m, 4H), 4.76−4.88 (m, 2H), 5.35−5.40 (m, 1H), 6.09 (d, 1H, J = 1.6 Hz), 6.40−6.50 (m, 1H), 6.82−6.88 (m, 1H), 7.25−7.37 (m, 5H), 8.28 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.8 (CH_2), 27.2 (CH_2), 39.4 (CH_2), 42.1 (CH_2), 43.0 (CH_2), 45.3 (CH_2), 45.8 (CH_3), 52.8 (CH_2), 53.1 (CH_2), 54.8 (CH_2), 56.4 (CH), 56.7 (CH), 59.5 (CH_2), 66.6 (CH_2), 115.4 (C), 122.1 (CH), 122.4 (CH), 123.6 (C), 125.5 (C), 126.4 (CH), 127.8 (CH), 128.9 (CH), 130.1 (C), 139.7 (C), 142.4 (CH), 143.4 (CH), 153.4 (CH), 156.9 (C), 165.5 (C), 166.0 (C). HRMS (EI): calcd for $C_{26}H_{32}N_6O_2S$ (M^+) 492.2307, found 492.2316.

(*E*)-4-(Dimethylamino)-1-(4-[(1*R*)-2-hydroxy-1-phenylethyl]-amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-2-buten-1-one (23). Compound **23** was synthesized in 68%

yield from **33b**, 4-bromocrotonoic acid, and *N*,*N*-dimethylamine in a manner similar to **19**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.14 (s, 3H), 2.33 (s, 3H), 2.97−3.20 (m, 4H), 4.08−4.09 (m, 2H), 4.73−4.96 (m, 4H), 5.36 (dd, 1H, J = 6.0, 10.4 Hz), 6.07 (d, 1H, J = 6.0 Hz), 6.48−6.72 (m, 1H), 6.81−6.86 (m, 1H), 7.23−7.39 (m, 5H), 8.28 (s, 1H). LCMS (ESI): m/z $[M + H]^+$ 438.1. HPLC purity 93.89%.

(*E*)-4-(Dimethylamino)-1-(4-[(1*R*)-1-phenylethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-2-buten-1-one (24). Compound **24** was synthesized in 79% yield from **33c**, 4-bromocrotonoic acid, and *N*,*N*-dimethylamine in a manner similar to **19**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.62 (d, 3H, J = 6.4 Hz), 2.27 (m, 6H), 3.03−3.11 (m, 4H), 3.93−3.99 (m, 2H), 4.80−4.89 (m, 2H), 5.32−5.40 (m, 1H), 5.52−5.53 (m, 1H), 6.48 (m, 1H), 6.91 (d, 1H, J = 14.8 Hz), 7.27−7.38 (m, 5H), 8.39 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.2 (CH_3), 25.9 (CH_2), 27.2 (CH_2), 39.4 (CH_2), 42.1 (CH_2), 42.9 (CH_2), 44.6 (CH_2), 45.3 (CH_3), 49.9 (CH), 60.1 (CH_2), 115.0 (C), 122.0 (CH), 123.4 (C), 125.2 (CH), 126.0 (CH), 127.4 (CH), 128.2 (C), 128.8 (CH), 129.7 (C), 142.7 (C), 143.2 (C), 153.7 (CH), 156.5 (C), 165.5 (C), 165.9 (C). HRMS (EI): calcd for $C_{23}H_{27}N_5OS$ (M^+) 421.1936, found 421.1941.

(*E*)-4-(Dimethylamino)-1-(4-[(1*S*)-1-phenylpropyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-2-buten-1-one (25). Compound **25** was synthesized in 67% yield from **33d**, 4-bromocrotonoic acid, and *N*,*N*-dimethylamine in a manner similar to **19**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.95 (t, 3H, J = 7.2 Hz), 1.85−2.04 (m, 2H), 2.17 (s, 3H), 2.37 (s, 3H), 3.13−3.17 (m, 4H), 3.84−4.03 (m, 2H), 4.80−4.89 (m, 2H), 5.30−5.46 (m, 2H), 6.45−6.60 (m, 1H), 6.89−6.94 (m, 1H), 7.25−7.36 (m, 5H), 8.37 (s, 1H). HRMS (EI): calcd for $C_{24}H_{29}N_5OS$ (M^+) 435.2093, found 435.2095.

(*E*)-1-(4-[(1*R*)-1-Benzyl-2-hydroxyethyl]amino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl)-4-(dimethylamino)-2-buten-1-one (26). Compound **26** was synthesized in 52% yield from **33e**, 4-bromocrotonoic acid, and *N*,*N*-dimethylamine in a manner similar to **19**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.27 (s, 3H), 2.33 (s, 3H), 2.49 (m, 1H), 2.77 (m, 1H), 2.92−2.95 (m, 1H), 3.07−3.15 (m, 3H), 3.60−3.93 (m, 4H), 4.46−4.49 (m, 1H), 4.76 (m, 1H), 4.85 (m, 1H), 5.37 (d, 1H, J = 11.6 Hz), 6.47 (dd, 1H, J = 16.0, 24.0 Hz), 6.87−7.00 (m, 1H), 7.27−7.36 (m, 5H), 8.36 (s, 1H). HRMS (EI): calcd for $C_{24}H_{29}N_5O_2S$ (M^+) 451.2042, found 451.2048.

(*E*)-4-(Dimethylamino)-1-4-[(2-hydroxyethyl)amino]-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-*d*]pyrimidin-7-yl-2-buten-1-one (27). Compound **27** was synthesized in 42% yield from **33f**, 4-bromocrotonoic acid, and *N*,*N*-dimethylamine in a manner similar to **19**. ^{1}H NMR (300 MHz, CD_3OD): δ 2.27 (s, 3H), 2.29 (s, 3H), 3.14−3.18 (m, 4H), 3.67−3.78 (m, 4H), 4.00 (dd, 2H, 6.0, 6.0 Hz), 4.83 (m, 2H), 6.63−6.88 (m, 2H), 8.25 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD): δ 26.6 (CH_2), 27.8 (CH_2), 40.5 (CH_2), 43.0 (CH_2), 44.0 (CH_2), 44.2 (CH_2), 45.3 (CH_3), 46.1 (CH_2), 61.2 (CH_2), 61.3 (CH_2), 116.6 (C), 124.1 (CH), 124.5 (CH), 126.5 (C), 127.3 (C), 129.3 (C), 129.9 (C), 143.3 (CH), 154.0 (CH), 158.7 (C), 166.0 (C), 167.3 (C). LCMS-ESI (m/z) 362.1 $[M + H]^+$. HPLC purity 93.35%.

EGFR WT and DM Kinase Inhibition Assay. GST-EGFR-KD^{WT} containing the EGFR kinase catalytic domain (residues 696−1022) and GST-EGFR-KD$^{L858RT/790M}$ containing the EGFR kinase catalytic domain (residues from 696 to 1022 and with L858R/T790M) were expressed in Sf9 insect cells transfected the baculovirus containing pBac-PAK8-GST-EGFR-KD plasmid, respectively. GST-EGFR-KD^{WT} protein expression and purification and kinase assay were done in a manner as reported earlier.[5] The EGFR$^{L858R/T790M}$ Kinase-Glo assays were carried out in 96-well plates at 37 °C for 60 min in a final volume of 50 μL including the following components: 200 ng GST-EGFR-KD$^{L858R/T790M}$ proteins, 25 mM HEPES, pH 7.4, 4 mM $MnCl_2$, 2 mM DTT, 10 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 10 μM poly(Glu,Tyr) 4:1, 0.5 mM Na_3VO_4, and 1 μM ATP. Following incubation, 50 μL Kinase-Glo Plus reagent (Promega) was added and the mixture was incubated at 25 °C for 20 min. A 70 μL aliquot of each reaction mixture was transferred to a black microtiter plate, and the luminescence was measured on Wallac Vector 1420 multilabel counter (PerkinElmer).

HCC827 Antiproliferation Assay. HCC827 cell viability was examined by the MTS assay (Promega, Madison, WI). One thousand HCC827 cells in 100 μL of RPMI1640 with 10% FBS medium were seeded in each well of a 96-well plate. After 96 h incubation with the test compound, the cells were further incubated with 20 μL of a MTS/PMS mixture (MTS/PMS ratio: 20:1) in each well of the 96-well plate for 2 h at 37 °C in a humidified incubator with 5% CO_2 to allow viable cells to convert the tetrazolium salt (MTS) into formazan. The amount/concentration of formazan, which indicates the number of live cells, was determined by measuring its absorbance at 490 nm using a PerkinElmer Victor2 plate reader (PerkinElmer, Shelton, CT).

Cell Extract Preparation and Western Blot Analyses. HCC827 cells were seeded into 12-well plates at a concentration of 2.5×10^5 cells per well. After 16 h of growth in serum-containing media, cells were incubated in serum-free media for 24 h. Compounds was then added for 1 h, at which time cells were stimulated for 5 min with 100 ng/mL EGF. Whole-cell lysates were prepared by suspending 2.5×10^5 cells in 100 μL lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v), sodium deoxycholate, 0.1% (w/v) SDS, 500 μM PMSF, 1 mM DTT, and 1 mM Na_3VO_4). The cell lysates were kept on ice and vigorously vortexed four times at 5 min intervals. The lysate was cleared by centrifugation at 15000*g* for 10 min at 4 °C. After adding SDS sample buffer, the supernatants were heated at 95 °C for 5 min and cell extract samples (25 μg) were resolved by 8% SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Sartorius Stedim Biotech). After transfer, the membrane was first incubated in TBST buffer (20 mM Tris, pH 7.6, 135 mM NaCl, and 0.1% (v/v) Tween 20) with 5% (w/v) skim milk powder, then incubated with the indicated primary antibody (dilution of antibodies was performed according to the manufacturer's instructions), washed, and blotted with horseradish-peroxidase-conjugated secondary antibody. Primary antibodies included the following: p-EGFR-(Y1068), EGFR (Cell Signaling Technology, Danvers, MA), and α-tubulin (Sigma, Natick, MA). The membrane was then developed using Western Lightning *plus*-ECL reagent (PerkinElmer, Shelton, CT) and exposed to X-ray film.

In Vivo Pharmacokinetics Evaluation of 19 in Rats. This study was approved by Institutional Animal Care and Use Committee of National Health Research Institutes. A solution of test compound (10 mg/mL) was prepared by dissolving appropriate amount compound in a mixture of PEG 400/dehydrated ethanol/Solutol (20:30:50, v/v/v) and was diluted with two parts of physiological saline to make the dosing solution of 3.3 mg/mL before dosing. Male Sprague−Dawley rats, weighing 250−350 g each (8−10 weeks old), were obtained from BioLASCO, Ilan, Taiwan. Compound **19** was administered to three male rats each intravenously (5 mg/kg dose) by a bolus injection to the jugular vein. The volume of dosing solution administered was adjusted according to the body weight recorded before dose administration. At 0 (prior to dosing), 2, 5, 15, and 30 min and at 1, 2, 4, 6, 8, and 24 h after dosing, a blood sample (~150 μL) was collected from each animal via the jugular-vein cannula and stored in ice (0−4 °C). Plasma was separated from the blood by centrifugation (14000*g* for 15 min at 4 °C in a Beckman model AllegraTM 6R centrifuge) and stored in a freezer (−60 °C). All samples were analyzed for the test compound by LC-MS/MS (ABI3000). Data were acquired via multiple reactions monitoring. Plasma concentration data were analyzed with standard noncompartmental method with WinNonLin software program (version 1.1, Pharsight Corporation, CA).

In Vivo HCC827 Xenograft Evaluation of 19 in Mice. Adult male nude mice (Nu-Fox1nu) were purchased from BioLasco, Taiwan Co., Ltd. Animals had access to food and water ad libitum. Experimental procedures using animals were approved by the Institutional Animal Care and Use Committees of The National Health Research Institutes. Human HCC827 cells were cultured in RPMI-1640 supplemented with 10% heat-inactived bovine serum (FBS) and incubated at 37 °C in humidified atmosphere consisting 5% CO_2. Eight-week-old nude mice were inoculated with HCC827 cells subcutaneously at 5×10^6 cells per mouse mixed with equal volume of Matrigel (Becton Bickinson) in 0.1 mL via a 24-gauge needle. Tumor volume was measured by using an electronic caliper and calculated with the formula length $\times$ width^2 $\times$ 0.5, twice a week. When the size of a growing tumor $\sim$200 mm^3, the HCC827 tumor bearing mice were administered compound **19** (dissolved in 10% DMSO + 20% Cremophor EL +70% normal saline) IV via the tail veins for 5 days per week for 2 consecutive weeks at 15 and 5 mg/kg dose (n = 7). Gefitinib was administered (dissolved in 1% Tween 80 in water) as reference compound periorally at 100 mg/kg dose (n = 8). Also a control group received vehicle alone (10% DMSO + 20% Cremophor EL +70% normal saline) as IV injection (n = 7). Tumor size and animal body weight were measured twice a week after drug treatments. At the end of the experiments, animals were euthanized with carbon dioxide, followed by cervical dislocation.

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