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Lead Optimization of N-3-Substituted 7-Morpholinotriazolopyrimidines as Dual Phosphoinositide 3-Kinase/Mammalian Target of Rapamycin Inhibitors: Discovery of PKI-402

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Herein we describe the identification and lead optimization of triazolopyrimidines as a novel class of potent dual PI3K/mTOR inhibitors, resulting in the discovery of **3** (PKI-402). Compound **3** exhibits good physical properties and PK parameters, low nanomolar potency against PI3Kα and mTOR, and excellent inhibition of cell proliferation in several human cancer cell lines. Furthermore, in vitro and in vivo biomarker studies demonstrated the ability of **3** to shut down the PI3K/Akt pathway and induce apoptosis in cancer cells. In addition, **3** showed excellent in vivo efficacy in various human cancer xenografts, validating suppression of PI3K/mTOR signaling as a potential anticancer therapy.

Introduction

The phosphoinositide 3-kinases (PI3K[a]) are a group of enzymes that phosphorylate the 3′-OH position of the inositol ring of phosphatidylinositol bisphosphate (4,5) (PIP2) to phosphatidylinositol triphosphate (3,4,5) (PIP3). Class I PI3Ks are divided into two groups, class IA (p110α, P110β, and p110δ isoforms) and class IB (p110γ). These isoforms are structurally similar but differ in subunit association and mode of activation (e.g., receptor tyrosine kinase vs G-protein-coupled receptor). Aberrant PI3K signaling caused by phosphoinositide 3-kinase α encoded gene (PIKCA) amplification or mutation, PIK3R1 mutation, phosphatase and tensin homologue (PTEN) loss, or receptor tyrosine kinase (RTK) dependent activation occurs in about 50% of all known solid tumors. Deregulated PI3K activity leads to elevated levels of PIP3, which acts as a second messenger responsible for the activation of the downstream kinase protein kinase B (Akt).[1] PIP3 is bound by the PH (pleckstrin homology) domains of both PDK1 and Akt serine/threonine (S/T) kinases resulting in their proximity at the inner cell membrane where 3-phosphoinositide-dependent kinase 1 (PDK1) phosphorylates and activates Akt (at T308).[2] Aberrantly activated Akt promotes growth, survival, proliferation, enhanced migration, and adhesion in cancer cells. Many Akt effects are mediated through the S/T kinase mammalian target of rapamycin (mTOR), which exists as either the mTOR complex-1 (TORC1) or mTOR complex-2 (TORC2) enzyme complexes. TORC1 regulates such processes as protein biosynthesis, while TORC2 fully activates Akt kinase by phosphorylating it at serine 473 (S473).[1,2] A dual PI3K/mTOR inhibitor could both prevent cancer cell proliferation and induce programmed cell death (apoptosis) by fully suppressing Akt activation.[1,4,7] Therefore, our goal was to identify potent small molecule dual PI3K/mTOR inhibitors, which we hypothesized would exert antitumor activity in a broad array of preclinical models. This hypothesis was validated by the Novartis dual PI3K/mTOR inhibitor **1** (NVP-BEZ-235, Figure 1).[3] Compound **1** is the most advanced PI3K/mTOR inhibitor reported and is currently undergoing phase II clinical trials.[4] In this publication, we describe the discovery of triazolopyrimidines and their lead optimization, culminating in the novel dual PI3K/mTOR inhibitor **3** (PKI-402).[5] Compound **3** displayed potent inhibition in all class I PI3Ks (e.g., IC_{50} vs PI3Kα = 1 nM) and mTOR. Compound **3** inhibited growth of a diverse set of human tumor lines in vitro, and it proved to be efficacious in three human tumor xenograft models.

The versatility of fused 4-morpholino-2-arylpyrimidines as PI3K/mTOR inhibitors has been demonstrated impressively by the discovery of **2** (GDC-0941), a PI3Kα isoform selective inhibitor built on a thieno[3,2,*d*]pyrimidine core,[6] and by the introduction of pyridofuranopyrimidines,[7] imidazolopyrimidines,[8] pyrrolopyrimidines[9] as dual PI3K/mTOR inhibitors or pyrazolopyrimidines[10] that function as highly selective mTOR inhibitors. However, despite the fact that highly selective PI3Kα[6] and mTOR[10] inhibitors have been introduced recently, only one preclinically validated dual PI3K/mTOR inhibitor, utilizing a fused pyrimidine scaffold bearing a morpholine, has been disclosed to date.[11] Herein, we report the discovery of the novel triazolopyrimidine scaffold and our lead optimization in this series, resulting in the discovery of **3**, a highly in vivo efficacious dual PI3K/mTOR inhibitor.

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[a] Abbreviations: PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; Akt, protein kinase B; PIP2, phosphatidylinositol bisphosphate (4,5); PIP3, phosphatidylinositol triphosphate (3,4,5); PTEN, phosphatase and tensin homologue; RTK, receptor tyrosine kinase; PH, pleckstrin homology; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; PDK1, 3-phosphoinositide-dependent kinase 1; S, serine; T, threonine; ATP, adenosine 5′-triphosphate; Her2+, human epidermal growth factor receptor 2+; ELISA, enzyme-linked immunosorbent assay; DELFIA, dissociation-enhanced lanthanide fluorescent.

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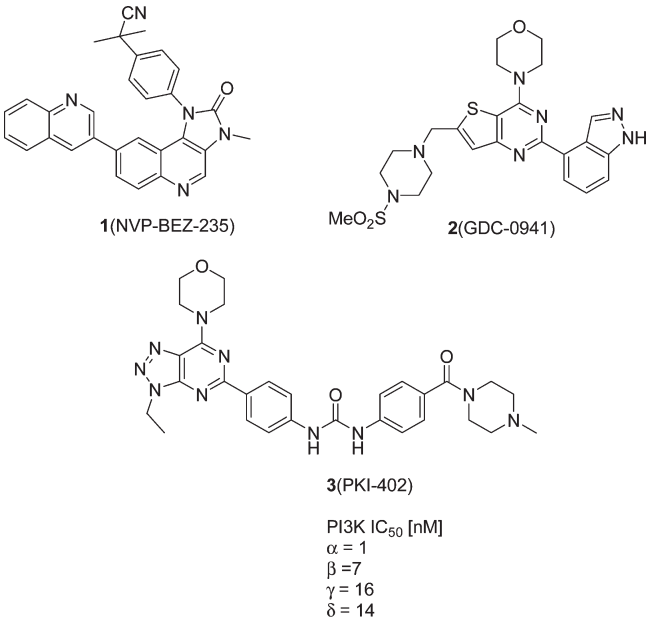


Figure 1. Structures of known PI3K inhibitors (**1** and **2**) and of novel compound **3**.

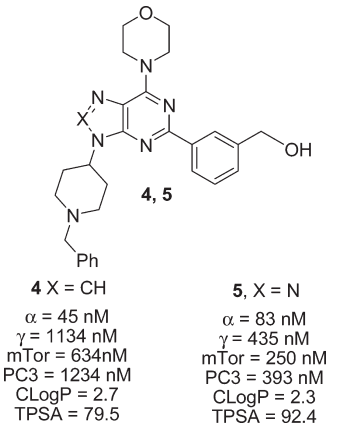


Figure 2. Structure and biological profile of initial discovery lead **4** and the triazolopyrimidine lead **5**.

Chemistry

The synthesis of triazolopyrimidine **5** (Figure 2) is shown in Scheme 1. Nitrobarbituric acid **6**[12] was chlorinated using $POCl_3$ and DMA to give 5-nitrotrichloropyrimidine **7** in 13% yield.[13] Pyrimidine **7** was reacted with morpholine at 0 °C to give the 4-morpholino-5-nitro-2,6-dichloropyrimidine **8**, which was aminated with 4-amino-1-benzylpiperidine in THF in the presence of NEt_3 to give **9**. Reduction[14] of **9** with Raney Ni and N_2H_4 gave the bis-amino analogue **10**, which was cyclized[14] using $NaNO_2$ in AcOH at 0 °C to yield triazolopyrimidine **11**.

For the introduction of C-2 aryl groups, Suzuki reaction of **11** with 3-hydroxymethylphenyl boronate was used to give **5** in good yield. This synthetic sequence can be applied to the synthesis of any 3-substituted 5-aryltriazolopyrimidine by varying the primary amine or arylboronic acid or ester.

A similar route was used to synthesize *N*-3-alkyltriazolopyrimidines **14**−**32** (Scheme 2). Compound **8** was reacted with the appropriate primary amines (in this case $MeNH_2$, $EtNH_2$, *i*-$PrNH_2$, and *c*-$PrNH_2$) and subsequently converted to **12a**−**d** as outlined in Scheme 1 for compound **11**. Then **12a**−**d** were converted to anilines **13a**−**d** by reaction with 4-aminophenylboronic acid pinacol ester under Suzuki conditions. Intermediates **13a**−**d** were condensed with the appropriate isocyanates to yield urea derivatives **14**−**32**. Alternatively, compounds **13a**−**d** were reacted with triphosgene and the appropriate aromatic and heteroaromatic amines to form ureido analogues **14**−**30**.

The synthesis of substituted amides is shown in Scheme 3. Compound **29** was saponified using NaOH in THF to obtain acid **31**, which was coupled with the appropriately substituted amines via HOBT and EDCI (or alternatively HBTU) activation to yield amides **3** and **32**−**38**.

Results and Discussion

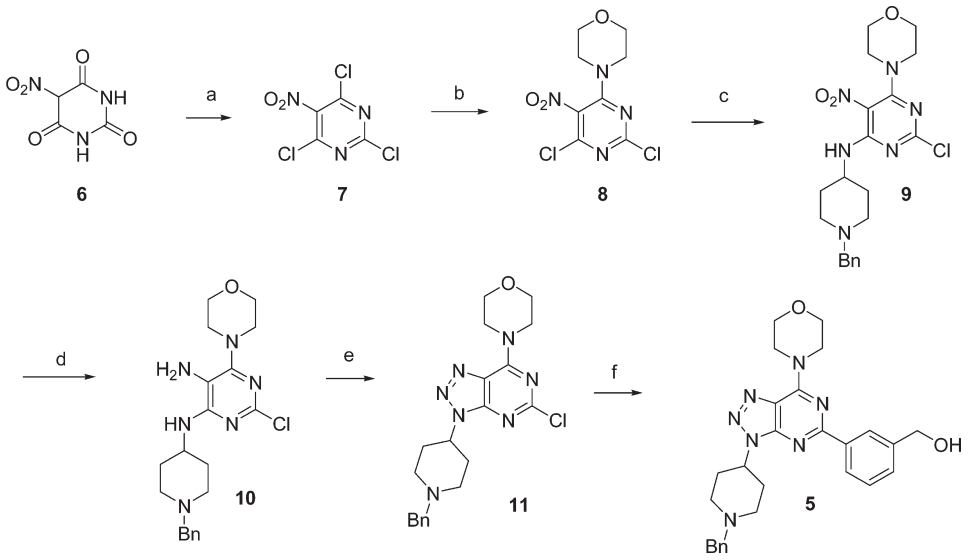
All final compounds **3** and **14**−**38** were tested in vitro against PI3Kα, PI3Kγ, and mTOR.

Our primary assay for inhibition of PI3Kα and PI3Kγ enzymes was a fluorescence polarization format assay.[15] Our primary optimization was directed at improving potency against PI3Kα. However, we are showing potency against PI3Kγ to illustrate the selectivity of our compounds over class IB isoforms, while the possible role of PI3Kγ in cancer remains unclear.[1] Our molecular modeling is based on cocrystals of inhibitors with PI3Kγ. Hence, predictions using molecular modeling only make sense when the series has good potency against PI3Kα and PI3Kγ. For mTOR enzyme potency, we used a dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) platform enzyme-linked immunosorbent assay (ELISA).[10] Cell growth inhibition was evaluated in MDA-361 [breast, human epidermal growth factor receptor (Her)2+/PI3KCA mutant] and PC3 (prostate, PTEN mutant) human tumor cell lines.

Because of the high degree of homology in the adenosine 5′-triphosphate (ATP) binding pocket and the similarity of their cores, all 4-morpholino-2 arylpyrimidines are expected to bind in the ATP binding pocket of mTOR, p110α, and p110γ in a similar binding mode, with the morpholine oxygen binding to the hinge region Val851 and the substituents at the aryl group in the 2-position interacting with Asp810 and Lys883. X-ray crystallography studies with PI3Kγ, molecular modeling and unpublished SAR results indicated that, in fact, those three interactions are crucial for potency against PI3Kα and mTOR. Therefore, we felt that modifying fused pyrimidines would give us the best chance to successfully develop a dual PI3K/mTOR inhibitor.

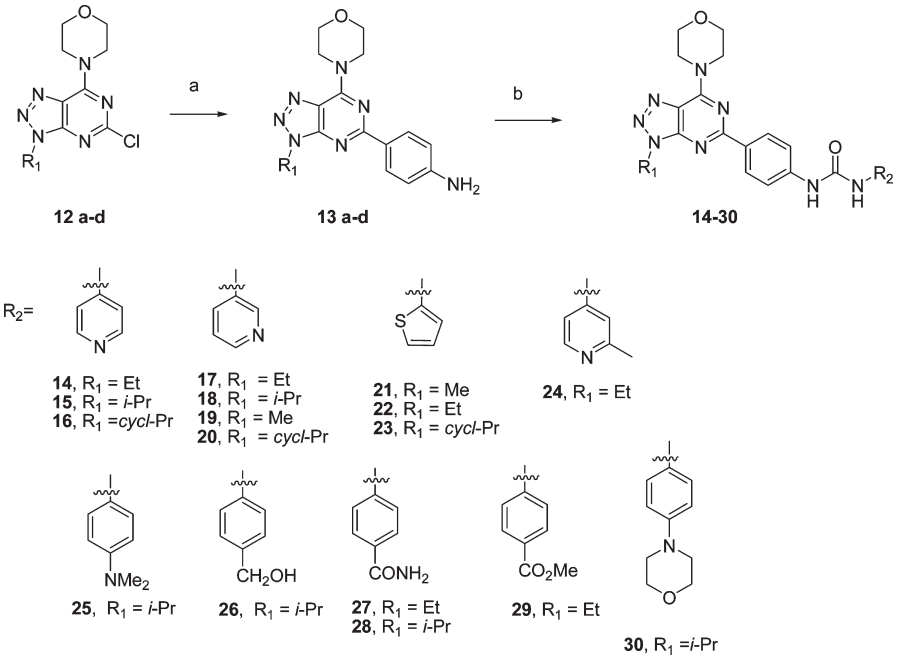
Imidazolopyrimidine **4** was identified as a potent and selective PI3Kα inhibitor. Replacing the "CH" of the imdazole ring **4** with a "N" led to triazolopyrimidine **5**, which exhibited a lower cLogP value and slightly higher topological polar surface area (TPSA) than **4**. In addition, **5** showed similar potency against PI3Kα but better potency against PC3 and MDA-361 cells relative to compound **4**. However, compound **5** showed limited solubility and poor microsomal stability caused by the OH group as a metabolic hot spot. A plethora of OH bioisosteres have already been published for fused pyrimidines.[6,10,16] Recently, it has been shown by Zask et al.[10] that incorporation of a urea appendage in the pyrazolopyrimidine core not only improved the potency against PI3Kα and mTOR but also increased metabolic stability. Hence, we explored this ureido appendage with triazolopyrimidines carrying small alkyl substitutents at the N-3 position to obtain potent PI3K/mTOR inhibitors with low molecular weight and good microsomal stability.

Scheme 1. Synthesis of Triazolopyrimidine **5**[a]



[a] Reagents and conditions: (a) $POCl_3$, $Me_2NC_6H_5$, $T < 30$ °C, 13%; (b) NEt_3, morpholine, 0 °C, 92%; (c) 4-amino-1-benzylpiperidine, NEt_3, CH_2Cl_2, 78%; (d) Raney Ni, MeOH, N_2H_4, 63%; (e) $NaNO_2$, AcOH, 0 °C, 54%; (f) $(Ph_3P)_4Pd$, 3-hydroxymethylphenylboronic acid, Na_2CO_3, DME, 140 °C, 1 h microwave, 45%.

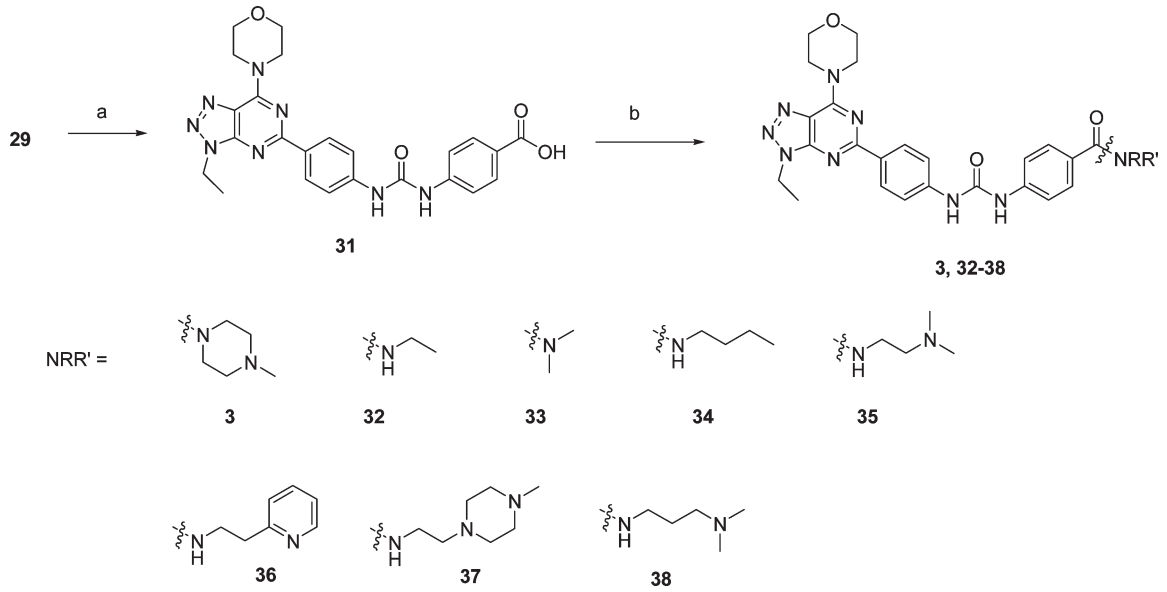
Scheme 2. Synthesis of *N*-3-Alkyl-2-arylureidotriazolopyrimidines **14**−**30**[a]



[a] Reagents and conditions: (a) 4-aminophenylboronic acid pinacol ester, $(Ph_3P)_4Pd$, Na_2CO_3, DME, 140 °C, 1 h, microwave, 57%; (b) triphosgene, R_2NH_2, Et_3N, THF or R_2NCO, THF.

The biological data for analogues **14**−**24** are compiled in Table 1. All heteroaryl derivatives (**14**−**24**) with small groups at N-3 showed potent inhibition of PI3Kα enzyme activity and potent inhibition of MDA-361/PC3-cell growth. The 4-pyridyl substituted urea analogues **14**−**16** exhibited good activity against PI3Kα, mTOR, PC3, and MDA-361. In terms of potent inhibition of PI3Kα enzyme activity and potent inhibition of MDA-361/PC3 cell growth, compounds are ranked in the order Et = *c*-Pr > *i*-Pr. However, the ethyl analogue **14** showed the best solubility compared to **15** and **16**. Compounds **14**−**16** also showed acceptable microsomal stability. Almost equally good potency was observed for the 3-pyridyl analogues **17**−**20**. The *i*-Pr analogue **18** exhibited the best cell potency in this subset, while *c*-Pr (**20**) and Et (**17**) were equipotent in enzyme and cells, and the Me substituted analogue **19** exhibited decreased potency against PI3Kα and cells. Interestingly, when an additional methyl group was introduced to the 3-pyridine core in **24**, we observed decreased cell potency compared to **17**. The 2-thiophene analogues **21**−**23** were highly potent against PI3Kα, mTOR, and cells. Here, ethyl analogue **22** showed the best potency against PI3Kα and against cells, establishing the order Et > *c*-Pr > Me. However, the thiophene analogues **21**−**23** showed only modest microsomal stability and poor solubility at

Scheme 3. Synthesis of 4-Benzamidoureido Analogues **3** and **32**−**38**[a]



[a] Reagents and conditions: (a) NaOH, THF, H_2O, 85%; (b) HOBT, EDCI (or HBTU), Et_3N, RR′NH, THF.

Table 1. Biological Data and Physical Properties for Heteroarylureidotriazolopyrimidines **14**−**24**

	IC_{50} [nM][a]						
compd	PI3Kα	PI3Kγ	mTOR	MDA-361	PC3	sol.[b] at pH 7.4	$t_{1/2}$(rat) [min][c]
14	5.0	18.5	0.63	35	49	18	27
15	5.0	21.5	0.51	54	67	0	> 30
16	6.0	13.5	0.84	31	70	0	21
17	9.5	25.0	0.89	74	96	0	19
18	10.5	48.5	0.57	39	91	1	24
19	10.5	49.0	2.95	198	281	1	12
20	10.5	48.5	0.57	67	90	1	24
21	6.5	22.0	3.70	184	366	2	9
22	2.5	11.5	3.00	81	133	1	10
23	3.0	19.5	3.05	73	149	0	15
24	8.0	37.0	21.00	196	252	3	11

[a] The values are an average of at least two separate determinations with a typical variation of less than ±30%. [b] In μg/mL. [c] Half-life of drug when incubating with rat liver microsomes.

Table 2. Biological Data and Physical Properties for 4-Arylureidotriazolopyrimidines **25**−**30**

	IC_{50} [nM][a]						
compd	PI3Kα	PI3Kγ	mTOR	MDA-361	PC3	sol.[b] at pH 7.4	$t_{1/2}$(rat) [min][c]
25	23.5	69.0	1.60	84	171	0	7
26	3.5	24.8	0.32	< 30	55	0	21
27	2.4	12.2	0.39	28	65	6	21
28	3.6	17.5	0.31	38	57	0	9
29	23.3	84.5	2.55	199	285	0	15
30	25.0	78.0	0.35	47	110	1	25

[a] The values are an average of at least two separate determinations with a typical variation of less than ±30%. [b] In μg/mL. [c] Half-life of drug when incubating with rat liver microsomes.

physiological pH. Other heteroaryl analogues such as 3-pyrrole, isoxazole, or 2-thiazole were screened as well, but they showed only moderate potency against PI3Kα and in cells and are not shown here. A number of compounds where R_2 = Ph demonstrated excellent potency against PI3Kα, mTOR, and PC3/MDA-361 cells. Especially, compound **26** appeared to be a potent inhibitor (Table 2). However, **26** showed poor solubility and only moderate microsomal stability. Amides **27** and **28** showed similar good cell and enzyme potency relative to **26**. In vitro biomarker studies revealed excellent suppression of Akt T308 phosphorylation (IC_{50} = 8 nM) in MDA-361 cells after 4 h of incubation with drug, which confirms correlation of the antiproliferative effect of **27** via PI3K/Akt pathway inhibition. For this study, MDA-361 cells were incubated with **27** for 4 h. In lieu of directly monitoring cellular PIP3 levels, compound inhibition of PI3K in MDA-361cells was demonstrated by potent suppression of Akt phosphorylation at T308. Suppression of pAkt-T308 linked the antiproliferative effect of **27** with PI3K/Akt pathway inhibition. Suppression of pAkt (T308) by **27** was

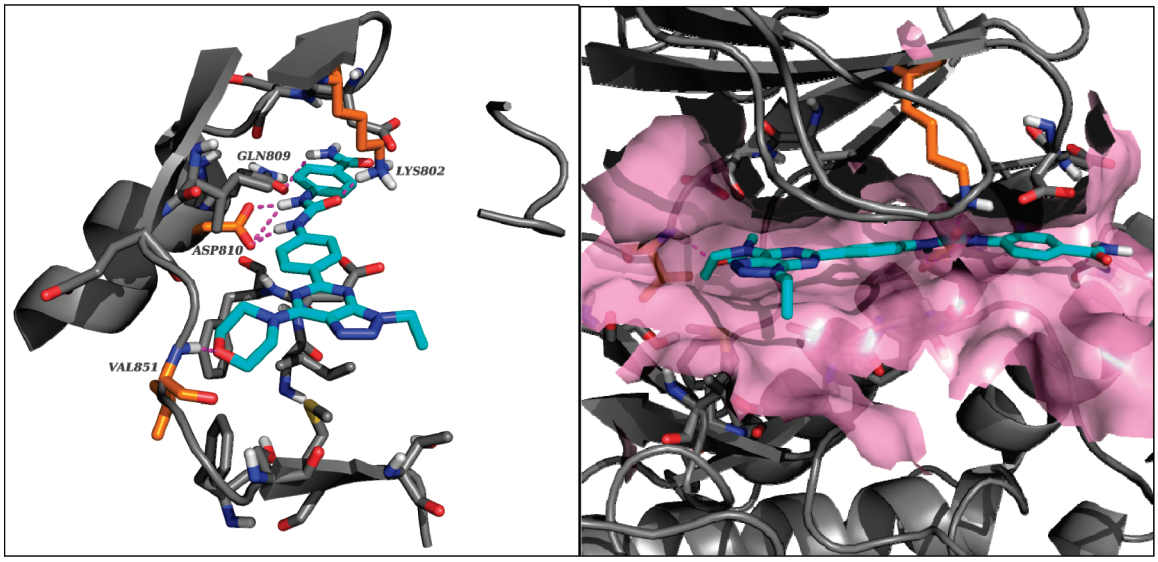


Figure 3. Modeling studies of analogue **27** docked in a homology model of PI3Kα, based on PI3Kγ crystal structures.[10]

Table 3. Biological Data and Physical Properties for 4-Benzamido Analogues **3** and **32**−**38**

	IC_{50} [nM][a]						sol.[b] at		$t_{1/2}$ (min)[c]		
compd	PI3Kα	PI3Kγ	mTOR	MDA-361	PC3	Akt T308[d]	pH 3.0	pH 7.4	human	nude mouse	rat
3	1.4	9.2	1.70	8	21	5	100	0	> 30	> 30	15
32	3.0	17.2	0.56	29	33	27	ND	1	> 30	29	16
33	1.4	12.3	0.75	16	17	8	ND	1	> 30	ND	14
34	6.0	33.0	2.70	68	96	47	ND	0	> 30	27	21
35	1.0	12.0	0.50	13	52	16	> 100	> 100	> 30	28	29
36	6.8	22.0	1.21	36	88	65	4.0	1	16	19	23
37	3.2	25.0	1.10	24	67	74	100	1	ND	28	17
38	2.4	9.0	2.20	41	116	44	100	100	30	26	26

[a] The values are an average of at least two separate determinations with a typical variation of less than ±30%. [b] In μg/mL. [c] Half-life of drug when incubating with liver microsomes of the species shown. [d] Phosphoblot IC_{50} values were determined by densitometric scans of Western blots.

measured by Western blot, using a phosphorylated Akt specific antibody (Cell Signaling Technology). Phosphoblot IC_{50} values were determined by densitometric scans of Western blots.

Attaching other solubilizing groups such as Me_2N (**25**) or morpholine (**30**) in the 4-position led to only moderately potent PI3Kα inhibitors with high potency against mTOR.

Among the various 4-substituted phenyl analogues, **27** and **28** looked the most interesting because of their high potency in enzyme and cell assays. Molecular modeling of compound **27** (Figure 3) shows the crucial hydrogen bonding interactions of the morpholine oxygen to the hinge region Val851 and of the urea to Asp810 and Lys802. Since the amide moiety is pointing away from the binding pocket toward the solvent, this is a good position to improve solubility and/or metabolic stability, with minimal impact on enzyme and cell potency. Table 3 shows the biological data for amido analogues **3** and **32**−**38** synthesized to probe this hypothesis.

As can be seen from the onset, introduction of an amido moiety at the 4-position of the phenyl in the urea appendage improved the enzyme and cell potencies and the microsomal stability. A comparison of the *N*-alkyamides **32** and **34** shows that introduction of alkyl substituents can increase cell potency of the amido analogues. However, this effect depends on the size of the alkyl group, so the *n*-Bu derivate (**34**) showed about 2-fold lower cell and enzyme potency relative to **32**. The tertiary amide **33** demonstrated increased cell potency over **32** but exhibited a similar stability profile. Excellent potency in PI3Kα, mTOR, and cells was observed for *N*,*N*-dimethylaminoethyleneamide **35**. This compound showed good aqueous solubility at pH 3 and pH 7 and very good stability in rat, nude mouse, and especially human microsomes. The piperazineamide **3** exhibited potency comparable to that of **35** against PI3Kα and mTOR. However, cell potency was further enhanced by 2-fold for **3**, with an IC_{50} value well below 10 nM against MDA-361 cells and an IC_{50} value of 21 nM against PC3 cells. Compound **3** also exhibited good pharmaceutical properties, such as good stability in nude mouse and human microsomes and aqueous solubility at pH 3. Replacing the *N*,*N*-dimethylaminoethyleneamide (**35**) with *N*,*N*-dimethylaminopropyleneamide (**38**) led to decreased (2- to 3-fold) potency in cell proliferation assays. Also, substituting the *N*,*N*-dimethylamino group with a 2-pyridyl group (**36**) or a 4-methylpiperazinyl group (**37**) led to lower potency against cells (MDA-361, PC3). In addition, **36** exhibited weaker potency against PI3Kα and mTOR than **35**, as well as low aqueous solubility and only modest microsomal stability in all species.

More importantly for amido analogues **3** and **32**−**38** the cell potency translated to the suppression of pAkt-T308, the most relevant PI3Kα specific biomarker. Among compounds **3** and **32**−**38**, the best in vitro biomarker suppression was found for **35** and **3**. Therefore, **35** and **3** were taken forward for in vivo biomarker studies in MDA-361 xenograft bearing mice. Figures 4 and 5 show both compounds **35** and **3** given at 50 mpk, iv, suppressed pAkt-T308, pAkt-S473, P70S6K, and

pS6 (pAkt-S473, P70S6K, and pS6 are mTOR specific biomarkers). Compound **3** administered at 50 and 100 mpk, iv, not only suppressed all the PI3K/mTOR biomarkers for at least 8 h (Figure 5) but also induced cleaved poly ADP-ribose polymerase (PARP), a marker for cells undergoing apoptosis.[17]

Pharmacokinetic analysis showed that the plasma concentration of **35** was 1229 ng/mL and tumor concentration was 2130 ng/mL at 4 h after a single 50 mg/kg iv dose. For compound **3**, plasma level was 525 ng/mL and tumor level was 2219 ng/mL at 8 h after a single 50 mg/kg iv dose. For both compounds **3** and **35** an excellent correlation between drug concentration in the plasma and tumor and biochemical response (biomarker pattern) was observed. However, **3** showed a better PK profile and longer lasting biomarker suppression in vivo than **35**, so **3** was taken forward for in vivo efficacy studies.

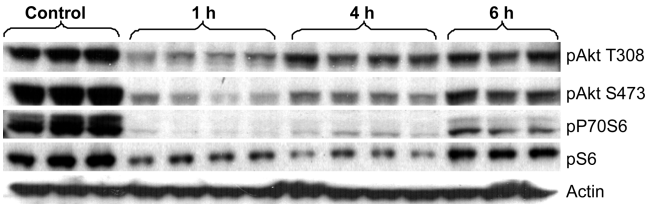


Figure 4. In vivo biomarker analysis of **35** given at 50 mpk (iv) to MDA-361 tumor bearing nude mice.

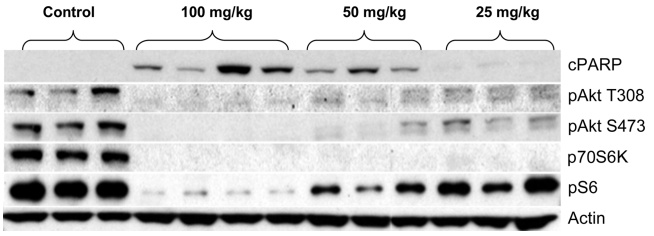


Figure 5. In vivo biomarker analysis at 8 h after **3** was administered at 25, 50, and 100 mpk (iv) to MDA-361 tumor bearing nude mice.

Evaluation of efficacy was performed in nude mice bearing MDA-361 human breast cancer tumors (Figure 6). When 100 mpk of **3** was given once daily for 5 days (one round), tumor regression was observed with no tumor regrowth until day 70 (not shown). At lower doses (25, 50 mg/kg), initial tumor regression was also observed, but tumor regrowth occurred between day 16 and day 20. Compound **3** also showed in vivo efficacy against other human tumor xenograft models, including U87 (glioma) and A597 (non-small-cell lung). In these studies, compound **3** was well tolerated, with no adverse side effects such as weight loss.

Additional analysis of compound **3** showed that it was Ames negative and selective for class I PI3Ks and mTOR. Selectivity of **3** was assessed against a panel of 236 human kinases (Invitrogene), where no off-target activity (>50% inhibition) was detected at 10 μM. Compound **3** is one of several compounds undergoing preclinical evaluation, and we will report further efficacy data in due course.

Conclusions

We have introduced a novel class of PI3K/mTOR inhibitors, namely, the triazolopyrimidines, which showed better potency than our related imidazolopyrimidines. Studies of various N-3-substitutions established the ethyl group as the best choice. Various aryl- and heteroarylureido appendages were screened resulting in the discovery of 4-benzamido analogues. This set of compounds showed especially high potency against PI3Kα and mTOR enzyme activity and excellent potency in tumor cell proliferation assays. By use

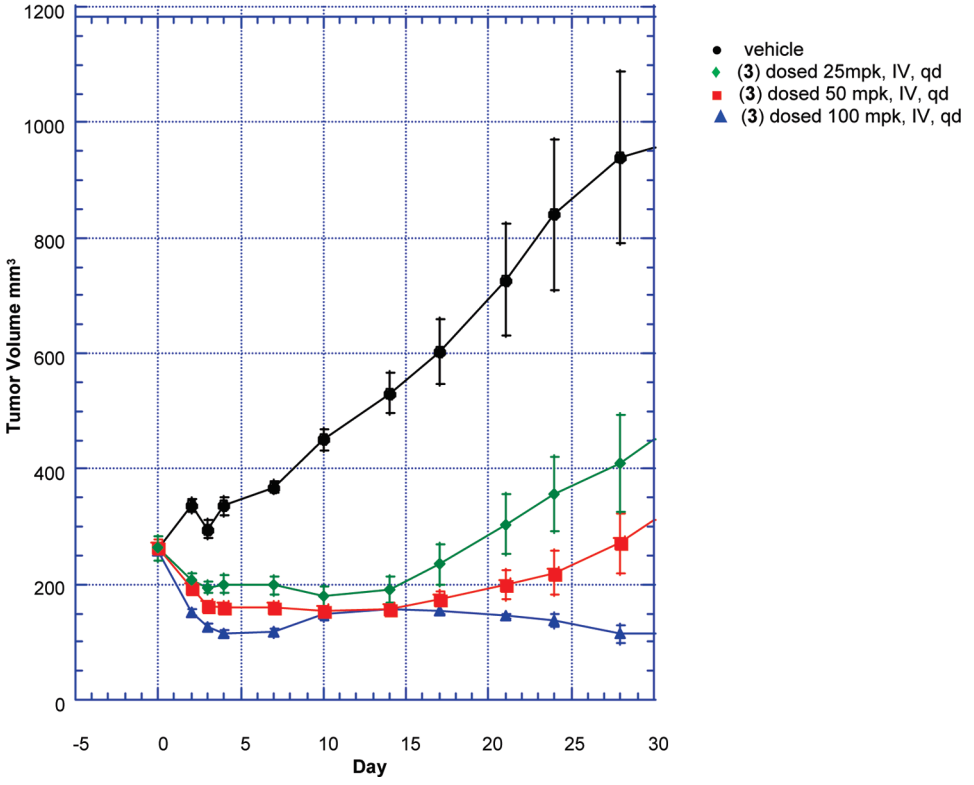


Figure 6. Antitumor efficacy of **3** in the MDA-361 (breast, Her2+/PIK3CA mutant) xenograft model.

of molecular modeling, water solubilizing amino substituents were designed and introduced, leading to the discovery of compounds **3** and **35**. These compounds had single digit nanomolar IC_{50} values in the PI3Kα and mTOR enzyme assays and in the tumor cell growth inhibition assays. For both compounds **3** and **35** cell growth inhibition translated well into both in vitro and in vivo biomarker suppression of pAkt-T308, pAkt-S473, P70S6K, and pS6. Additionally, both compounds **3** and **35** induced cleaved PARP (a marker for cells undergoing apoptosis) in vitro. Compound **3** demonstrated superior in vivo biomarker suppression profile (Figure 5) in terms of both duration (8 h) and induction of cleaved PARP. These data suggest that complete and sustained pAkt suppression induced apoptosis in MDA-361 tumor cells in vivo. Subsequently, an in vivo efficacy study with MDA-361 xenograft tumors showed that compound **3** administered at 100 mg/kg, iv, dx5 (one round) caused both initial tumor regression and no tumor regrowth for 70 days. We are currently evaluating the antitumor efficacy of compound **3** in other models.

Experimental Section

General Methods. Melting points were determined in open capillary tubes on a Mel-temp melting point apparatus and are uncorrected. ^{1}H NMR spectra were determined with a Bruker DPX-400 spectrometer at 400 MHz. Chemical shifts are reported in parts per million (δ) relative to residual chloroform (7.26 ppm), TMS (0 ppm), or dimethyl sulfoxide (2.49 ppm) as an internal reference with coupling constants (J) reported in hertz (Hz). The peak shapes are denoted as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Electrospray (ES) mass spectra were recorded in positive or negative mode on a Micromass Platform spectrometer. Electron impact (EI) and high-resolution mass spectra were obtained on a Finnigen MAT-90 spectrometer. Combustion analyses were obtained using Perkin-Elmer series II 2400 CHNS/O analyzer. The purity of compound **3** was determined by combustion analysis. The purity of compounds **18**, **25**, **28**, **30**, **34**, **37** was determined by analytical LC/MS using an Agilent 1100 LC, equipped with an Agilent MSD mass spectrometer, and by analytical LC using X-Bridge BEH column, MeOH/H_2O eluent at 1 mL/min flow (containing 0.05% NH_4OAc), 20 min gradient of 10% MeOH to 90% ACN, monitored by UV absorption at 215 nm. The purity of compounds **3**, **5**, **13a**−**d**, **14**−**17**, **19**−**24**, **26**, **27**, **29**, **31**−**33**, **35**, **36**, and **38** was determined by analytical LC on a Aquasil C18 column, using MeCN/H_2O eluent at 0.8 mL/min flow (containing 0.1% formic acid), 5.5 min gradient of 0% MeCN to 100% MeCN, monitoring UV absorption at 254 nm. The purity of all the above-mentioned compounds **3**, **5**, **13a**−**d**, **14**−**38** was found to be >95%. Reverse phase HPLC purifications were performed on a Gilson preparative HPLC system controlled by Unipoint software using a Phenomenex Gemini 100 mm × 30.0 mm. Thin-layer chromatography (TLC) was performed on Merck PLC prescored plates $_{60}F_{254}$.The terms "concentrated" and "evaporated" refer to removal of solvents using a rotary evaporator at water aspirator pressure with a bath temperature equal to or less than 40 °C. Unless otherwise noted, reagents were obtained from commercial sources and were used without further purification.

Preparation of 5-Nitro-2,4,6-trichloropyrimidine (7). An amount of 19.2 g (111 mmol) of anhydrous 5-nitrobarbituirc acid (**6**) was suspended in 77 mL (833 mmol, 7.5 equiv) of $POCl_3$. To the suspension were added slowly 53 mL (333 mmol, 3 equiv) of *N*,*N*-diethylaniline so that the reaction temperature never exceeded 45 °C. A water bath at 28 °C was used to cool the reaction mixture during the addition. (Caution, reaction is strongly exothermic!!!). When addition was completed, the mixture was heated for another 30 min to 45 °C. The reaction mixture was cooled to room temperature and poured onto 600 g of ice−water. The resulting mixture was stirred for 5 min and extracted with Et_2O (3 × 800 mL). The combined ethereal layers were dried over $MgSO_4$. Filtration for removal of $MgSO_4$ and evaporation of Et_2O gave a red oil, which was purified by column chromatography on silica gel using 100% CH_2Cl_2. The product fractions were unified and the solvent was evaporated to give **7** as a white solid (3.45 g, 13% yield). ^{13}C NMR ($CDCl_3$) δ 154.4, 159.3 ppm. MS (EI) m/z 228.

Preparation 2,6-Dichloro-5-nitro-4-morpholinopyrimidine (8). To a solution of 2,4,6-trichloronitropyrimidine **7** (6.20 g, 27.2 mmol) in CH_2Cl_2 (170 mL) at 0 °C was added a solution of morpholine (2.34 g, 27.2 mmol) and NEt_3 (2.74 g, 27.2 mmol) in CH_2Cl_2 (70 mL) over a period of 1 h. The reaction mixture was stirred for another 1 h at 0 °C and allowed to warm to 20 °C and stirred for 12 h to drive the reaction to completion. For purification, silica gel (20 g) was added to the reaction mixture and the solvent was removed so that product was adsorbed on the silica gel. The resultant residue was placed on a silica gel column, and the crude material was purified by flash chromatography using CH_2Cl_2 eluent. After concentration, the product **8** was obtained as yellow solid (6.90 g, 91% yield). ^{1}H NMR ($CDCl_3$) δ 3.62 (m, 4H), 4.17 (m, 4H) ppm. MS (ESI) m/z 279.

Prepration of 5-Chloro-3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidine (12b). Step 1. To a cooled solution of ethylamine (2 M solution in THF) (3.94 mL, 7.89 mmol) and NEt_3 (796 mg, 7.89 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added a solution of 2,6-dichloro-5-nitro-4-morpholinopyrimidine **8** (2.0 g, 7.2 mmol) dissolved in CH_2Cl_2 (10 mL) over a period of 0.5 h. The reaction mixture was stirred for another 1 h at 0 °C and allowed to warm to 20 °C and stirred for 1−4 h to drive the reaction to completion. The product was purified by flash chromatography on silica gel by eluting it with CH_2Cl_2 followed by CH_2Cl_2/EA (10:1) to give the product as yellow solid (2.1 g, 100% yield). MS (ESI) m/z 288.

Step 2. In a three-neck flask was suspended under nitrogen atmosphere 2-chloro-N^4-ethyl-6-morpholin-4-ylpyrimidine-4,5-diamine (6 g, 21.87 mmol) and Raney Ni (15 g) in methanol (600 mL). To the stirring reaction mixture was added slowly $N_2H_4 \cdot H_2O$ (3 mL, 9 mmol, 9 equiv), and the stirring was continued for 1 h to drive the reduction to completion. The reaction mixture was filtered over Celite and the filtrate was evaporated to obtain the product (N^4-ethyl-2-chloro-6-morpholin-4-ylpyrimidine-4,5-diamine) as light-brown solid (4.1 g, 73% yield). MS (ESI) m/z 258.

Step 3. To a stirred solution of N^4-ethyl-2-chloro-6-morpholin-4-ylpyrimidine-4,5-diamine (4.1 g, 15.89 mmol) in acetic acid/water (1:1) (82 mL) at 0 °C was added aqueous (0.5 N) $NaNO_2$ solution (41 mL, 20.5 mmol), and the reaction mixture was allowed to stir for 2 h. The off-white solid was collected by filtration and dried in vacum to give the 5-chloro-3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine **12b** (3.26 g, 76% yield). ^{1}H NMR (DMSO) δ 1.47 (t, J = 7.3 Hz, 3H), 3.74 (m, 2H), 3.81 (m, 2H), 3.94 (m, 2H), 4.53 (q, 7.3 Hz, 2H), 4.56 (m, 2H) ppm. MS (ESI) m/z 269. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 98.9% purity.

Preparation of 5-Chloro-3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine (12a). 5-Chloro-3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine (**12a**) was synthesized starting from 2,6-dichloro-5-nitro-4-morpholinopyrimidine **8** (2.75 g, 10 mmol) and methylamine in THF solution (2.5 mL, 10 mmol) as outlined for compound **12b**. 5-Chloro-3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine **12a** was obtained as yellow solid (1.3 g, 51% yield). Mp 168 °C. ^{1}H NMR ($CDCl_3$) δ 3.87 (m, 4H), 4.07 (m, 2H), 4.17 (s, 3H), 4.68 (m, 2H) ppm. MS (APCI) m/z 255.2.

Preparation of 5-Chloro-3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine (12c). 5-Chloro-3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine (**12c**) was synthesized starting

from 2,6-dichloro-5-nitro-4-morpholinopyrimidine (2 g, 7.19 mmol) and isopropylamine (424 mg, 7.19 mmol) as outlined for compound **12b** to obtain 5-chloro-3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine **12c** as an off-white solid. The product was found to be pure enough for further transformations (1.5 g, 74% yield). ^{1}H NMR (DMSO) δ 1.58 (d, J = 6.5 Hz, 6H), 3.75 (m, 2H), 3.81 (m, 2H), 3.93 (m, 2H), 4.56 (m, 2H), 5.03 (hep, J = 6.5 Hz, 1H) ppm. MS (ESI) m/z 283.2. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 99.5% purity.

Preparation of 5-Chloro-3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidine (12d). Starting from 2,6-dichloro-5-nitro-4-morpholinopyrimidine **8** (1.38 g, 5 mmol) and cyclopropylamine (54 mg, 6 mmol) and following the procedures as outlined for compound **12b**, 5-chloro-3-cyclopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine **12d** was obtained as an off-white solid (940 mg, 61%). MS (ESI) m/z 283.2.

General Procedure for the Preparation of 2-Aryl-7-morpholinotriazolopyrimidines by Suzuki Reaction. To a microwave processing tube was added dimethoxyethane (1.6 mL), aqueous Na_2CO_3 (2 M solution) (0.4 mL, 0.8 mmol, 2 equiv), $(Ph_3P)_4Pd$ (46 mg, 0.08 mmol), and the appropriately substituted boronic acid or ester (0.75 mmol, 2 equiv) and the 2 chloro-3-alkyl-7-morpholinotriazolopyrimidines (0.38 mmol), and the vessel was sealed. The mixture was heated to 140 °C for 45 min. The solvents were distilled on a rota-evaporator, and the crude compound was purified by silica gel chromatography CH_2Cl_2/EA (10:1) to give the product as an off-white solid (76−97% yield).

Preparation of 4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine (13b). 4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine **13b** was prepared from 5-chloro-3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidine **12b** (1.45 g, 5.40 mmol) and 4-(4,4,5,5-tetramethyl-[1,3,2]dioxaborolan-2-yl)phenylamine (1.53 g, 7.03 mmol) following the above general Suzuki procedure to give the titled product **13b** (1.63 g, 92% yield). ^{1}H NMR (DMSO) δ 1.52 (t, J = 7.6 Hz, 3H), 3.79 (m, 4H), 4.12 (m, 2H), 4.58 (q, J = 7.6 Hz, 2H), 4.59 (m, 2H), 5.65 (s, 2H), 6.62 (d, J = 8.3 Hz, 2H), 8.15 (d, J = 8.3 Hz, 2H) ppm. MS (ESI) m/z 326. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 99.5% purity.

Preparation of 4-(3-Methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenylamine (13a). 4-(3-Methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine **13a** was prepared from 5-chloro-3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine **12a** (1.3 g, 5.1 mmol) and 4-(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-yl)phenylamine (2.2 g, 10 mmol) following the general Suzuki procedure to give 4-(3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)aniline **13a** as a brown solid (900 mg, 56%yield). Mp 153 °C. ^{1}H NMR ($CDCl_3$) δ 3.91 (m, 6H), 4.23 (m, 1H), 4.23 (s, 3H), 4.69 (m, 2H), 6.74 (d, J = 8.8 Hz, 2H), 8.31 (d, J = 8.8 Hz, 2H) ppm. MS (ESI) m/z 312.3. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 97.3% purity.

Preparation of 4-(3-Isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine (13c). 4-(3-Isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)-phenylamine **13c** was prepared from 5-chloro-3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine **12c** (1.50 g, 5.3 mmol) and 4-(4,4,5,5-tetramethyl[1,3,2]dioxaborolan-2-yl)phenylamine (1.74 g, 7.97 mmol) following the general Suzuki procedure to give the titled product (1.22 g, 74% yield). ^{1}H NMR (DMSO) δ 1.64 (d, J = 6.8 Hz, 6H), 3.80 (m, 4H), 4.11 (m, 2H), 4.55 (m, 4H), 5.14 (hep, J = 6.8 Hz, 1H), 6.72 (d, J = 9.5 Hz, 2H), 8.19 (d, J = 9.5 Hz, 2H) ppm. MS (ESI) m/z 340. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 95.3% purity.

Preparation of (3-Cyclopropyl-7-morpholino-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)aniline (13d). 4-(3-Cyclopropyl-7-morpholino-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)aniline **13d** was prepared from 4-(5-chloro-3-cyclopropyl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-7-yl)morpholine **12d** (600 mg., 2.14 mmol) and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline following the general Suzuki procedure to give the product as an off-white solid (700 mg, 97% yield). ^{1}H NMR (DMSO) δ 1.24 (m, 2H), 1.35 (m, 2H),3.52 (m, 2H), 3.79 (m, 4H), 3.99 (hep, J = 3.8 Hz, 1H) 4.12 (m, 2H), 4.52 (m, 2H), 6.66 (d, J = 9.0 Hz, 2H), 8.17 (d, J = 9.0 Hz, 2H) ppm. MS (ESI) m/z = 338.3. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 96.6% purity.

Preparation of Methyl 4-({[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzoate (29). To a stirred solution of 4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine **13b** (2.6 g, 8.0 mmol) in anhydrous CH_2Cl_2 (65 mL) was added methyl 4-isocyanatobenzoate (1.55 g, 8.8 mmol) in one portion. The mixture was stirred for 8 h, hexane (50 mL) was added, and the solid was collected by filtration. The filter cake was washed with hexane (10 mL) and dried in a vacuum oven to give the product **29** as an off-white solid (3.06 g, 76% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.3 Hz, 3H), 3.83 (s, 7H), 4.16 (m, 2H), 4.61 (m, 2H), 4.63 (q, J = 7.3 Hz, 2H), 7.61 (m, 4H), 7.91 (d, J = 9.3 Hz, 2H), 8.39 (d, J = 8.6 Hz, 2H), 9.13 (s, 1H), 9.18 (s, 1H) ppm. MS (ESI) m/z 503.3. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 95.7% purity.

Preparation of 4-({[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzoic Acid (31). In a one-neck flask equipped with reflux condenser were suspended methyl 4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]-pyrimidin-5-yl)phenyl]carbamoyl}amino)benzoate **29** (3.54 g, 7.1 mmol) in THF (20 mL), methanol (5 mL), and NaOH (5N)(5 mL, 25 mmol). The mixture was refluxed for 2 h and cooled to 0 °C and acidified (pH < 1) with HCl (6 N). During the acidification a white solid was formed, which was collected by filtration. The filter cake was washed with water (10 mL) and dried in a vacuum oven to give the product **31** as an off-white solid (3.34 g, 98% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.3 Hz, 3H), 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.63 (q, J = 7.3 Hz, 2H), 7.60 (m, 4H), 7.88 (d, J = 8.3 Hz, 2H), 8.39 (d, J = 8.8 Hz, 2H), 9.24 (s, 1H), 9.27 (s, 1H) ppm. MS (ESI) m/z 489.3. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 97.3% purity.

Preparation of 1-[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]-3-{4-[(4-methylpiperazin-1-yl)carbonyl]-phenyl}urea (3). 4-({[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzoic acid **31** (1.26 g, 2.57 mmol), 1-methylpiperazine (335 mg, 3.35 mmol), NEt_3 (464 μL, 3.35 mmol), and HOBT (452 mg, 3.35 mmol) were suspended in anhydrous THF (8 mL), and EDCI (640 mg, 3.35 mmol) was added. The mixture was stirred for a minimum of 6 h. The solvent was removed on a rota-evaporator, and the crude mixture was dissolved in DMSO (15 mL) and purified by semipreparative HPLC using (ACN/water/NH_3) to give after combining product fractions and solvent removal (1.15 g, 79% yield) 1-[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-{4-[(4-methylpiperazin-1-yl)carbonyl]phenyl}urea **3** as a white solid. ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.3 Hz, 3H), 2.20 (s, 3H), 2.33 (m, 4H), 3.50 (m, 4H), 3.83 (m, 4H), 4.15 (m, 2H), 4.62 (m, 2H), 4.63 (q, J = 7.3 Hz, 2H), 7.35 (d, J=8.6 Hz, 2H), 7.54 (d, J = 8.6 Hz, 2H),

7.61 (d, J = 8.8 Hz, 2H), 8.39 (d, J = 8.8 Hz, 2H), 8.97 (s, 1H), 9.07 (s, 1H) ppm. Anal. Calcd for $C_{29}H_{34}FN_{10}O_3$ (%): C, 61.04; H, 6.01; N, 24.54. Found: C, 60.85; H, 6.40; N, 24.25. MS (ESI) m/z 571.3. HRMS: calcd 571.288 82, found 571.287 38. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, UV absorption at 215 nm, showed 99.3% purity.

Preparation of (1-Benzylpiperidin-4-yl)-(2-chloro-6-morpholin-4-yl-5-nitropyrimidin-4-yl)amine (9). To a cooled solution of 4-amino-1-benzylpiperidine (1.25 g, 6.58 mmol) and NEt_3 (796 mg, 7.89 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added, over a period of 0.5 h, a solution of 2,6-dichloro-5-nitro-4-morpholinopyrimidine **8** (1.5 g, 6.58 mmol) dissolved in CH_2Cl_2 (10 mL). The reaction mixture was stirred for another 1 h at 0 °C and allowed to warm to 20 °C and stirred for 4 h to drive the reaction to completion. The product was purified by silica gel column chromatography by eluting it with CH_2Cl_2 followed by CH_2Cl_2/EA (10:1) to give the product **9** as a yellow solid (2.0 g, 70% yield). ^{1}H NMR ($CDCl_3$) δ 1.62 (m, 2H), 2.01 (m, 2H), 2.24 (t, J = 10.8 Hz, 2H), 2.81 (m, 2H), 3.54 (m, 6H), 3.77 (m, 4H), 4.18 (m, 1H), 7.33 (m, 5H), 8.44 (d, J = 7.8 Hz, 1H) ppm. MS (ESI) m/z 433.1.

Preparation of N^4-(1-Benzylpiperidin-4-yl)-2-chloro-6-morpholin-4-ylpyrimidine-4,5-diamine (10). In a three-neck flask was suspended under nitrogen atmosphere (1-benzylpiperidin-4-yl)-(2-chloro-6-morpholin-4-yl-5-nitropyrimidin-4-yl)amine (1.0 g, 2.3 mmol) and Raney Ni (1 g) in methanol (100 mL). To the stirring reaction mixture was added slowly $N_2H_4 \cdot H_2O$ (1.2 mL, 21 mmol, 9 equiv), and the stirring was continued for 1 h to drive the reduction to completion. The reaction mixture was filtered over Celite and the filtrate was evaporated to obtain the product **10** as light-brown solid (900 mg, 97% yield). ^{1}H NMR (DMSO) δ 1.50 (m, 2H), 1.87 (m, 2H), 2.05 (m, 2H), 2.82 (m, 2H), 2.96 (m, 4H), 3.48 (m, 2H), 3.71 (m, 4H), 4.31 (m, 2H), 6.45 (m, 1H), 7.32 (m, 2H) ppm. MS (ESI) m/z 403.1.

Preparation of 3-(1-Benzylpiperidin-4-yl)-5-chloro-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine (11). To a stirred solution of N^4-(1-benzylpiperidin-4-yl)-2-chloro-6-morpholin-4-ylpyrimidine-4,5-diamine **10** (500 mg, 1.24 mmol) in acetic acid/water (1:1) (5 mL) at 0 °C was added aqueous (0.5 N) $NaNO_2$ solution (5 mL, 2.5 mmol), and the reaction mixture was allowed to stir for 2 h. The off-white solid was collected by filtration and dried in vacuum to give **11** (510 mg, 100% yield). MS (ESI) m/z 414.2

Preparation of {3-[3-(1-Benzylpiperidin-4-yl)-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl]phenyl}methanol (5). {3-[3-(1-Benzylpiperidin-4-yl)-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl]phenyl}methanol **5** was prepared from 3-(1-benzylpiperidin-4-yl)-5-chloro-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidine (145 mg, 0.35 mmol) and 3-hydroxymethylphenylboronic acid (106 mg, 0.7 mmol) following the above general procedure for Suzuki reactions. The crude product was dissolved in DMSO (2 mL), filtered, and purified by semipreparative HPLC using ACN/water/NH_3 as mobile phase. After unification of the product fraction and solvent removal, the product was obtained as a white solid (80 mg, 47% yield). ^{1}H NMR (DMSO) δ 2.10 (m, 2H), 2.31 (m, 4H), 2.99 (m, 2H), 3.58 (s, 2H), 3.82 (m, 4H), 4.16 (m, 2H), 4.60 (m, 2H), 4.60 (d, J = 5.8 Hz, 2H), 4.86 (m, 1H), 5.32 (t, J = 5.8 Hz, 1H), 7.27 (m, 1H), 7.34−7.40 (m, 4H), 7.47 (d, J = 5.1 Hz, 2H), 8.32 (td, J = 5.8, 2.1 Hz, 1H), 8.39 (s, 1H) ppm. MS (ESI) m/z 486.4. HRMS: calcd 486.261 75, found 486.2607. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 97.7% purity.

Preparation of 1-[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-4-ylurea (14). To a stirred solution of triphosgene (68 mg, 0.23 mmol) in CH_2Cl_2 (5 mL) was added 4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]-pyrimidin-5-yl)phenylamine **13b** (100 mg, 0.46 mmol) at 0 °C. The reaction mixture was stirred for 15 min, and 4-aminopyridine (40 mg, 0.46 mmol) and NEt_3 (64 μL, 0.46 mmol) were added. The reaction mixture was stirred for additional 1 h. The solvents were distilled on a rota-evaporator and the crude mixture was purified by semipreparative HPLC (NH_3-method) to give 1-[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-4-ylurea **14** (22 mg, 11% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.3 Hz, 3H), 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.64 (q, 7.3 Hz, 2H), 7.48 (d, J = 5.8 Hz, 2H), 7.61 (d, J = 7.8 Hz, 2H), 7.37−7.42 (m, 4H), 9.23 (s, 1H), 9.27 (s, 1H) ppm. MS (ESI) m/z 446. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 99.2% purity.

Preparation of 1-[4-(3-Isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-4-ylurea (15). To a stirred solution of triphosgene (39 mg, 0.13 mmol) in CH_2Cl_2 (1 mL) was added 4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine **13c** (50 mg, 0.14 mmol) at 25 °C. The reaction mixture was stirred for 15 min, 4-aminopyridine (42 mg, 0.44 mmol) and NEt_3 (62 μL, 0.44 mmol) were added, and the reaction mixture was stirred for an additional 1 h. The solvents were removed in a nitrogen stream and the crude mixture was purified by semipreparative HPLC (TFA method) to give 1-[4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-4-ylurea **15** (22 mg, 57% yield). MS (ESI) m/z 460. HRMS: calcd 460.220 40, found 460.219 84. Analytical LC/MS, using Aquasil C18 column, ACN/H_2O eluent at 0.8 mL/min flow (containing 0.1% formic acid), 5.5 min gradient 0% ACN to 100% ACN, monitored by UV absorption at 254 nm, showed 99.5% purity. Analytical LC, using X-Bridge BEH column, MeOH/H_2O eluent at 1 mL/min flow (containing 0.05% NH_4OAc), 20 min gradient 10% MeOH to 90% ACN, monitored by UV absorption at 215 nm, showed 99.4% purity.

Preparation of 1-(4-(3-Cyclopropyl-7-morpholino-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl)-3-(pyridin-4-yl)urea (16). To a solution of triphosgene (66 mg, 0.223 mmol) in CH_2Cl_2 (1 mL) was added 4-(3-cyclopropyl-7-morpholino-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)aniline **13d** (150 mg, 0.445 mmol), and the mixture was stirred for 30 min. Then pyridin-4-amine (126 mg, 1.34 mmol) and Et_3N (187 uL, 1.34 mmol) in CH_2Cl_2-(1.5 mL) were added and the mixture was stirred overnight. The solvents were removed in a nitrogen stream, and the residue was purified by semipreparative HPLC (TFA method) to give the product **16** as a white solid (TFA salt) (120 mg, 47% yield). ^{1}H NMR (DMSO) δ 1.28(m, 2H), 1.39 (m, 2H), 3.82 (m, 4H), 4.04 (hep, J = 3.8 Hz, 1H), 4.15 (m, 2H), 4.58 (m, 2H), 7.67 (d, J = 9.0 Hz, 2H), 7.92 (d, J = 7.0 Hz, 2H), 8.43 (d, J = 9.0 Hz, 2H), 8.61 (d, J = 7.0 Hz, 2H), 9.64 (s, 1H), 10.66 (s, 1H) ppm. MS (ESI) m/z = 458.3. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 96.3% purity.

Preparation of 1-[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-3-ylurea (17). To a stirred solution of 4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]-pyrimidin-5-yl)phenylamine **13b** (150 mg, 0.46 mmol) in anhydrous $CHCl_3$ (2 mL) were added pyridine 3-isocyanate (83 mg, 0.69 mmol) and NEt_3 (97 μL, 0.69 mmol). The mixture was stirred for 18 h and the solvents were removed in vacuo to obtain the crude product, which was purified by semipreparative HPLC (NH_3 method), to give 1-[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-3-ylurea **17** as an off-white solid (55 mg, 26% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.6 Hz, 3H), 3.83 (m, 4H), 4.18 (m, 2H), 4.62 (m, 2H), 4.64 (q, J = 7.6 Hz, 2H), 7.33 (dd, J = 8.3, 4.0 Hz, 1H), 7.60 (d, J = 8.8 Hz, 2H), 7.97 (ddd, J = 8.3, 2.5, 1.5 Hz, 1H), 8.21 (dd, J = 4.8, 1.5 Hz, 1H), 8.39 (d, J = 8.8 Hz, 2H), 8.62 (d, J = 2.5 Hz, 1H), 8.93 (s, 1H), 9.13 (s, 1H) ppm. MS (ESI) m/z 446.4. Analytical LC, using Prodigy ODS3 column,

ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 99.1% purity.

Preparation of 1-[4-(3-Isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-3-ylurea (18). The compound was prepared as described for compound **16** using triphosgene (39 mg, 0.13 mmol), 4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine (50 mg, 0.14 mmol), 3-aminopyridine (42 mg, 0.44 mmol), and NEt_3 (62 μL, 0.44 mmol) in CH_2Cl_2 (1 mL) to give 1-[4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-3-ylurea **18** (18 mg, 47% yield). MS (ESI) *m*/*z* 460. HRMS: calcd 460.220 40, found 460.219 78. Analytical LC/MS, using Aquasil C18 column, ACN/H_2O eluent at 0.8 mL/min flow (containing 0.1% formic acid), 5.5 min gradient 0% ACN to 100% ACN, monitored by UV absorption at 254 nm, showed >99.5% purity. Analytical LC, using X-Bridge BEH column, MeOH/H_2O eluent at 1 mL/min flow (containing 0.05% NH_4OAc), 20 min gradient 10% MeOH to 90% ACN, monitored by UV absorption at 215 nm, showed 96.4% purity.

Preparation of 1-[4-(3-Methyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-3-ylurea (19). 4-(3-Methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)aniline **13a** (60 mg, 0.19 mmol) and 3-pyridylisocyanate (25 mg, 0.20 mmol) were suspended in THF (2 mL) to obtain 1-[4-(3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)-phenyl]-3-pyridin-3-ylurea as a solid. The solid was suspended in diethylether and filtered. It was found to be pure enough (60 mg, 72% yield). Mp 272 °C. ^{1}H NMR (DMSO) δ 3.82 (m, 4H), 4.18 (m, 2H), 4.20 (s, 3H), 4.58 (m, 2H), 7.33 (dd, J = 8.8, 4.8 Hz, 1H), 7.61 (d, J = 8.8 Hz, 2H), 7.97 (m, 1H), 8.21 (m, 1H), 8.40 (d, J = 8.8 Hz, 2H), 8.62 (s, 1H), 8.92 (s, 1H), 9.13 (s, 1H) ppm. MS (ESI) *m*/*z* 432.46. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 97.8% purity.

Preparation of 1-(4-(3-Cyclopropyl-7-morpholino-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl)-3-(pyridin-3-yl)urea (20). To a solution of 4-(3-cyclopropyl-7-morpholino-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)aniline **13d** (120 mg, 0.35 mmol) in $CHCl_3$ (2 mL) were added Et_3N (93 μL, 0.668 mmol) and 3-isocyanatopyridine (80 mg, 0.668 mmol). The mixture was stirred overnight and the solvent was evaporated and purified by HPLC (TFA method) to give the product **20** as a white solid (TFA salt) (112 mg, 55% yield). ^{1}H NMR (DMSO) δ 1.27 (m, 2H), 1.38 (m, 2H), 3.82 (m, 4H), 4.04 (hep, J = 3.8 Hz, 1H), 7.63 (d, J = 8.8 Hz, 2H), 7.66 (m, 1H), 8.19 (m, 1H), 8.38 (m, 1H), 8.40 (d, J = 8.8 Hz, 2H), 8.89 (m, 1H), 9.42 (s, 1H), 9.43 (s, 1H) ppm. MS (ESI) *m*/*z* = 458.3. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 95.7% purity.

Preparation of [4-(3-Methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]-3-(2-thienyl)urea (21). To a solution of 4-(3-methyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)aniline **13a** (60 mg, 0.19 mmol) in $CHCl_3$ (2 mL) was added 2-thienyl isocyanate (20 mg, 0.20 mmol). The mixture was stirred overnight. The solvent was evaporated in a N_2 stream and the crude mixture was purified by semipreparative HPLC (NH_3 method) to give the product **21** as a white solid (62 mg, 72% yield). Mp 182 °C. ^{1}H NMR (DMSO) δ 3.83 (m, 4H), 4.16 (m, 2H), 4.19 (s, 3H), 4.59 (m, 2H), 6.56 (dd, J = 3.5, 1.5 Hz, 1H), 6.82 (dd, J = 5.5, 3.5 Hz, 1H), 6.89 (dd, J = 5.5, 1.5 Hz, 1H), 7.59 (d, J = 8.8 Hz, 2H), 8.39 (d, J = 8.8 Hz, 2H), 9.35 (s, 1H), 9.99 (s, 1H) ppm. MS (ESI) *m*/*z* = 437.5. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 96.1% purity.

Preparation of 1-[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]-3-(2-thienyl)urea (22). To a stirred solution of 4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]-pyrimidin-5-yl)phenylamine **13b** (150 mg, 0.46 mmol) in anhydrous $CHCl_3$ (2 mL) was added thienyl 2-isocyanate (87 mg, 0.69 mmol) and NEt_3 (97 μL, 0.69 mmol). The mixture was stirred for 18 h and the solvent was evaporated in a N_2 stream to obtain the crude product, which was purified by semipreparative HPLC (NH_3 method), to give 1-[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]-3-(2-thienyl)urea **22** as an off-white solid (90 mg, 43% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.3 Hz, 3H), 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.63 (q, J = 7.3 Hz, 2H), 6.56 (dd, J = 3.5, 1.5 Hz, 1H), 6.83 (dd, J = 5.5, 3.5 Hz, 1H), 6.89 (dd, J = 5.5, 1.5 Hz, 1H), 7.59 (d, J = 8.8 Hz, 2H), 8.38 (d, J = 8.8 Hz, 2H), 9.03 (s, 1H), 9.67 (s, 1H) ppm. MS (ESI) *m*/*z* 451.4. HRMS: calcd 451.165 92, found 451.165 62. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 97.8% purity.

Preparation of 1-(4-(3-Cyclopropyl-7-morpholino-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl)-3-(thiophen-2-yl)urea (23). To a solution of 4-(3-cyclopropyl-7-morpholino-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)aniline **13d** (75 mg, 0.222 mmol) in $CHCl_3$ (1 mL) was added Et_3N (46 uL, 0.0.333 mmol) and 2-isocyanatothiophene (42 mg, 0.333 mmol). The mixture was stirred overnight, and the solvent was evaporated in a N_2 stream. The crude material was purified by HPLC (NH_3 method) to give the product as a white solid (51 mg, 50% yield). ^{1}H NMR (DMSO) δ 1.27 (m, 2H), 1.38 (m, 2H), 3.82 (m, 4H), 4.04 (hep, J = 3.8 Hz, 1H), 4.15 (m, 2H), 4.57 (m, 2H), 6.56 (dd, J = 3.5, 1.5 Hz, 1H), 6.83 (dd, J = 5.5, 3.5 Hz, 1H), 6.90 (dd, J = 5.5, 1.5 Hz, 1H), 7.60 (d, J = 8.8 Hz, 2H), 8.38 (d, J = 8.8 Hz, 2H), 9.05 (s, 1H), 9.69 (s, 1H) ppm. MS (ESI) *m*/*z* = 463.3. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 95.7% purity.

Preparation of 1-[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]-3-(2-methylpyridin-4-yl)urea (24). The compound was prepared as described for **16** using triphosgene (74 mg, 0.25 mmol), 4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine **13b** (100 mg, 0.31 mmol), 4-amino-2-methylpyridine (100 mg, 0.93 mmol), and NEt_3 (430 μL, 0.44 mmol) in CH_2Cl_2 (3 mL) to give 1-[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-(2-methylpyridin-4-yl)urea **24** (13 mg, 9% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.6 Hz, 3H), 2.63 (s, 3H) 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.64 (q, J = 7.6 Hz, 2H), 7.66 (d, J = 8.8 Hz, 2H), 7.77 (m, 1H), 7.81 (m, 1H), 8.44 (d, J = 8.8 Hz, 2H), 8.50 (d, J = 6.5 Hz, 1H), 9.88 (s, 1H), 10.53 (s, 1H) ppm. MS (ESI) *m*/*z* 460. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 95.0% purity.

Preparation of 1-(4-Dimethylaminophenyl)-3-[4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-urea (25). The compound was prepared as described for compound **16** using triphosgene (39 mg, 0.13 mmol), 4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine **13c** (50 mg, 0.14 mmol), 4-*N*,*N*-dimethylaniline (60 mg, 0.44 mmol), and NEt_3 (62 μL, 0.44 mmol) in CH_2Cl_2 (1 mL) to give 1-(4-dimethylaminophenyl)-3-[4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]urea **25** (26 mg, 63% yield). Analytical LC/MS, using Aquasil C18 column, ACN/ H_2O eluent at 0.8 mL/min flow (containing 0.1% formic acid), 5.5 min gradient 0% ACN to 100% ACN, monitored by UV absorption at 254 nm, showed 99.5% purity. Analytical LC, using X-Bridge BEH column, MeOH/H_2O eluent at 1 mL/min flow (containing 0.05% NH_4OAc), 20 min gradient 10% MeOH to 90% ACN, monitored by UV absorption at 215 nm, showed 96.7% purity. MS (ESI) *m*/*z* 502. HRMS: calcd 502.267 35, found 502.265 93.

Preparation of 1-(4-Hydroxymethylphenyl)-3-[4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]urea (26). The compound was prepared as described for compound **16** using triphosgene (39 mg, 0.13 mmol), 4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine **13c** (50 mg, 0.14 mmol), 4-aminobenzyl alcohol (54 mg, 0.44 mmol), and NEt_3 (62 μL, 0.44 mmol) in CH_2Cl_2 (1 mL) to give 1-[4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-3-ylurea **26** (18 mg, 47% yield). ^{1}H NMR (DMSO) δ 1.66 (d, J = 6.3 Hz, 6H), 3.83 (m, 4H), 4.16 (m, 2H), 4.43 (s, 3H), 4.60 (m, 2H), 5.19 (hep, J = 6.3 Hz, 1H), 7.24 (d, J = 8.8 Hz, 2H), 7.42 (d, J = 8.8 Hz, 2H), 7.59 (d, J = 8.8 Hz, 2H), 8.37 (d, J = 8.8 Hz, 2H), 8.70 (s, 1H), 8.96 (s, 1H) ppm. MS (ESI) m/z 489. HRMS: calcd 489.235 72, found 489.236 10. Analytical LC/MS, using Aquasil C18 column, ACN/H_2O eluent at 0.8 mL/min flow (containing 0.1% formic acid), 5.5 min gradient 0% ACN to 100% ACN, monitored by UV absorption at 254 nm, showed 99.5% purity.

Preparation of 1-[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-4-ylurea (27). To a stirred solution of triphosgene (39 mg, 0.13 mmol) in CH_2Cl_2 (1 mL) was added 4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]-pyrimidin-5-yl)phenylamine **13b** (50 mg, 0.14 mmol) at 25 °C. The reaction mixture was stirred for 15 min, 4-aminobenzamide (59 mg, 0.44 mmol) and NEt_3 (62 μL, 0.44 mmol) were added, and the reaction mixture was stirred for an additional 1 h. The solvents were removed in a nitrogen stream and the crude mixture was purified by semipreparative HPLC (NH_3 method) to give 1-[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-pyridin-4-ylurea **27** (22 mg, 57% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.6 Hz, 3H), 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.64 (q, J = 7.6 Hz, 2H), 7.20 (s, 1H), 7.53 (d, J = 8.8 Hz, 2H), 7.60 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 3H), 8.39 (d, J = 8.8 Hz, 2H), 8.99 (s, 1H), 9.07 (s, 1H) ppm. MS (ESI) m/z 488. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 96.4% purity.

Preparation of 4-{3-[4-(3-Isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]ureido}benzamide (28). The compound was prepared as described for compound **16** using triphosgene (100 mg, 0.33 mmol), 4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine (140 mg, 0.41 mmol), 4-aminobenzamide (163 mg, 1.2 mmol), and NEt_3 (567 μL, 4.1 mmol) in CH_2Cl_2 (5 mL) to give 4-{3-[4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-ureido}benzamide **28** (68 mg, 33% yield). ^{1}H NMR (DMSO) δ 1.66(d, J = 6.3 Hz, 6H), 3.83 (m, 4H), 4.16 (m, 2H), 4.60 (m, 2H), 5.19 (hep, J = 6.3 Hz, 1H), 7.20 (s, 1H), 7.53 (d, J = 8.8 Hz, 2H), 7.60 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 3H), 8.39 (d, J = 8.8 Hz, 2H), 9.00 (s, 1H), 9.08 (s, 1H) ppm. MS (ESI) m/z 502. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 95.4% purity.

Preparation of 1-[4-(3-Isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-(4-morpholin-4-ylphenyl)urea (30). The compound was prepared as described for compound **16** using triphosgene (39 mg, 0.13 mmol), 4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenylamine **13c** (50 mg, 0.14 mmol), 4-morpholinylaniline (79 mg, 0.44 mmol), NEt_3 (62 μL, 0.44 mmol) in CH_2Cl_2 (1 mL) to give 1-[4-(3-isopropyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-3-(4-morpholin-4-ylphenyl)urea **30** (14 mg, 31% yield). Analytical LC/MS, using Aquasil C18 column, ACN/H_2O eluent at 0.8 mL/min flow (containing 0.1% formic acid), 5.5 min gradient 0% ACN to 100% ACN, monitored by UV absorption at 254 nm, showed 98.5% purity. Analytical LC, using X-Bridge BEH column, MeOH/H_2O eluent at 1 mL/min flow (containing 0.05% NH_4OAc), 20 min gradient 10% MeOH to 90% ACN, monitored by UV absorption at 215 nm, showed 97.1% purity. MS (ESI) m/z 544. HRMS: calcd 544.277 92, found 544.277 21.

Preparation *N*-Ethyl-4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzamide (32). A solution of 4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzoic acid **31** (50 mg, 0.102 mmol), Hunig's base (79 mg, 0.612 mmol), HBTU (116 mg, 0.306 mmol), and NMP (1 mL) was stirred for 1 h at room temperature, and ethylamine (2 M in THF) (77 μL, 0.15 mmol) was added. The stirring was continued overnight. The solvents were removed in a nitrogen stream and the crude mixture was purified by semipreparative HPLC (TFA method) to give the product **32** as a white solid (16 mg, 30% yield). ^{1}H NMR (DMSO) δ 1.12 (t, J = 7.3 Hz, 3H), 1.55 (t, J = 7.6 Hz, 3H), 3.28 (m, 1H), 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.64 (q, J = 7.6 Hz, 2H), 7.54 (d, J = 8.8 Hz, 2H), 7.60 (d, J = 8.8 Hz, 2H), 7.80 (d, J = 8.8 Hz, 2H), 8.30 (t, J = 53 Hz, 3H), 8.39 (d, J = 8.8 Hz, 2H), 9.00 (s, 1H), 9.08 (s, 1H) ppm. MS (ESI) m/z 516.2. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 96.3% purity.

Preparation of 4-({[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)-*N*,*N*-dimethyl-benzamide (33). A solution of 4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)-benzoic acid **31**(50 mg, 0.102 mmol), Hunig's base (79 mg, 0.612 mmol), HBTU (116 mg, 0.306 mmol), and NMP (1 mL) was stirred for 1 h at room temperature, and $HNMe_2$ (2 M in THF) (77 μL, 0.15 mmol) was added. The stirring was continued overnight. The solvents were removed in a nitrogen stream and the crude mixture was purified by semipreparative HPLC (TFA method) to give the product **33** as a white solid (9 mg, 17% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.6 Hz, 3H), 2.96 (s, 6H), 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.63 (q, J = 7.6 Hz, 2H), 7.37 (d, J = 8.8 Hz, 2H), 7.52 (d, J = 8.8 Hz, 2H), 7.60 (d, J = 8.8 Hz, 2H), 8.39 (d, J = 8.8 Hz, 2H), 8.94 (s, 1H), 9.05 (s, 1H) ppm. MS (ESI) m/z 516.3. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 99.0% purity.

Preparation of *N*-Butyl-4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzamide (34). A solution of 4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzoic acid **31** (50 mg, 0.102 mmol), Hunig's base (79 mg, 0.612 mmol), HBTU (116 mg, 0.306 mmol), and NMP (1 mL) was stirred for 1 h at room temperature, and *n*-butylamine (14 mg, 0.15 mmol) was added. The stirring was continued overnight. The solvents were removed in a nitrogen stream and the crude mixture was purified by semipreparative HPLC (TFA method) to give the product **34** as a white solid (30 mg, 54% yield). Analytical LC/MS, using Aquasil C18 column, ACN/H_2O eluent at 0.8 mL/min flow (containing 0.1% formic acid), 5.5 min gradient 0% ACN to 100% ACN, monitored by UV absorption at 254 nm, showed 95.5% purity. Analytical LC, using X-Bridge BEH column, MeOH/H_2O eluent at 1 mL/min flow (containing 0.05% NH_4OAc), 20 min gradient 10% MeOH to 90% ACN, monitored by UV absorption at 215 nm, showed 95.5% purity. MS (ESI) m/z 544.3. HRMS: calcd 544.277 92, found 544.278 98.

Preparation of *N*-[2-(Dimethylamino)ethyl]-4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-carbamoyl}amino)benzamide (35). A suspension of 4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzoic acid **31** (200 mg, 0.40 mmol), *N*,*N*-dimethylethylendiamine (87 μL, 0.8 mmol), NEt_3 (112 μL, 0.8 mmol), HOBT (110 mg, 0.8 mmol), and EDCI (154 mg, 0.8 mmol) in anhydrous THF (3 mL) gave *N*-[2-(dimethylamino)ethyl]-4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzamide **35** as free base. The free base was treated with MeOH/HCl to form the HCl salt of **35** (89 mg, 37% yield). ^{1}H NMR (DMSO) δ 1.55

(t, J = 7.6 Hz, 3H), 2.84 (d, J = 5.0 Hz, 6H), 3.26 (dd, J = 11.3 5.0 Hz, 2H), 3.61 (dd, J = 11.3 5.0 Hz, 1H), 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.63 (q, J = 7.6 Hz, 2H), 7.58 (d, J = 8.8 Hz, 2H), 7.61 (d, J = 8.8 Hz, 2H), 7.86 (d, J = 8.8 Hz, 2H), 8.39 (d, J = 8.8 Hz, 2H), 8.63 (t, J = 5 Hz, 1H), 9.48 (m, 2H), 9.68 (s, 1H) ppm. MS (ESI) m/z 559.3. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 99.0% purity.

Preparation of 4-({[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)-*N*-(2-pyridin-2-ylethyl)benzamide (36). A solution of 4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-carbamoyl}amino)benzoic acid **31** (50 mg, 0.102 mmol), Hunig's base (79 mg, 0.612 mmol), and HBTU (116 mg, 0.306 mmol) in NMP (1 mL) was stirred for 1 h at room temperature, and 2-(2-aminoethyl)pyridine (19 mg, 0.15 mmol) was added. The stirring was continued overnight. The solvents were removed in a nitrogen stream and the crude mixture was purified by semipreparative HPLC (NH_3 method) to give the product **36** as a white solid (44 mg, 61% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.5 Hz, 3H), 3.01(t, J = 6.8 Hz, 2H), 3.61 (q, J = 6.8 Hz, 2H), 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.63 (q, J = 7.5 Hz, 2H), 7.28 (m, 1H), 7.31 (d, J = 8.3 Hz, 1H), 7.54 (d, J = 8.8 Hz, 2H), 7.61 (d, J = 8.8 Hz, 2H), 7.77 (m, 3H), 8.39 (d, J = 8.8 Hz, 2H), 8.46 (t, J = 5.7 Hz, 1H), 8.56 (d, J = 5.0 Hz, 1H), 9.02 (m, 2H), 9.10 (s, 1H) ppm. MS (ESI) m/z 593.3. HRMS: calcd 593.2732, found 593.2727. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 98.2% purity.

Preparation of 4-({[4-(3-Ethyl-7-morpholin-4-yl-3*H*-[1,2,3]-triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)-*N*-[2-(4-methylpiperazin-1-yl)ethyl]benzamide (37). The compound was prepared as described in the example above using 4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)-phenyl]carbamoyl}amino)benzoic acid **31** (50 mg, 0.1 mmol), 4-methylpiperazinylethanamine (20 mg, 0.2 mmol), NEt_3 (30 μL, 0.2 mmol), HOBT (30 mg, 0.2 mmol), and EDCI (40 mg, 0.2 mmol) in anhydrous THF (1 mL) to give 4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-carbamoyl}amino)-*N*-[2-(4-methylpiperazin-1-yl)ethyl]benzamide **38** (34 mg, 55% yield). Analytical LC/MS, using Aquasil C18 column, ACN/H_2O eluent at 0.8 mL/min flow (containing 0.1% formic acid), 5.5 min gradient 0% ACN to 100% ACN, monitored by UV absorption at 254 nm, showed >99.5% purity. Analytical LC, using X-Bridge BEH column, MeOH/H_2O eluent at 1 mL/min flow (containing 0.05% NH_4OAc), 20 min gradient 10% MeOH to 90% ACN, monitored by UV absorption at 215 nm, showed 98.4% purity. MS (ESI) m/z 614.3. HRMS: calcd 614.3310, found 614.3303.

Preparation of *N*-[3-(Dimethylamino)propyl]-4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-carbamoyl}amino)benzamide (38). The compound was prepared as described in the example above using 4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidin-5-yl)phenyl]-carbamoyl}amino)benzoic acid **31** (100 mg, 0.2 mmol), *N*,*N*-dimethylpropyldiamine (40 mg, 0.4 mmol), NEt_3 (56 μL, 0.4 mmol), HOBT (55 mg, 0.4 mmol), and EDCI (77 mg, 0.4 mmol) in anhydrous THF (2 mL) to give *N*-[3-(dimethyl-amino)propyl]-4-({[4-(3-ethyl-7-morpholin-4-yl-3*H*-[1,2,3]triazolo-[4,5-*d*]pyrimidin-5-yl)phenyl]carbamoyl}amino)benzamide **38** as free base. The free base was treated with MeOH/HCl to form the HCl salt of **38** (39 mg, 32% yield). ^{1}H NMR (DMSO) δ 1.55 (t, J = 7.3 Hz, 3H), 1.90 (m, 2H), 2.76 (d, J = 4.8 Hz, 6H), 3.08 (m, 2H), 3.32 (6, J = 6.3 Hz, 2H), 3.83 (m, 4H), 4.16 (m, 2H), 4.62 (m, 2H), 4.63 (q, J = 7.3 Hz, 2H), 7.56 (d, J = 8.8 Hz, 2H), 7.61 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H), 8.38 (d, J = 8.8 Hz, 2H), 8.54 (t, J = 5.8 Hz, 1H), 9.58 (s, 2H), 9.89 (s, 1H) ppm. MS (ESI) m/z 573.4. Analytical LC, using Prodigy ODS3 column, ACN/H_2O eluent at 1 mL/min flow (containing 0.05% TFA), 20 min gradient 5% ACN to 95% ACN, monitored by UV absorption at 215 nm, showed 98.4% purity.

Biology Methods. Enzyme Assays. Enzyme assays were done in fluorescent polarization (FP) format, adapted from the Echelon K-1100 PI3K FP assay kit protocol (1). PIP2 and TAMRA-labeled detector (PIP3-analogue) were purchased from Echelon. Human PI3Kα and PI3Kγ were expressed in SF9 insect cells and purified by GST and/or FLAG affinity columns. Human PI3Kβ, PI3Kδ and the two most common mutant forms of PI3Kα (E545K and H1047R) were purchased from Upstate Biotech. Mouse GRP1 was produced as a GST fusion protein in *E. coli* and isolated by GST-Sepharose. Assay reaction buffer was 20 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 0.05% CHAPS, and 0.01% βME. Assay STOP/detection buffer was 100 mM Hepes, pH 7.5, 4 mM EDTA, 0.05% CHAPS. FP assays were run in Nunc 384-well black polypropylene fluor plates. The FP reaction was run in 20 μL of reaction buffer containing 20 μM PIP2, 25 μM ATP, and >4% DMSO (compound solvent). The reaction was run for 30 min at room temp. The reaction was stopped with 20 μL of STOP/detection buffer containing 10 nM probe and 40 nM GST-GRP. Assay plates were incubated for 2 h, and fluorescence polarization was measured in a Perkin-Elmer Envision plate reader with TAMRA-FP filters.

Selectivity of **3** was evaluated in a 236 human kinase panel (Invitrogen) at ATP K_m for each enzyme.

Cell Culture and Growth Inhibition Assay. The following cell lines were obtained from ATCC: MDA361, U87MG, A549, and PC3. All cell lines were propagated at 37 °C in 5% CO_2 incubators in growth media recommended by ATCC supplemented with penicillin/streptomycin and 10% fetal calf serum.

Cell growth inhibition was determined using the CellTiter 96 Aqueous nonradioactive cell proliferation assay from Promega. This homogeneous colorimetric method determined the number of viable cells in proliferation assays. The assay was carried out in 96-well format following manufacturer's instructions, with cell number numbers per well being adjusted on the basis of growth characteristics of the various cell lines used. Assay end point data were quantitated after 72 h of compound exposure using a Victor2 V (Wallac) model 1420 multilabel HTS counter.

Cell Lysis and Western Blotting. Cell lysis was done to enable biochemical analysis of PI3K/mTOR signaling pathway proteins after exposure of various cell lines to **3**. Briefly, 3×10^6 cells were seeded onto 6-well microtiter plates (Nunc) 24 h prior to being exposed to these compounds in complete growth medium. Cells were exposed to these compounds for 4 h (unless indicated otherwise in the figures). After exposure to compounds, cell growth medium was removed and cells were washed twice with cold (4 °C) PBS. Cell lysis buffer (0.2 mL) was then added to each microtiter plate well with sufficient mixing to ensure complete cell lysis. Cell lysis buffer consisted of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/mL leupeptin. Cell lysates were then spun in a microfuge for 30 s at 14 000 rpm. Supernatant (75 μL) was then combined with 30 μL of 3× protein gel loading buffer [187.5 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 0.03% (w/v) bromophenol blue, and 125 mM DTT]. Samples were boiled for 5 min and then subjected to SDS−PAGE, transferred to nitrocellulose, and probed with various antibodies specific to the protein and phosphoprotein components of the PI3K/Akt/mTOR signaling pathway. Antibodies were obtained from Cell Signaling Technology, and they were anti(α)-Akt, α-phospho(p)-Akt at T308, α-p-Akt at S473, α-4EBP1, α-p-4EBP1 at T37/46, α-p70 S6 kinase, α-p-p70 S6 kinase at T389, α-PARP, α-cleaved PARP at D214, α-p-ENOS at S1177, α-p-GSK3α/β at S21/9, and α-p-PRAS40 at T246. All antibodies were used as recommended by the manufacturer, and specific antigen/antibody interactions were identified by a

horseradish peroxidase (HRP) conjugate secondary antibody (Ab) that enabled chemiluminescent detection. **3** inhibition of protein phosphorylation was quantified from Western blots analyzed on the BioRad Fluor-S MultiImager (Hercules, CA), using Quantity One Analysis software.

Establishment of Xenograft Tumors, Efficacy Studies, and Biomarker Analysis. Establishment of tumors, group randomization, tumor, and body weight recording during efficacy studies was described elsewhere.[17] Briefly, all in vivo studies using nude mice were conducted under an approved Institutional Animal Care and Use Committee protocol. Generally, tumor cells were suspended at 5×10^7 cells/mL in cell growth medium and the cell suspension (0.2 mL) was injected subcutaneously (sc) into the flank of a ~8 week old female nude mice (Charles River, Wilmington, MA). Mice with tumors greater than 100 mm^3 after 1 week were administered vehicle or compound by intravenous (iv) tail vein injection at various dose levels. Injection volumes for **3** were 0.2 mL in vehicle containing 0.5% methylcellulose and 0.4% polysorbate 80 (Tween-80), with vehicle pH adjusted to 3.5 by lactic acid to fully solubilize **3**. In tumor bearing mice tumor weight and body weight were monitored two or three times weekly. Tumor weight was calculated by the following formula: tumor weight (mg) = $(d^2 \times d/2)$, where d and d are the shortest and longest diameters of the tumor, respectively, measured in millimeters. Statistical analysis (Student's t test) of the log of the relative tumor growth compared treated groups with the control group. Statistically significant reduction in the tumor growth of treated groups compared to controls (vehicle) was defined as $p < 0.05$.

Pharmacodynamic (biomarker) measurements were done on tumor bearing female nude mice administered **3** or vehicle by iv injections as above. Tumor tissue and normal tissue samples were collected from euthanized animals, and tissue was homogenized, washed twice with cold (4 °C) PBS, and then treated with cell lysis buffer. Cell lysis buffer was the same as that described above. As with in vitro samples, cell lysates combined with gel loading buffer were boiled for 5 min and then subjected to SDS−PAGE, transferred to nitrocellulose, and probed with various antibodies specific to the protein and phosphoprotein components of the PI3K/Akt/mTOR signaling pathway.

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