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LETTER

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Discovery of 3,5-Diamino-1,2,4-triazole Ureas as Potent Anaplastic Lymphoma Kinase Inhibitors

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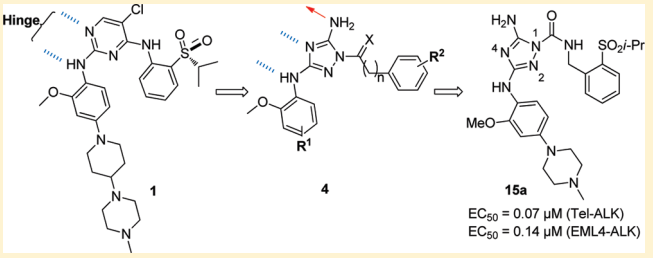
Supporting Information

ABSTRACT: A series of novel 3,5-diamino-1,2,4-triazole benzyl ureas was identified as having potent anaplastic lymphoma kinase (ALK) inhibition exemplified by **15a**, **20a**, and **23a**, which exhibited antiproliferative IC_{50} values of 70, 40, and 20 nM in Tel-ALK transformed Ba/F3 cells, respectively. Moreover, **15a** and **23a** potently inhibited the growth and survival of NPM-ALK positive anaplastic large cell lymphoma cell (SU-DHL-1) and neuroblastoma cell lines (KELLY, SH-SY5Y) containing the F1174L ALK mutation. These compounds provide novel leads for the development of small-molecule ALK inhibitors for cancer therapy.



KEYWORDS: ALK, 3,5-diamino-1,2,4-triazole urea

Anaplastic lymphoma kinase (ALK) was first identified as part of the nucleophosmin (NPM)-ALK fusion protein derived from a chromosomal translocation detected in the majority (60%) of anaplastic large cell lymphoma (ALCL) patients.[1−3] Echinoderm microtubule-associated protein like 4 (EML4) was discovered as a novel fusion partner with ALK in approximately 5% of patients with nonsmall-cell lung cancer (NSCLC).[4,5] Chromosomal translocations involving the *ALK* gene at 2p23 with numerous partner genes result in constitutive activation of the kinase domain and in an "oncogene-addicted" state in several tumors, including inflammatory myofibroblastic tumors (IMT),[6,7] diffuse large B cell lymphoma (DLBCL),[8] and squamous cell carcinoma. Recently, it has also been discovered that germline mutations in ALK are the cause of the majority of hereditary neuroblastoma cases and that ALK activation by mutation and/or gene amplification is functionally relevant in high-risk sporadic neuroblastoma.[9,10] Pharmacological studies using the potent ALK inhibitor, 5-chloro-N^4-(2-(isopropylsulfonyl)phenyl)-N^2-(2-methoxy-4-(4-(4-methylpiperazin-1-yl)-piperidin-1-yl)phenyl)pyrimidine-2,4-diamine (**1**, TAE684), have provided preclinical validation for targeting ALK kinase activity for the treatment of NPM-ALK, EML4-ALK, and point mutation driven ALK-dependent tumors.[10−13] Altogether, these findings suggest that development of small-molecule ALK inhibitors would provide efficacious therapeutics for ALK-driven hematological malignancies and solid tumors.[2]

Currently, no small-molecule ALK inhibitor is approved for clinical cancer therapy; however, a dual c-Met/ALK inhibitor [(*R*)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)-5-(1-(piperidin-4-yl)-1*H*-pyrazol-4-yl)pyridin-2-amine, **2**, PF-2341066] is currently being investigated in a phase II/III clinical trial in ALCL, NSCLC, and neuroblastoma.[14] To date, clinical activity has been observed in EML4-ALK NSCLC and ALK-translocated IMT.[15,16] As compound **2** was originally developed as a c-Met inhibitor, its cellular potency against ALK is only moderate (IC_{50} ∼ 200 nM), and several resistance mutations have recently been reported.[17,18] Therefore, the development of potent and selective inhibitors of wild-type and mutant ALK for treating ALK-positive cancers is urgently needed. In this letter, we report the design and synthesis of 3,5-diamino-1,2,4-triazole benzyl ureas as potent adenosine triphosphate (ATP)-competitive ALK inhibitors. Cell-based structure−activity relationship (SAR) studies guided the discovery of **15a**, **20a**, and **23a**, which exhibit potent inhibitory activity in ALK-transformed Ba/F3 cells, NPM-ALK-positive ALCL cells, and ALK-mutated neuroblastoma cells.

Recently, two independent groups reported the crystal structure of the ALK kinase domain in complex with ATP competitive

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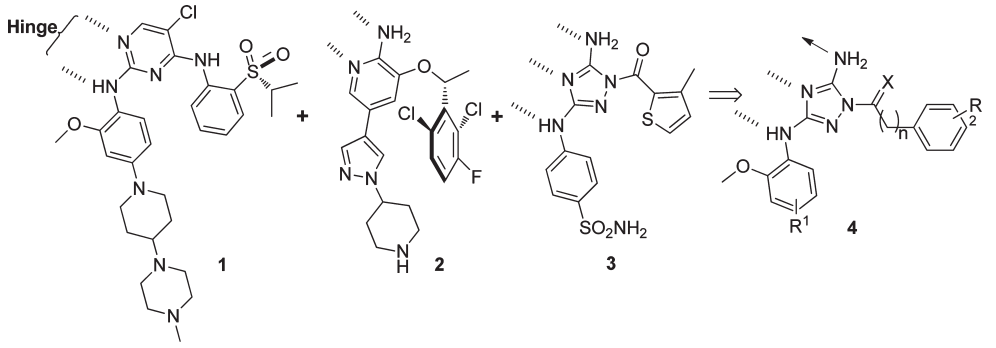
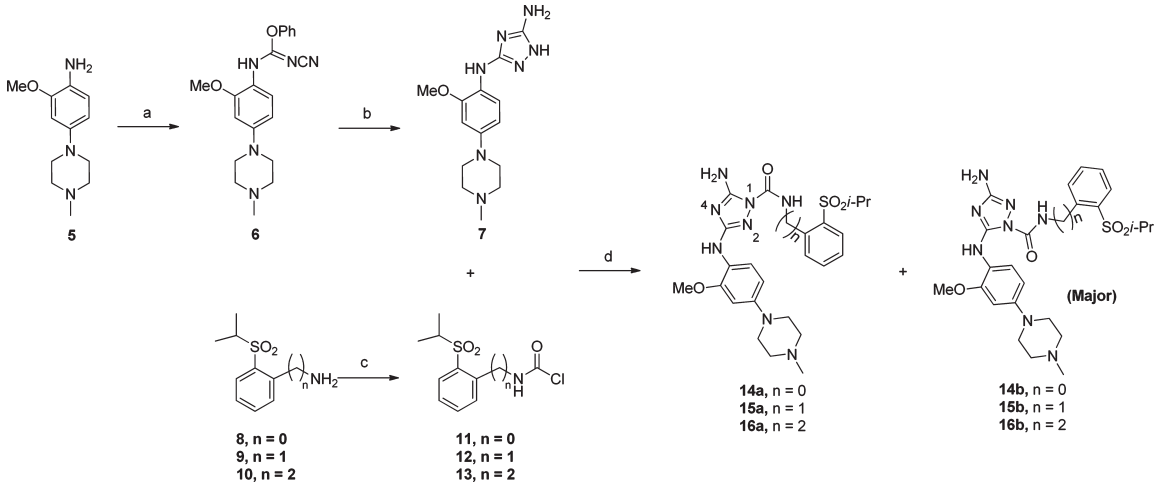


Figure 1. Scaffold design strategy.

Scheme 1[a]



[a] Reagents and conditions: (a) Diphenyl cyanocarbonimidate, THF, reflux. (b) Hydrazine, THF, 0−75 °C. (c) Triphosgene, dioxane, microwave, 150 °C, 20 min. (d) Pyridine/DMF, room temperature.

inhibitors.[19,20] To date, small-molecule ALK inhibitors have been described from the aminopyridine, pyridone, indolocarbazole, dianilinopyrimidine, acylamino-indazole, and *1H*-pyrrolo[2,3-*b*]-pyrazine classes.[2,14,21] To design a new class of ALK inhibitor, we explored 3,5-diamino-1,2,4-triazole ureas, which can be viewed as a molecular amalgam of the 2,4-dianilinopyrimidines, such as **1**,[11] and 1-acyl-1*H*-[1,2,4]-triazole-3,5-diamine **3**[22] (Figure 1). Compound **1** is a highly potent inhibitor of NPM-ALK-Ba/F3 cell proliferation (IC_{50} = 3 nM). Originally, modeling studies and subsequently cocrystal structures (PDB code: 2XB7) demonstrated that **1** occupies the ATP-binding site and uses the aminopyrimidine motif to form two hydrogen bonds to the ALK "hinge" segment.[11] Compound **3** was discovered as a potent cyclin-dependent kinase 1 (CDK1) inhibitor with an IC_{50} of 4.8 nM, but we hypothesized that it might exhibit affinity to ALK due to reported modest potency (IC_{50} = 2.4 μM) against the highly homologous insulin receptor kinase (InsR).[22] The chemotype **4** was designed as a hybrid of aminopyrimidine **1**, aminopyridine **2**, and 1,2,4-triazole-3,5-diamine **3**. The 1,2,4-triazole-3,5-diamine was used as the core scaffold with the potential for forming three hydrogen bonds with the hinge segment. The acyl appendage of **4** was intended to be capable of reaching either toward the front analogous to the isopropyl phenyl sulfone of **1** or toward the back of the ATP binding pocket analogous to the dichlorophenyl moiety of **2**.

To validate our design strategy, a small set of 3,5-diamino-1,2,4-triazole urea analogues representing the chemotype **4** was synthesized using a concise four-step synthetic route (Scheme 1). The ortho methoxyaniline was reacted with diphenylcyanocarbonimidate, and the resulting intermediate was then cyclized by reacting with hydrazine to give the corresponding triazole **7**. This triazole was then acylated with the substituted benzylcarbamic chlorides to yield one major regioisomer and one minor isomer. The structures of these two regioisomeric products were assigned based on literature,[22] NMR spectroscopy, and the X-ray crystallographic analysis of a representative analogue **29** (Table 3). In contrast to literature reports,[22] the major regioisomer turned out to be the 2-acylated isomer for the majority of analogues that are reported below.

The compounds **14**−**16** were tested against Tel-ALK-Ba/F3, EML4-ALK-Ba/F3, and parental Ba/F3 cell lines. We were surprised by the complete lack of ALK inhibitory activity of the 1,2,4-triazole aniline urea exemplified by compounds **14a** and **14b**. On the basis of molecular modeling and the potential for conformation restricting intramolecular hydrogen bonds, we had anticipated that these compounds would possess some level of ALK inhibition. However, introducing an additional one-carbon spacer into the aniline urea side chain resulted in 1-acylated regioisomer **15a**, which possessed IC_{50} values of 70 and 140 nM against Tel-ALK-Ba/F3 and EML4-ALK-Ba/F3 cell, respectively,

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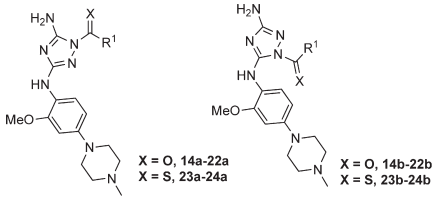
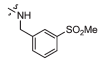
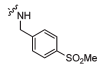
Table 1. SAR of 1-Acyl Moiety for ALK

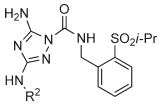


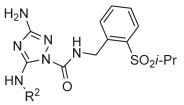
Structure (R^1)	Compound ID	Tel-Alk[a]	EML4-ALK[a]	Ba/F3[a]
	**14a**	8.2	ND[b]	>10
	14b	>10	ND[b]	>10
	**15a**	0.07	0.14	>10
	15b	7.3	4.6	>10
	**16a**	0.45	0.80	>10
	16b	1.9	3.1	>10
	**17a**	ND[b]	0.61	>10
	17b	ND[b]	1.4	>10
	**18a**	7.6	>10	>10
	18b	>10	>10	>10
	**19a**	>10	>10	>10
	19b	>10	>10	>10
	**20a**	0.04	0.26	3.5
	20b	0.48	3.26	>10
	**21a**	0.76	>10	>10
	21b	6.9	2.0	1.9
	**22a**	7.37	>10	>10
	22b	>10	>10	>10
	**23a**	0.02	0.03	2.9
	23b	0.02	0.68	0.76
	**24a**	0.01	0.08	1.03
	24b	2.3	0.81	2.6

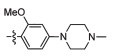
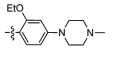
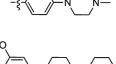
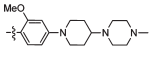
[a] Antiproliferative activity (IC_{50}, μM) on Tel-Alk-Ba/F3, EML4-Alk-Ba/F3, and parental Ba/F3, respectively; values are means of two experiments, and the standard deviation is less than 10% of means. [b] Not determined.

and was not cytotoxic to parental Ba/F3 cell (IC_{50} > 10 μM). Compound **16a**, possessing a two-carbon spacer in the urea side chain, displayed 6-fold decreased Tel-ALK potency (IC_{50} = 450 nM). Both of the 2-acylated regio-isomers **15b** and **16b** were much less potent with IC_{50} values of 7300 and 1900 nM, respectively. These results indicated that proper length of the spacer and position of acylation are the key determinants to achieve potent ALK inhibitory activity. We reasoned that the benzyl amine urea side chain acylated at the 1-position perhaps

Table 2. SAR of Substitution on 3-Amino for ALK





Structure (R^2)	Compound ID	Tel-Alk[a]	EML4-ALK[a]	Ba/F3[a]
	**15a**	0.07	0.14	>10
	15b	7.3	4.6	>10
	**25a**	0.21	0.12	>10
	25b	>10	8.9	>10
	**26a**	0.43	0.67	>10
	26b	>10	8.0	>10
	**27a**	0.22	0.10	9.6
	27b	0.91	0.77	3.7
	**28a**	0.52	0.86	4.4
	28b	>10	4.9	7.1
	**29a**	4.0	>10	>10
	29b	>10	>10	>10

[a] Antiproliferative activity (IC_{50}, μM) on Tel-Alk-Ba/F3, EML4-Alk-Ba/F3, and parental Ba/F3 respectively; values are means of two experiments, and the standard deviation is less than 10% of means.

provided the best fit to the hydrophobic back pocket when the central heterocyclic ring is reduced from six (as in compounds **1** and **2**) to five (as in compound **4**).

Encouraged by this result, a series of 3,5-diamino-1,2,4-triazole ureas were synthesized and tested for antiproliferative potency in Tel-ALK-Ba/F3 and EML4-ALK-Ba/F3 cells. We first investigated the acyl moiety of this scaffold by substituting the methylsulfonyl group at different positions (ortho, meta, and para) of the benzyl ring, resulting in compounds **17a**−**19a** and **17b**−**19b**. Whereas **17a** maintained moderate potency against EML4-ALK-Ba/F3 cell (IC_{50} = 0.61 μM), the others lost ALK inhibitory potency substantially (Table 1). This suggested that ortho substitution of the benzyl ring is critical for achieving potent ALK inhibition. When 2-isopropylsulfonylbenzyl amine was replaced with 2,6-dichlorobenzyl amine, the resulting compound **20a** exhibited improved potency with an IC_{50} of 40 nM against Tel-ALK transformed Ba/F3 cells, but it also inhibited parental Ba/F3 cells with an IC_{50} of 3.5 μM, suggesting that additional targets were being engaged that contributed to nonspecific cytotoxicity. Interestingly, the 2-acylated product **20b** also displayed inhibitory activity (IC_{50} = 480 nM) albeit with 10-fold reduced potency. Compound **21a** possessing a 2,6-dichloro-3-fluoro-methylbenzyl amine side chain adopted from compound **1**[23] exhibited approximately 10-fold decreased Tel-ALK potency. Replacement of the benzyl urea NH with an N-methyl substituent (**22a**) resulted in a sharp decrease in cellular potency (IC_{50} = 7.4 μM), suggesting that this group may be responsible for a critical contact with the enzyme. The corresponding thio urea compounds (**23a**, **23b**, and **24a**) exhibited potent ALK inhibitory activity, but both **23b** and **24a** also possessed potent nonspecific cytotoxicity toward parental Ba/F3 cells. As discussed further below, this may be related to the thio ureas exhibiting a significantly broadened kinase selectivity profile.

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Next, we investigated the consequence of varying the aniline side chain at the 3-position of the 1,2,4-triazole (Table 2). Here, we discovered that 2-alkyloxy substituent on the aniline aromatic ring served as a handle for controlling kinase selectivity as reported for **1**[11] and (*R*)-4-((8-cyclopentyl-7-ethyl-5-methyl-6-oxo-5,6,7,8-tetrahydropteridin-2-yl)amino)-3-methoxy-*N*-(1-methylpiperidin-4-yl)benzamide (BI-2536)[24] (see the kinase selectivity discussion). Progressing from 2-methoxy (**15a**) to 2-ethoxy (**25a**) to 2-isoproxy (**26a**) resulted in a gradual decrease in cellular Tel-ALK potency. Using the aniline tail adopted from **1**, compound **27a** displayed slightly decreased Tel-ALK potency (IC_{50} = 220 nM). Replacement with 4-methoxycarbonyl-2-methoxy aniline and 4-bromo-2-methoxy aniline resulted in compounds **28a** and **29a** with IC_{50} values of 520 and 4000 nM, respectively. This suggested that the 4-*N*-methyl piperazine functional group is important for achieving cellular potency. Again, only the 1-acylated regioisomers exhibited cellular activity, and most of the 2-acylated regioisomers (**15b**, **25b**, **26b**, **28b**, and **29b**) were inactive except for **27b**, which possessed an IC_{50} of 1 μM. To corroborate the structure assignment, we successfully crystallized both isomers of **29a** and **29b**, which possess a heavy bromine atom. Structural assignment for the other compounds was made by comparing the ^{1}H NMR signals of protons of the 3-amino (NH) and 5-amino (NH_2) groups to the corresponding protons of **29**.

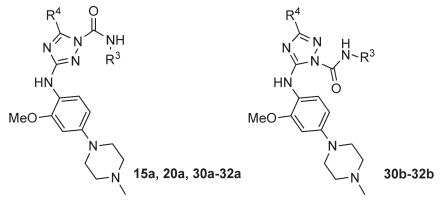
The function of the 5-amino group (NH_2) was also investigated in the context of the 2-(isopropylsulfonyl)benzyl and 2,6-dichlorobenzyl compound series (Table 3). Because of the difficulty of isomer separation, the 5-*N*-isopropyl analogues (**30** and **31**) and des-amino analogues (**32**) were tested as a mixture of both isomers. All of the compounds displayed dramatically reduced potency, suggesting that the 5-amino group may make an additional hydrogen bond to the kinase hinge.

The SAR exploration of 3,5-diamino-1,2,4-triazole urea scaffold revealed that the one carbon spacer of the urea side chain (n = 1), 1-acyl substitution, and the 2-methoxy group of the aniline side chain with *N*-methylpyparazine were key structural features required to achieve potent cellular activity against Tel-ALK and EML4-ALK. To better understand the structure feature effect, we performed a molecular modeling study using Glide[25] based upon the recently reported cocrystal structure of ALK with **1** (PDB code: 2XB7)[20] (please see the Supporting Information for a detailed modeling study).

To evaluate the inhibitory potency of these new ALK kinase inhibitors against different ALK fusion and mutant ALK kinases, the most potent compounds (**15a**, **20a**, and **23a**), as well as **1** and **2**, were tested against a panel of cell lines including NSCLC-related cell lines[5] (EML4-ALK-Ba/F3, EML4-ALK (F1174L)-Ba/F3, and EML4-ALK (L1196M)-Ba/F3), a NPM-ALK positive ALCL cell line (SU-DHL-1),[26] and neuroblastoma cell lines [KELLY (F1174L), SH-SY5Y (F1174L), and SMS-KCN (R1275Q)] (Table 4). These selected cell lines were sensitive to the growth inhibitory activity of **15a**, **20a**, and **23a** but to different extents. This likely reflects a combination of kinase selectivity profiles of these compounds and the degree of addiction to ALK kinase potency in these different cells. Compounds **15a** and **23a** possessed submicromolar IC_{50} values across the entire panel of cell lines with the exception of SMS-KCN (R1275Q), which was resistant to compound **1**.

With the potent antiproliferative activities of these new ALK inhibitors in hand, we assessed the selectivity of this scaffold using the KINOME*scan* methodology across a panel of 402 kinases (Ambit Biosciences, San Diego, CA).[27] Five compounds,

Table 3. SAR of Substitution on 5-Amino for ALK



Structure R^3	R^4	Compound ID	a/b ratio	Tel-Alk[a]	EML4-ALK[a]	Ba/F3[a]
	NH_2	**15a**	-	0.07	0.14	>10
		**30**	1/8	>10	3.3	2.8
	NH_2	**20a**	-	0.04	0.26	3.5
		**31**	5/9	2.5	10.0	>10
	H	**32**	1/5	3.0	10.0	>10

[a] Antiproliferative activity (IC_{50}, μM) on Tel-Alk-Ba/F3, EML4-Alk-Ba/F3, and parental Ba/F3 respectively; values are means of two experiments, and the standard deviation is less than 10% of means.

Table 4. Antiproliferative Activity of Selected Compounds against a Diverse Panel of ALK-Positive Cell Lines

		IC_{50} (μM)[a]				
cell line	histology	**15a**	**20a**	**23a**	**1**	**2**
Tel-ALK-Ba/F3		0.07	0.04	0.02	0.001	0.19
EML4-ALK-Ba/F3	NSCLC	0.14	0.26	0.03	0.02	0.28
EML4-ALK (F1174L)-Ba/F3	NSCLC	0.72	2.1	0.29	0.06	0.62
EML4-ALK (L1196M)-Ba/F3	NSCLC	0.62	2.3	0.11	0.08	2.2
Kelly (F1174L)	neuroblastoma	0.18	0.25	0.07	0.38	0.42
SH-SY5Y (F1174L)	neuroblastoma	0.68	2.0	0.23	0.16	0.53
SMS-KCN (R1275Q)	neuroblastoma	3.8	4.0	1.3	0.52	0.91
SU-DHL-1 (NPM-ALK)	ALCL	0.01	0.08	0.001	ND[b]	0.01

[a] The data are expressed as the required compound concentration for inhibiting cell growth at 50%; values are means of two experiments, and the standard deviation is less than 10% of means. [b] Not determined.

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15a, **20a**, **24a**, **25a**, and **26a**, were screened at a concentration of 10 μM, which revealed a significant number of potential kinase targets for this inhibitor class (please see the Supporting Information Ambit profiling data for details). Compound **20a** has slightly better potency than compound **15a**, but **20a** exhibits less selectivity with the KINOME*scan* selectivity score S_{10} of 0.31 (123/402) as compared to **15a** with the S_{10} of 0.21. Similarly, as compared to **20a**, the thio urea **24a** has better potency against ALK but also possesses dramatically decreased selectivity with the S_{10} of 0.62, which could be the reason for its cytotoxicity to parental Ba/F3 cells. The 2-alkyloxy substituent on the aromatic ring of 3-aniline side chain serves as the selectivity handle evidenced by the S_{10} of **15a**, **25a**, and **26a**, which are 0.21, 0.13, and 0.06, respectively. This is consistent with the finding that the ortho methoxy group attached to the 2-aniline substituent in **1** offering its selectivity of ALK over other tested kinases.[11] For comparison, the 3,5-diamino-1,2,4-triazole urea scaffold possesses overall improved selectivity when compared with the 2,4-dianilinopyrimidine scaffold exemplified by **1** [S_{10} = 0.66 (231/353)].

In conclusion, **15a**, **20a**, and **23a** represent a new chemotype capable of potent ALK inhibition. The strong inhibitory effects across a panel of clinical relevant cell lines with ALK mutation were observed, suggesting the potential of this chemical series for ultimately developing drugs for the treatment of diseases including NSCLC, ALCL, and neuroblastoma. Despite the relatively large number of kinases that can be potently targeted by this scaffold, compounds like **15a**, **20a**, and **23a** are not general cytotoxic agents as evidenced by lack of cytotoxicity toward parental Ba/F3 cells. Several challenges must be overcome to further develop this chemical series including kinase selectivity, chemical stability of the acyl triazole linkage, and synthetic methods to produce the desired regioisomer.

■ ASSOCIATED CONTENT

Supporting Information. Procedures and characterization data for all compounds, procedures for cellular assay, crystal structures of **29a** and **29b**, and kinase selectivity profiling data for **1**, **15a**, **20a**, **24a**, **25a**, and **26a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

⊥These authors contributed equally.

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■ ABBREVIATIONS

ALK, anaplastic lymphoma kinase; ALCL, anaplastic large cell lymphoma; ATP, adenosine triphosphate; CDK1, cyclin-dependent kinase 1; DLBCL, diffuse large B cell lymphoma; EML4, echinoderm microtubule-associated protein-like 4; IMT, inflammatory myofibroblastic tumors; InsR, insulin receptor kinase; NSCLC, nonsmall cell lung cancer.

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