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LETTER

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Discovery of a benzo[e]pyrimido-[5,4-b][1,4]diazepin-6(11H)-one as a Potent and Selective Inhibitor of Big MAP Kinase 1

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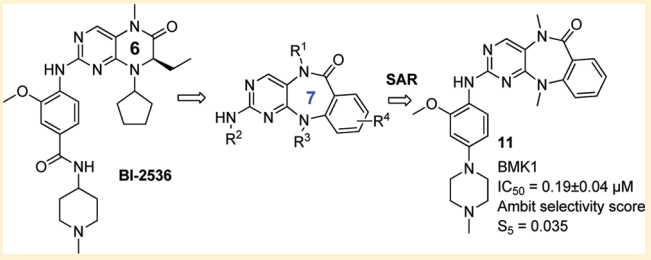
Supporting Information

ABSTRACT: Kinome-wide selectivity profiling of a collection of 2-amino-pyrido[2,3-d]pyrimidines followed by cellular structure−activity relationship-guided optimization resulted in the identification of moderately potent and selective inhibitors of BMK1/ERK5 exemplified by **11**, **18**, and **21**. For example, **11** possesses a dissociation constant (K_d) for BMK1 of 19 nM, a cellular IC_{50} for inhibiting epidermal growth factor induced BMK1 autophosphorylation of 0.19 ± 0.04 μM, and an Ambit KINOME*scan* selectivity score (S_5) of 0.035. Inhibitors **18** and **21** are also potent BMK1 inhibitors and possess favorable pharmacokinetic properties which enable their use as pharmacological probes of BMK1-dependent phenomena as well as starting points for further optimization efforts.



KEYWORDS: MAPK, BMK1, ERK5

The mitogen-activated protein kinases (MAPKs) are crucial components of signaling cascades that regulate numerous physiological processes.[1,2] Four MAPK pathways have been identified thus far, including extracelluar-signal-regulated kinase 1/2 (ERK1/2), c-Jun-amino-terminal kinase (JNK), p38, and BMK1.[2] As a new member of the MAPKs, BMK1 is activated by a wide variety of mitogens and stress stimuli.[3−6] A recently reported link between abnormal levels of BMK1 expression and cancer has greatly increased interest in this signaling pathway.[7] Deregulation of the BMK1 pathway has been shown to be associated with various malignant properties of human cancers including increased metastatic potential of prostate cancer cells,[8] sustained malignant growth of ErbB2 overexpressing mammary carcinomas,[9] and chemoresistance of breast cancer cells.[10] Since BMK1 was cloned in 1995, only two dual inhibitors of BMK1 and its upstream kinase MEK5, ((Z)-3-(((3-((dimethylamino)-methyl)-phenyl)amino)-(phenyl)methylene)-2-oxoindoline-6-carboxamide (BIX02188) and (Z)-3-(((3-((dimethylamino)-methyl)phenyl)amino)(phenyl)-methylene)-*N*,*N*-dimethyl-2-oxoindoline-6-carboxamide (BIX02189)), have been reported (Figure 1).[11] Therefore, the development of potent and selective small molecule BMK1 inhibitors for use in investigating and validating the BMK1 pathway as a target are urgently needed.

In this Letter, we report the design and synthesis of novel compounds to explore the BMK1 structure−activity relationship (SAR) using a 2-amino pyrido[2,3-d]pyrimidine template. Iterative rounds of medicinal chemistry and biological evaluation of these compounds led to the discovery of **11**, **18**, and **21** as potent and selective BMK1 inhibitors.

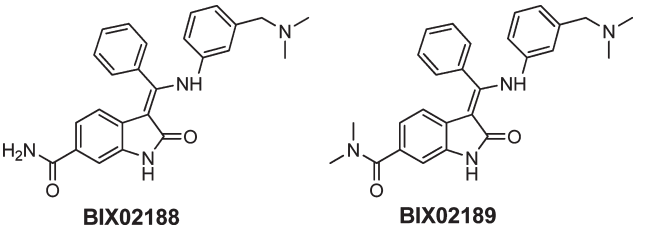


Figure 1. Dual inhibitors of BMK1 and MEK5.

Efforts to identify inhibitors of a new kinase target often begin with a screen of archived inhibitors that were developed for previous kinase projects.[12] We initiated our chemistry effort by utilizing a small library of compounds containing a pyrido[2,3-d]pyrimidine template. The pyrido[2,3-d]pyrimidine core can be classified as a privileged scaffold with respect to targeting the ATP-site of kinases.

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Examples of kinase inhibitors containing this core scaffold include BI-2536, a selective polo-like kinase family (PLK1, PLK2, and PLK3) inhibitor[13,14] and 2-((3,5-difluoro-4-hydroxyphenyl)-amino)-8-isopentyl-5,7-dimethyl-7,8-dihydropteridin-6(5H)-one (BI-D1870), an inhibitor of RSK.[15]

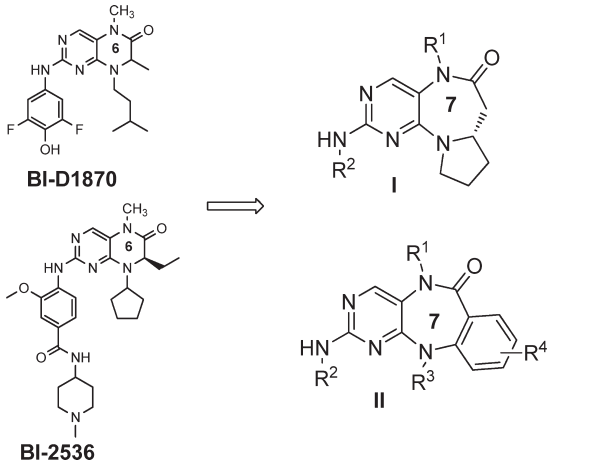
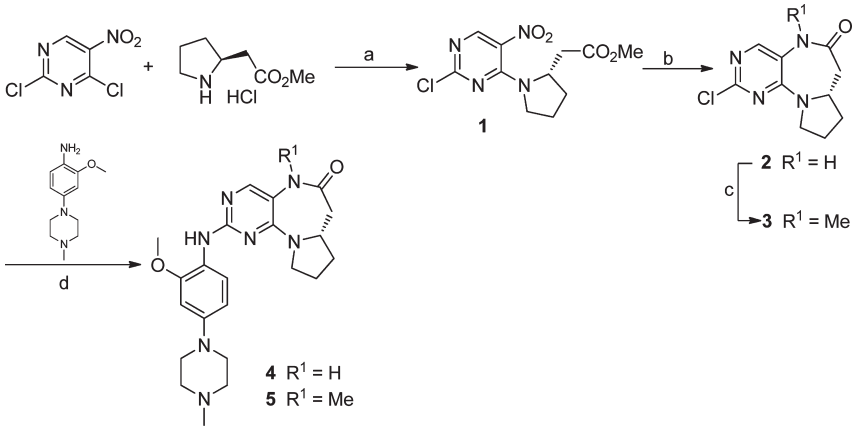


Figure 2. Pyrido[2,3-d]pyrimidine scaffold expansion.

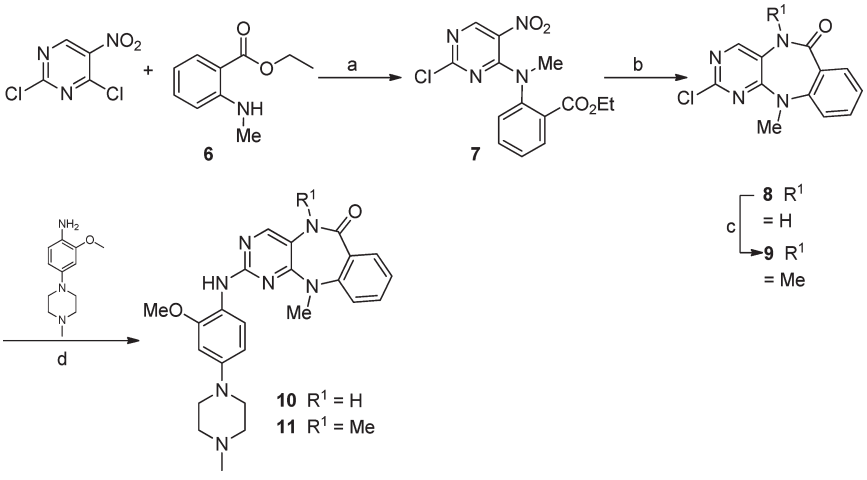
Using information gleaned from these examples,[13−15] we designed the potential kinase inhibitor chemotypes **I** and **II** by expanding the 6-membered pyridone ring of BI-2536 to a 7-membered lactam ring (Figure 2). An efficient four-step synthetic route was developed to enable the synthesis of several compounds (**4**, **5**, **10**, and **11**) derived from scaffolds **I** and **II**. The synthesis of **4** and **5** is outlined in Scheme 1. First, 2,4-dichloro-5-nitropyrimidine was reacted with (*S*)-methyl 2-(pyrrolidin-2-yl)acetate hydrochloride using diisopropylethyl amine as base at room temperature to give the amination product **1** in moderate yield. This reaction was followed by iron-mediated reduction of **1** and in situ cyclization in acetic acid at 50 °C to afford the 7-member lactam intermediate **2** in good yield. Compound **5** was obtained via methylation of **2** and followed by palladium mediated amination of **3** with 2-methoxy-4-(4-methylpiperazin-1-yl)-benzenamine. Similarly, compound **4** was synthesized via palladium mediated amination of intermediate **2**.

Scheme 1. Synthesis of 2-Amino-7a,8,9,10-tetrahydro-5H-pyrimido[5,4-b]pyrrolo [1,2-d][1,4]diazepin-6(7H)-one 4[a]



[a] Reagents and conditions: (a) DIEA, 2-PrOH, RT; (b) Fe/HOAc, 50 °C; (c) MeI/NaH, DMA, 0 °C; (d) X-Phos (9% mol), $Pd_2(dba)_3$ (6% mol), K_2CO_3 (3.0 equiv), *t*-BuOH, 100 °C.

Scheme 2. Synthesis of 2-Amino-5,11-dimethyl-5H-benzo[e]pyrimido[5,4-b][1,4]diazepin-6(11H)-one[a]



[a] Reagents and conditions: (a) DIEA, dioxane, 50 °C; (b) Fe/HOAc, 50 °C; (c) MeI/NaH, DMA, 0 °C; (d) X-Phos (9% mol), $Pd_2(dba)_3$ (6% mol), K_2CO_3 (3.0 equiv), *t*-BuOH, 100 °C.

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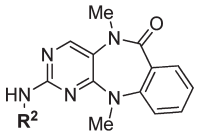
Table 1. Kinase Hits for 10 and 11

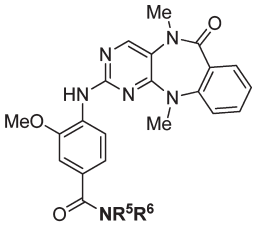
compd ID	kinase hits[a]
10	ABL1(F317I), BRK, C-FMS, DCAMKL1, DCAMKL2, **ERK5**, FRK, GAK, KIT(L576P), MYLK4, TNK1, TNK2
11	DCAMKL1, DCAMKL2, **ERK5**, FRK, MNK2, PKD1, PKD2, RSK4∼b, TAO1, TAO3, TNK1, TNK2, ULK1, ULK2

[a] Kinases that exhibited greater than 95% displacement from an immobilized ligand by the tested compounds. Kinase designations are those used by Ambit; Erk5 is another name for BMK1. RSK4∼b stands for RPS6KA6(Kin.Dom.2-C-terminal).

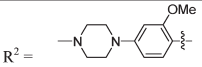
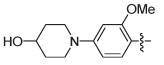
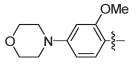
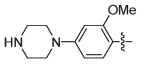
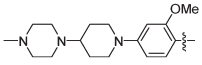
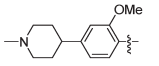
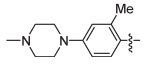
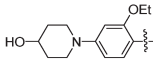
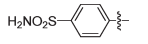
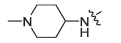
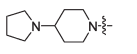
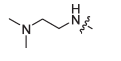
Table 2. SAR of 2-Amino Moiety for BMK1



11-19



20-22

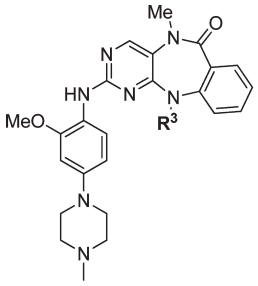
Compound ID	Structure	Inhibition of BMK1 activity (%)[a]	IC_{50} (μM)[b]
11	R^2 = 	82	0.19±0.04
12		73	0.24±0.04
13		65	0.26±0.03
14		75	0.23±0.04
15		80	0.20±0.03
16		67	0.26±0.04
17		60	0.28±0.04
18		76	0.24±0.04
19		<50	0.31±0.06
20	R^5R^6N = 	<50	0.32±0.05
21		92	0.13±0.03
22		74	0.24±0.03

[a] Compounds were tested at a concentration of 0.5 μM. The data are expressed as inhibition of kinase activity in the presence of the inhibitors relative to DMSO control. [b] IC_{50}s were calculated using densitometry of phospho-BMK1 separatated by BMK1 by SDS gel electrophoresis using 6-point titration curves ± standard error of mean.

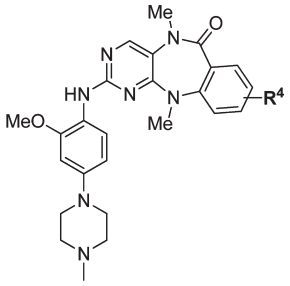
Similarly, **10** and **11** were synthesized by using *N*-methyl anthranilic ethyl ester **6** as starting material with higher yields (Scheme 2).

These four compounds were screened against a diverse panel of 402 kinases (Ambit KINOME*scan*) using an in vitro ATP-site competition binding assay at a concentration of 10 μM.[16,17] Given the generic nature of the pyrimidine-derived hinge interacting motif, we were surprised to discover that these compounds exhibited highly selective profiles (see the Supporting Information for complete profiling data). Kinases that exhibited greater than 95% displacement from an immobilized ligand by compound **10** and **11** are listed in Table 1.

Table 3. SAR of Substituents of Anthranilic Acid Moiety for BMK1



23-25



26-29

Compound ID	Structure	Inhibition of BMK1 activity (%)[a]	IC_{50} (μM)[b]
11	R^3 = 	82	0.19±0.04
23		79	0.20±0.04
24		77	0.23±0.03
25		79	0.20±0.04
26	R^4 = 5-Me	<50	3.10±0.62
27	4-F	<50	1.32±0.22
28	5-Cl	<50	1.24±0.24
29	4-Cl	<50	11.38±2.04

[a] Compounds were tested at a concentration of 0.5 μM. The data are expressed as inhibition of kinase activity in the presence of the inhibitors relative to DMSO control. [b] IC_{50}s were calculated using densitometry of phospho-BMK1 separatated by BMK1 by SDS gel electrophoresis using 6-point titration curves ± standard error of mean.

Because of our interest in the BMK1 signaling pathway, we were drawn to the strong inhibition effect of **10** and **11** against BMK1 with ambit scores of 0.5 and 0, respectively.[17] Measurement of dissociation constants revealed that compound **11** exhibited much better affinity toward BMK1 with a measured dissociation constant (K_d) of 19 nM, while **10** possessed a K_d of 670 nM. The other kinase targets potently inhibited by **11** were DCAMKL1 (K_d = 1.2 nM), DCAMKL2 (K_d = 10 nM), GAK (K_d = 450 nM), TNK1 (K_d = 29 nM), and TNK2 (K_d = 440 nM). The KINOME*scan* selectivity score (S_5) calculated at a threshold of 5% of the dimethyl sulfoxide (DMSO) control for **11** is 0.035 (14 out of 402 kinases) is comparable to that calculated for BI-2356 (S_5 = 0.025, 9 out of 353) which represents a high level of selectivity.[18] Next, we examined the inhibitory activity against cellular BMK1 by measuring the ability of the

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compounds to inhibit epidermal growth factor (EGF)-stimulated autophosphorylation of BMK1 in HeLa cells. The cellular data demonstrated that **11** (but not **10**) significantly inhibited BMK1 autophosphorylation at a concentration of 1 μM. These cellular results are consistent with the ambit biochemical data, which reveals that **11** is a promising lead having the ability to inhibit BMK1 both in vitro and in cells.

Using **11** as a lead, the SAR for the 2-amino-5,11-dimethyl-5H-benzo[e]pyrimido[5,4-b][1,4]diazepin-6(11H)-ones was explored, utilizing inhibition of BMK1 autophosphorylation in HeLa cells as a readout. HeLa cells were serum starved overnight followed by treatment with inhibitors for 1 h. Cells were then stimulated with epidermal growth factor (EGF, 20 ng/mL) for 17 min, and BMK1 activation was detected by mobility retardation.[19] We first explored the 2-position of this scaffold by replacing the aniline substituent of **11** with different 2-methoxy-4-substituent anilines (Table 2). Introduction of 4-hydroxyl-piperidin-1-yl, piperazin-1-yl, and 4-(4-methylpiperazin-1-yl)piperidin-1-yl groups at the 4-position of the 2-methoxy aniline resulted in compounds **12**, **14**, and **15**, respectively. These modifications all yielded compounds possessing similar activities (Table 2). Compounds **13** and **16** containing substitutents with morpholino and 1-methylpiperidin-4-yl groups, respectively, exhibited slight decreases in activity. Substitution of the 2-methoxy group (**11**) with a methyl group (**17**) led to a sharp decrease in activity, while the 2-ethoxy substituted compound **18** maintained similar activity. These results indicated that the 2-alkyloxy group contributes significantly to the overall potency of this series of analogues. When 2-methoxy-4-(4-methylpiperazin-1-yl)-aniline was replaced with 4-sulfonamideaniline, the resulting compound (**19**) was less effective. Substitution with various amides at the 4-position resulted in the synthesis of compounds **20**−**22** with **21** exhibiting the best activity within this set of compounds.

Table 4. Antiproliferative Activity of 21 against a Diverse Panel of Selected Cancer Cell Lines

cell line	organ	histology	EC_{50} (μM)[a]
PC-3	prostate	adenocarcinoma	3.6
BPH-1	prostate	benign prostate hyperplasia	1.6
MCF7	breast	adenocarcinoma	>10
AU565	breast	adenocarcinoma	>10
SK-N-AS	brain	neuroblastoma	4.4
NCI-H1299	lung:NSCLC	nonsmall cell lung cancer	4.2
NCI-H522	lung:NSCLC	nonsmall cell lung cancer	0.95

[a] The cells were treated with **21** at nine concentrations and incubated for 72 h. The data are expressed as the required compound concentration for inhibiting cell growth at 50%.

We next investigated the effects of modification to the N-substituent ($\mathbf{R^3}$) of the anthranilic acid and to the aryl ring ($\mathbf{R^4}$) (Table 3). N-Substituents ($\mathbf{R^3}$) ranging from methyl, ethyl, isopropyl, to cyclopentyl are exemplified by **11**, **23**, **24**, and **25**, and all maintained similar activity suggesting tolerance for further elaboration at this position. There appears to be limited tolerance for substitution on the aryl ring of anthranilic acid, as the 5-methyl, 4-fluoro, 5-chloro, and 4-chloro analogues (**26**, **27**, **28**, and **29** respectively) all exhibited a dramatic loss in activity. While these analogues might suggest a limited tolerance for further elaboration of the aryl ring, modifications at corresponding positions of other kinase scaffolds has enabled access to an additional hydrophobic binding pocket as a result of a switch to the DFG-out conformation.[20,21] Further investigation will be required to examine whether "type-II" BMK1 inhibitors can be accessed through this approach.

The SAR exploration of the 2-amino-5,11-dimethyl-5H-benzo[e]pyrimido[5,4-b][1,4]diazepin-6(11H)-one scaffold revealed that N-methyl substitution at lactam position ($\mathbf{R^1}$), the 2-methoxy group of 4-substituted aniline, and the absence of substituents ($\mathbf{R^4}$ = H) on the aryl ring of anthranilic acid were key structural features required to achieve potent cellular inhibitory activity against BMK1. The two most active compounds **11** and **21** inhibited EGF induced BMK1 autophosphorylation with submicromolar cellular IC_{50} values of 0.19 ± 0.04 and 0.13 ± 0.03 μM, respectively.

For a preliminary evaluation of the antiproliferative activity of these new BMK1 kinase inhibitors against cancer cell lines, **11** was profiled against a panel of 252 cancer cell lines which represent diverse tumor types (see the Supporting Information for a detailed list). Then the most active compound **21** was tested in the subset of cancer cell lines that exhibited more than 50% of cell growth inhibition at a concentration of 5.0 μM of **11** (Table 4). These selected cell lines were sensitive to the growth inhibitory activity of **21** but to different extents. Most of the selected cell lines exhibited EC_{50} values in the single-digit micromolar range consistent with BMK1 signaling contributing to proliferation of cancer cell lines but more potent compounds are needed to establish what correlation exists between inhibition of BMK1 autophosphorylation and antiproliferative activity.[8−10]

The pharmacokinetic properties of **18** and **21** were also evaluated following intravenous and oral delivery in rats and mice, respectively. These studies demonstrated that **18** and **21** exhibit favorable pharmacokinetic properties with a $T_{1/2}$ of 2.0 and 2.6 h, AUC of 903 and 7871 h·ng/mL, and %F of 68.6 and 86.6, respectively (Table 5).

Inhibitor **18** has been used as a "tool" compound to investigate the BMK1-mediated inhibition of the tumor suppressor activity of the promyelocytic leukemia protein in tumor cells.[22] The in vivo efficacy of **18** was examined in a HeLa cell xenograft model. The tumor-bearing mice were injected intraperitoneally with **18**

Table 5. Pharmacokinetic Parameters of 18 and 21[a]

compd	route	dose (mg/kg)	T_{max} (h)	C_{max} (ng/mL)	$AUC_{0-\infty}$ (hr·ng/mL)	$T_{1/2}$ (hr)	CL (mL/min/kg)	V_{ss} (L/kg)	F (%)
18	IV	1	0.08	439	658	2.0	25.5	3.4	
	PO	2	0.50	247	903	3.4	37.0		68.6
21	IV	1			907	2.62	18.4	5.36	
	PO	10	2.0	1287	7871				86.6

[a] IV = intravenous injection, PO = oral delivery, T_{max} = time of maximum plasma concentration, C_{max} = maximum plasma concentration, AUC = area under the curve (measure of exposure), $T_{1/2}$ = half-life, CL = plasma clearance, V_{ss} = volume of distribution, F = oral bioavailability.

(50 mg/kg) or vehicle solution twice a day for 28 days. The in vivo experimental results showed that inhibition of BMK1 by **18** significantly suppressed tumor growth by 95%, demonstrating the efficacy and tolerability of BMK1-targeted cancer treatment in animals (for a more comprehensive in vivo efficacy study, please see ref 22).

In conclusion, **11**, **18**, and **21** represent a new chemotype exhibiting potent and highly selective BMK1 activities. They were discovered using kinome-wide profiling followed by cellular BMK1-guided SAR study. Given their excellent kinase selectivity, favorable pharmacokinetic parameters, and efficacy in xenograft tumor models, the 2-amino-5,11-disubstituted-5H-benzo[e]pyrimido [5,4-b]-[1,4]diazepin-6(11H)-ones may represent a privileged scaffold for the development of therapeutic agents targeting BMK1.

■ ABBREVIATIONS

BMK1, big MAP kinase 1; DIEA, *N*,*N*-diisopropylethylamine; DMA, *N*,*N*-dimethylacetamide; EGF, epidermal growth factor; ERK1/2, extracelluar-signal-regulated kinase 1/2; ERK5, extracelluar-signal-regulated kinase 5; ErbB-2/HER2, human epidermal growth factor receptor 2; JNK, c-Jun-amino-terminal kinase; MAPK, mitogen-activated protein kinase; MEK5, MAP kinase kinase 5; $Pd_2(dba)_3$, tris(dibenzylideneacetone)dipalladium-(0); PLK, polo-like kinase; RSK, ribosomal S6 kinase; SAR, structure−activity relationship; X-phos, 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl.

■ ASSOCIATED CONTENT

Supporting Information. Procedures and characterization data for all compounds; procedures for biochemical assays and cellular assay, ambit profiling data for **4**, **5**, **10**, **11**, **18**, and BI-2536, and cancer cell line profiling data for **11**. 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. N.S.G., X.D. and T.S. designed the chemistry scaffold. X.D. and N.K. performed the chemical synthesis and characterization. Q.Y., J.-D.L. and N.S.G. designed the biological experimental research. Q.Y. performed the biological experiment and analysis. U.M. and J.E.S. performed the cancer cell lines profiling and analyzed the data. X.D. and N.S.G co-wrote the paper. All authors read and edited the manuscript.

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