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Discovery of novel imidazo[1,2-*a*]pyrazin-8-amines as Brk/PTK6 inhibitors

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ABSTRACT

A series of substituted imidazo[1,2-*a*]pyrazin-8-amines were discovered as novel breast tumor kinase (Brk)/protein tyrosine kinase 6 (PTK6) inhibitors. Tool compounds with low-nanomolar Brk inhibition activity, high selectivity towards other kinases and desirable DMPK properties were achieved to enable the exploration of Brk as an oncology target.

Breast tumor kinase (Brk, also known as PTK6) has been cloned from metastatic breast tumor samples and from cultured human melanocytes.[1,2] Brk is normally expressed in the differentiating epithelial cells of the intestine, skin, prostate, and oral cavity[3–6] where it has been shown to promote cellular differentiation, apoptosis, and more recently to mediate migration/wound healing.[7] In tumors which over express Brk this Src-family, nonreceptor protein–tyrosine kinase has been implicated as a mediator of cancer cell phenotypes, including increased proliferation, survival, and migration.[8] Potential Brk substrates include RNA-binding proteins: Sam68,[9] SLM-1,[10] SLM-2,[11] and PSF[12]; transcription factors: STAT3[13] and STAT5a/b[14]; and a variety of signaling molecules: p190RhoGAP,[15] paxillin,[16] Akt,[17] IRS-4,[18] BKS/STAP-2,[19] and KAP3A.[20]

Studies have shown that Brk interacts with the ErbB family signaling pathway. Early work by Kamalati et al. demonstrated an interaction between Brk and the ErbB family member EGFR and over expression of Brk enhanced EGF-induced proliferation.[21] Additional ErbB family members such as ErbR2, ErbR3, and ErbR4 showed similar interactions with Brk.[22–24] Brk was also found to enhance ERK1/2 activation in ErbB over-expressing cells.[22]

A report from Kamalati et al. showed that over expression of Brk potentially activates the PI3k/Akt pathway.[23] Brk has also been shown to play a role in the activation of STAT signaling pathway.[14,13] Several developing lines of evidence suggest that Brk is involved directly in the processes of migration and invasion that characterize metastatic breast malignancy. For example, Chen et al. reported that Brk is upstream of paxcillin, and CrkII and Rac1 activate migration and invasion programs in skin (A431) and breast (MDA-MB231) cancer cell lines.[15] Lange et al. showed that breast cancer cells are dependent upon Brk expression for their migratory response to EGF and heregulin-β1.[8] It was also reported that the Brk/Rac1/p38 MAPK pathway is required for EGF or heregulin-β1 induced breast cancer cell migration.[25] Recently it was reported that Brk protects breast cancer cells from autophagic cell death induced by loss of anchorage.[26]

All these findings suggested that Brk plays a divergent role as a regulatory proto-oncogene in selected tissues and oncogenic role in malignant settings. The specific cellular mechanisms of the Brk pathway are very important in the rational drug design of cancer therapy. Specific inhibition of Brk kinase activity may provide a potentially novel approach to inhibit the growth of selected tumors, sensitize the response of the tumor cells to other chemotherapeutics and prevent/inhibit metastasis of cancer with enhanced therapeutic windows.

There are no specific Brk/PTK6 inhibitors reported to date. Dasatinib (**3**, Fig. 1), a Src/Abl multi-kinase inhibitor that is approved for chronic myelogenous leukemia (CML), is a potent PTK6/Brk (IC_{50} = 9 nM).[27]

Focused library screening using a HTRF-format biochemical assay[28a] as well as ALIS[29] screening of our internal compound library against the Brk enzyme yielded several classes of compounds, including the imidazo[1,2-*a*]pyrazin-8-amine series (**1**, Fig. 1) described herein. Compound **1** had a moderate inhibitory activity (IC_{50} = 500 nM) to Brk.

The imidazo[1,2-*a*]pyrazine series is known to have strong affinity to Aurora kinases.[30] In addition, Brk itself is highly homologous to other Src family kinases. Therefore our primary objectives

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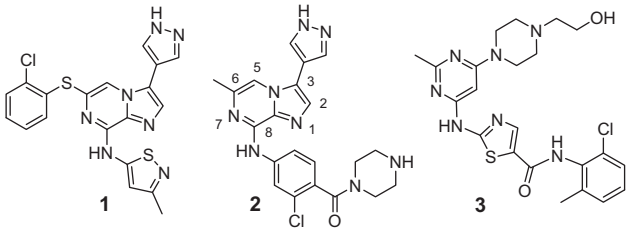


Figure 1. Brk screening hits and Dasatinib (**3**, Sprycel).

were to improve biochemical potency and mechanism-based cellular activity (inhibition of phosphorylation of SAM68) as well as selectivity towards Aurora and Src family kinases (i.e., Lck). An analysis of compounds in our internal file and subsequent follow-up screening resulted in the identification of compound **2** (Fig. 1), which had relatively potent enzymatic activity (Brk IC_{50} = 7 nM), moderate cellular activity (*p*-SAM68 IC_{50} = 156 nM) and some selectivity towards both Aurora B (IC_{50} = 754 nM) and Lck (IC_{50} = 420 nM). In comparison, Dasatinib inhibited Aurora B and Lck with IC_{50} = 6485 and 3 nM, respectively.

A computational model was generated by docking compound **2** into a homology model of BRK (Fig. 2) using SRC as the template and predicted that the series bound to BRK in the ATP pocket. According to this model the pyrazole ring resides inside the enzyme while the C-2 nitrogen and the C-8 NH form hydrogen bonds with the hinge region of Brk and is similar to that of published Aurora A/B inhibitors.[30] The 2-chlorobenzoic amide interacts within the hydrophobic region in front of the ATP binding pocket and extends to the solvent front. A distinct hydrogen bond was also expected between the amide carbonyl group and the side chain of Arg-195. Aurora kinases prefer an amino-isothiazole ring in this region to stabilize the bioactive conformation through a polar interaction between the 7-position core nitrogen and the isothiazole sulfur atom.[30] This difference suggested that the core orientation can be changed through substitutions on the benzene or piperazine ring; or that the ring could be replaced with other heterocycles to improve selectivity toward Aurora kinases.

The general synthetic route for compound **2** and its analogs are shown in Scheme 1. Condensation of 3,5-dibromopyrazin-2-amine and 2-chloroacetaldehyde in *i*-PrOH at reflux afforded the imidazo[1,2-*a*]pyrazine core. An 8-Br moiety was substituted with a methylthiol group to give intermediate **5** (for **2**, R^2 = H, **5a**). Iodization at the C-3 position provided the key intermediate **6** (for **2**, R^2 = H, R^6 = Me **6a**), which facilitated installation of different

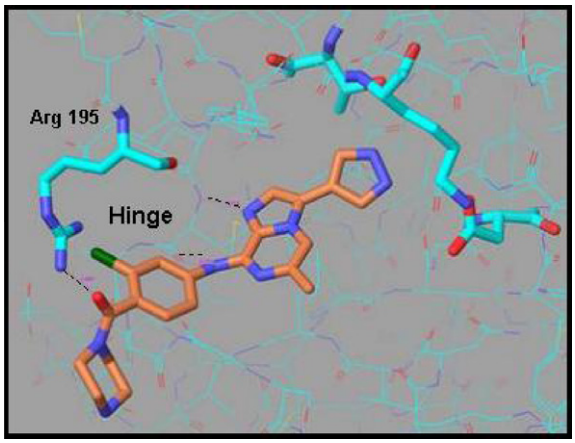
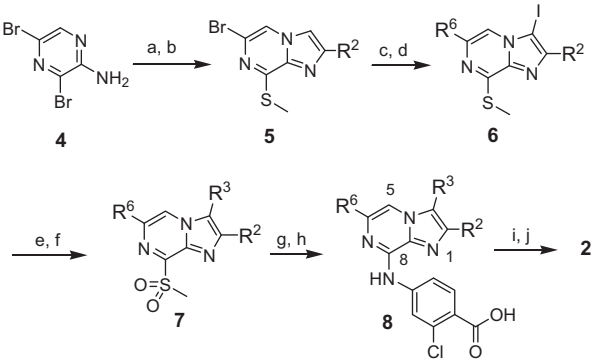


Figure 2. Docking of compound **2** into the homology models of BRK using SRC as the template (2src.pdb).



Scheme 1. Reagents and conditions: (a) 50% chloraldehyde in water, IPA, reflux, 79%; (b) NaSMe, MeOH, rt, 93%; (c) trimethylboroxine, 5 mol % $Pd(Ph_3P)_4$, K_2CO_3, DMF, 100 °C, 54%; (d) *N*-iodosuccinimide, DMF, 60 °C, 97%; (e) SEM-pyrazoloboronic acid pinacol ester, 5 mol % $Pdcl_2(dppf)$, K_3PO_4, 1,4-dioxane/water (9:1), 100 °C, 92%; (f) m-CPMA, DCM, rt, 90%; (g) methyl 4-amino-2-chlorobenzoate, NaH, DMF, rt, 83%; (h) LiOH, THF/water (1:1) rt; (i) Boc-piperazine, HATU, DIEA, DMF; (j) 4 N HCl in dioxane, 40 °C.

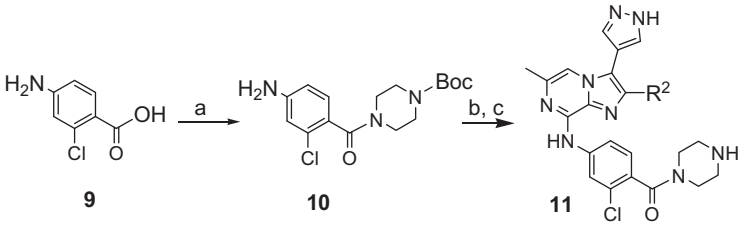
functional groups at C-3 by coupling with readily available boronic acids. The methylthiol group was then converted to methylsulfone by using *m*-CPBA to give key intermediate **7** (for **2**, R^2 = H, R^3 = 3-pyrazol, R^6 = Me, **7a**), which could be replaced with a variety of nucleophilic amines. Reaction of **7a** with methyl 4-amino-2-chlorobenzoate followed by hydrolysis gave key intermediate **8** (for **2**, R^2 = H, R^3 = 3-pyrazol, R^6 = Me, **8a**). Coupling of acid **8** with various amines generated amide-based analogs and global deprotection of acid-labile protecting groups with HCl gave the final product **2**.

Syntheses of the C-2 substituted analogs **11a–d** (Scheme 2.) started with **4** and corresponding 2-chloro-aldehydes followed by subsequent steps to provide the key intermediate sulfones **7** (R^2 = Me, Et, *c*-Pr and *i*-Pr and R^3 = 3-pyrazol, R^6 = Me). Coupling of 2-chloro-4-aminobenzoic acid with 4-Boc-piperazine afforded intermediate **10** and replacement of the sulfone group with **10** followed by global deprotection resulted in compounds **11a–d**.

Early data (Table 1, compounds **11a–d**) revealed that the space at the C-2 position of the imidazo[1,2-*a*]pyrazine core was very limited. Only a hydrogen was tolerated (compound **2**) and all other larger groups caused substantial loss of inhibitory activity toward Brk. In some cases compounds found to be relatively weak in the enzyme assay were not tested further in the cellular assay (denoted as not determined or N.D.).

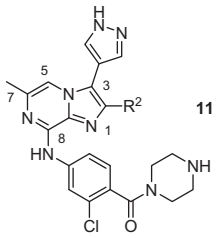
Variation of the C-3 group (Table 2) indicated that the 3-*N*-H-pyrazole was an optimal group for BRK inhibitory activity; and other more basic heterocycles (**12d–f**) resulted in a loss of Brk activity. Methylation of the pyrazole (**12a**) decreased the potency (Brk IC_{50} = 256 nM). Analog **12b** containing a 3-thiophene group gave a moderate Brk inhibitory potency (Brk IC_{50} = 51 nM).

Starting with intermediate **4**, Pd-mediated coupling reactions were employed to install different functional groups at C-6. The resulting products were then carried through similar steps as shown in Scheme 1 to give final compounds **13a–d** in Table 3. The hydrogen analog **13a** only gave moderate potency to Brk. The SAR showed that small alkyl groups, such as ethyl (**13b**) and cylcopropyl (**13c**) were tolerated and preferred. These functional groups not only improved the cell-based activity, but also increased the selectivity window toward both kinases Aurora B and Lck. Inhibitor **13c** demonstrated a favorable cell-based profile with a *p*-SAM68 IC_{50} of 22 nM and more than a 300-fold selectivity window to both kinases Aurora B and Lck.



Scheme 2. Reagents and conditions: (a) 4-Boc-piperazine, HATU, DIEA, DMF, rt, 96%; (b) sulfone 7, NaH, DMF, rt, 81%; (c) 4 N HCl in dioxane, 40 °C.

Table 1
Brk and *p*-SAM68 inhibition data for **11a–d**



11

Compds	R^2	IC_{50} (nM)	
		Brk	*p*-SAM68
11a	—Me	1524	N.D.
11b	—Et	>10,000	N.D.
11c	—*c*-Pr	>10,000	N.D.
11d	—*i*-Pr	>10,000	N.D.

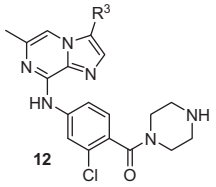
Assay conditions listed in Ref. 28.

The clear SAR at C-2, C-3, and C-6 helped us to focus on the potential at the C-8 amide region as we mentioned early in the proposed binding mode. Starting with intermediate **6**, replacement of the sulfone with amines followed by hydrolysis, amide formation and global deprotection provided final compounds **14a–e** in Table 4. Initial results (Table 4) showed that the *para*-orientation of the amide to the 8-amino group on the benzene ring was required to maintain the potency. Free acid **14a** and meta amide **14b** gave moderate inhibitory activity to Brk (IC_{50} = 94 and 104 nM, respectively). Saturation of the middle ring (**14c**) caused loss of activity, possibly due to an unfavorable angle change of the amide carbonyl group relative to the piperazine or phenyl ring. Replacement of the benzene ring of the amide with other five-member rings, such as thiophene (**14d**) and thiazole (**14e**) maintained the potency (Brk IC_{50} = 10 and 12 nM, respectively). However, this class of compounds possessed potent activity against Aurora B (Aur B IC_{50} less than 13 nM, the limit of the assay); consistent with results reported in publications from the Aurora kinase program.[30]

As shown in Table 5, replacement of the benzoic amide of **2** with substituted pyridines (**15a–c**) gave moderate to good inhibitory activity in both biochemical and cell-based assays. This is consistent with the proposed binding mode wherein a hydrophobic ring was anticipated to be preferred in this region. Replacement of the benzene ring with a non-amide substituted thiazole (**15d**) and thiophene (**15e**) showed very good potency to Brk (IC_{50} = 13 and 10 nM) as well as to Aurora B (IC_{50} <13 nM).[30] Therefore we shifted our efforts towards replacing the original chlorine on the benzene ring.

Data in Table 6 demonstrated that a variety of functional groups can be tolerated at the ortho position adjacent to the amide carbonyl group. Hydrogen (**16a**) and fluorine (**16b**) substitutions gave similar results as chlorine substitution. Substitutions with CF_3 (**16c**), methoxy (**16d**), cyclopropyl (**16e**), and isopropyl (**16f**) all

Table 2
Brk and *p*-SAM68 inhibition data for **12a–f**

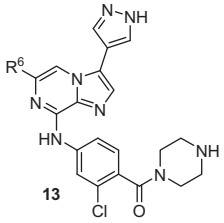


12

Compds	R^3	IC_{50} (nM)	
		Brk	*p*-SAM68
12a		256	>3000
12b		51	N.D.
12c		177	N.D.
12d		355	N.D.
12e		319	N.D.
12f		2643	N.D.

Assay conditions listed in Ref. 28.

Table 3
Brk and *p*-SAM68 inhibition and counti-screening data for **13a–d**

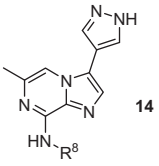


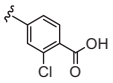
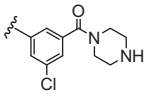
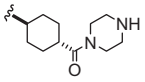
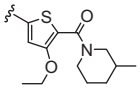
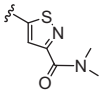
13

Compds	R^6	IC_{50} (nM)			
		Brk	*p*-SAM68	Aur B	Lck
2		7	157	754	420
13a		391	N.D.	N.D.	N.D.
13b		10	38	653	722
13c		6	22	2541	1830
13d		477	N.D.	N.D.	N.D.

Assay conditions listed in Ref. 28.

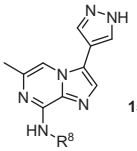
Table 4
Brk and *p*-SAM68 inhibition and counti-screening data for **14a–e**

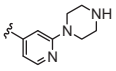
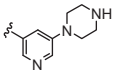
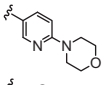
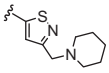
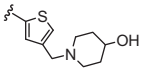


Compds	R^8	IC_{50} (nM)			
		Brk	*p*-SAM68	Aur B	Lck
14a		97	N.D.	N.D.	N.D.
14b		104	N.D.	N.D.	N.D.
14c		799	N.D.	N.D.	N.D.
14d		10	N.D.	<13	330
14e		12	N.D.	N.D.	N.D.

Assay conditions listed in Ref. 28.

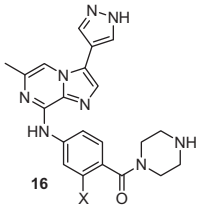
Table 5
Brk and *p*-SAM68 inhibition and counti-screening data for **15a–e**



Compds	R^8	IC_{50} (nM)			
		Brk	*p*-SAM68	Aur B	Lck
15a		11	109	561	3710
15b		75	244	N.D.	N.D.
15c		495	N.D.	N.D.	N.D.
15d		13	94	<13	N.D.
15e		10	N.D.	<13	N.D.

Assay conditions listed in Ref. 28.

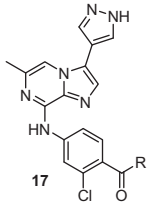
Table 6
Brk and *p*-SAM68 inhibition and counti-screening data for **16a–e**

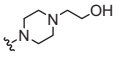


Compds	X	IC_{50} (nM)			
		Brk	*p*-SAM68	Aur B	Lck
16a	H	15	167	232	603
16b	F	32	153	143	N.D.
16c	CF_3	7	35	84	722
16d	OMe	23	22	837	N.D.
16e	*c*-Pr	38	40	128	169

Assay conditions listed in Ref. 28.

Table 7
Brk and *p*-SAM68 inhibition and counti-screening data for **17a–j**



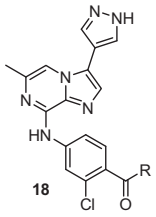
Compds	R	IC_{50} (nM)			
		Brk	*p*-SAM68	Aur B	Lck
17a		8	>600	N.D.	N.D.
17b		3	>3000	N.D.	N.D.
17c		16	>3000	N.D.	N.D.
17d		68	132	N.D.	1200
17e		93	608	N.D.	N.D.
17f		8	254	43	2000
17g		379	N.D.	N.D.	N.D.
17h		72	120	N.D.	N.D.
17i		60	137	N.D.	10
17j		34	113	N.D.	70

Assay conditions listed in Ref. 28.

increased cell-based activity to Brk. However, the selectivity windows to Aurora B and Lck both dropped to within 10-fold.

Docking experiments suggested the piperazine ring of **2** was oriented toward the solvent front (Fig. 2). Replacement with various functional groups gave diverse results (Table 7, **17a–j**). Methylation (**17a**), lactamation (**17b**), and amidation (**17c**) of the piperazine amine maintained the biochemical activity, but lost target-engagement activity in the cell. Attachment of a linear alcohol (**17d**), replacement with piperidine (**17e**) or alcohol (**17h**) or methoxy (**17i**) substituted piperidines afforded moderate potency in the *p*-SAM68 assay. A morpholine-based replacement (**17f**) gave moderate cell-based activity (*p*-SAM68 IC_{50} = 254 nM) but poor selectivity toward Aurora B (IC_{50} = 43 nM). Interestingly, **17f** also showed good PK in rats (PO, 10 mpk, AUC = 12.6 μM h, C_{6h} = 1 μM). Surpris-

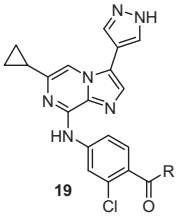
Table 8
Brk and *p*-SAM68 inhibition and counti-screening data for **18a–f**



Compds	R	IC_{50} (nM)			
		Brk	*p*-SAM68	Aur B	Lck
18a		10	23	1227	2262
18b		16	42	194	4648
18c		18	19	305	3417
18d		19	62	11,000	539
18e		7	47	339	1501
18f		11	177	242	3208

Assay conditions listed in Ref. 28.

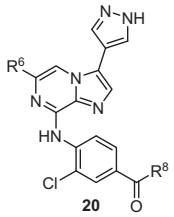
Table 9
Data for combination compounds **19a–g**



Compds	R	IC_{50} (nM)			
		Brk	*p*-SAM68	Aur B	Lck
19a		2	5	2252	180
19b		10	14	2987	597
19c		3	47	4588	476
19d		13	33	2501	945
19e		5	45	2739	284
19f		10	17	2517	365
19g		124	>600	8484	10,400

Assay conditions listed in Ref. 28.

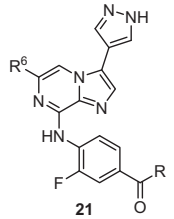
Table 10
Data for selected 2-Cl substituted analogs **20a–d**



Compds	R^6	R^8	IC_{50} (nM)			
			Brk	*p*-SAM68	Aur B	Lck
20a			64	N.D.	>20,000	>20,000
20b			63	N.D.	>20,000	>20,000
20c			156	N.D.	>20,000	>20,000
20d			156	N.D.	>20,000	>20,000

Assay conditions listed in Ref. 28.

Table 11
Data for selected 2-F substituted analogs **21a–d**



Compds	R^6	R	IC_{50} (nM)			
			Brk	*p*-SAM68	Aur B	Lck
21a			10	54	>20,000	>20,000
21b			3	7	>20,000	>20,000
21c			70	N.D.	>20,000	>20,000
21d			30	52	>20,000	>20,000
21e			9	46	>20,000	>20,000
21f			3	7	>20,000	14,114
21g			10	38	>20,000	>20,000
21h			6	<7	>20,000	>20,000

Assay conditions listed in Ref. 28.

ingly, homopiperazine analog **17j** showed only moderate potency to Brk (IC_{50} = 34 nM) and picked up Lck activity (IC_{50} = 70 nM).

As shown in Table 8, compounds **18a–f** with direct substitution and bridge-linking on the piperazine ring dramatically improved cell-based activity to the low double-digit nM range. Geminal dimethyl substitution on the piperazine ring (**18a**) not only improved the cell-based potency, but also lifted the selectivity window to more than 100-fold for both Aurora B and Lck.

Experience suggested that combination compounds with these substituted piperazines (from Table 7) and the cyclopropyl group we discovered at C-6 of the core (from Table 3) might further improve the potency and selectivity window. As shown in Table 9, compounds **19a–f** all demonstrated single-digit nM enzymatic potency and low double-digit nM cell-based activity. Compound **19a** even demonstrated single-digit nM cell-based activity, so far one of the best on the program. However, the selectivity window to Lck unexpectedly dropped to 50- to 100-fold. Surprisingly, the morpholine analog **19g** showed only moderate enzymatic potency

Table 12
Mouse PK data for compound **21a**

Parameter (units)	IP	PO	PO	PO
Number of animals (N)	15	15	15	15
Dose (mg/kg)	30	10	30	100
AUC (0–6 h) (ng h/mL)	7205	42	606	3388
AUC (0–6 h) (μM h)	17.1	0.1	1.4	8.1
C_{max} (ng/ml)	7513	18	176	675
C_{max} (μM)	17.9	0.04	0.42	1.60
T_{max} (h)	0.5	6.0	2.0	4.0

(IC_{50} = 124 nM) and greater than 600 nM cell-based activity, although the selectivity windows toward Aurora and Lck were maintained.

Moving the chlorine on the benzene ring to the next position caused the enzymatic potency to drop (Table 10, compounds **20a–d**). Surprisingly, fluorine substitution at this position provided the final top compounds (Table 11, compounds **21a–h**). Almost all of these compounds showed single-digit to low double-digit nM cell-based activity. The enhanced activity might be due to the improved hydrogen bond between the amide carbonyl group and Arg-195 as we proposed in the binding mode in Figure 2. The selectivity windows to Aurora B and Lck were both significantly improved to greater than 300-fold. For compound **21a**, it had clean CYP inhibition profile (all >20 μM) and low intrinsic clearance in hepatocyte-based clearance assays (human = 2.0 μL/min/mill cells and rat = 5.3 μL/min/mill cells). The mouse PK results are shown in Table 12. The bioavailability of **21a** was only about 5.8% and is likely attributed to poor permeability (CACO-2: 0 nm/s). Compound **21d** showed a similar potency and selectivity profile as **21a**. Surprisingly, **21d** had much better permeability (CACO-2: 314 nm/s) and PK profile (rat, po, 10 mpk, AUC_{0-6h} = 31.1 μM h, C_{6h} = 3.5 μM). These results suggested that compounds **21a** and **d** may be appropriate tool compounds to evaluate the in vivo activity of Brk inhibitors in xenograft breast tumor models to further validate the potential of Brk/PTK6 as an oncology target.

In summary, we have discovered imidazo[1,2-*a*]pyrazin-8-amines as potent Brk kinase inhibitors. The overall SAR suggested that this type of inhibitors probably bind to the enzyme as shown in Figure 2. Several inhibitors, with single-digit nanomolar target-engagement cell-based activity and an appealing overall DMPK profile, could be used as tool compounds to further validate Brk/PTK6 as a potential target for cancer treatment.

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28. (a) Brk/PTK6 kinase assay was in HTRF format and the CisBio HTRF kinase TK (62TK0PEJ) kit was used. Assay reagents are solubilized as per kit protocol. Compounds are diluted and pre-incubated with PTK6 enzyme (Invitrogen) in assay plate for 30 min at RT while shaking. Substrate is added followed by ATP to start reaction in 384 well Corning assay plates. Reaction is stopped after one hour by addition of kit supplied XL665/antibody Cryptate solution. Plate is read after 1 h using Pherastar Microplate reader. (b) *p*-Sam68 alpha-screen assay. 293 WT-PTK6 cells are plated overnight in 96 well TC plates containing 10% DMEM with supplements and G418. Media is flicked off and complete media containing compound is added and incubated for 3 h at 37 °C. Media is aspirated and cells are rinsed with PBS. Cells are lysed in 50 μL lysis buffer (50 mM Tris, pH 7.4; 250 mM NaCl; 5 mM EDTA; 50 mM NaF; 1 mM Na_3VO_4; 1% Nonidet P40 (NP40); 0.02% NaN_3; 2 mM Na_3VO_4) is added and placed on a plate shaker for 5 min. Lysates are now ready for the assay. Five micro-liter of lysate and 5 μl dilution buffer (25 mM HEPES, pH 7.5; 100 mM NaCl; 0.01% T-20) were added per well (Perkin Elmer). Ten microliter of 3X PT66 acceptor beads were then added to each well (Final = 20 μg/ml, Perkin Elmer), and the plat was put on a shaker for 90 min. Subsequently, 5 μL of 6x Biotinylated-SAM68. (Final = 0.2 μg/mL, Santa Cruz) was added and the plate was out on a shaker for 60 min before addition of 5 μl of 6x Donor beads (Final: 20 μg/mL). The plate was put on a shaker for 30 min before reading by Envision Plate Reader (Perkin Elmer).
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