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Letter

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[3*a*,4]-Dihydropyrazolo[1,5*a*]pyrimidines: Novel, Potent, and Selective Phosphatidylinositol-3-kinase β Inhibitors

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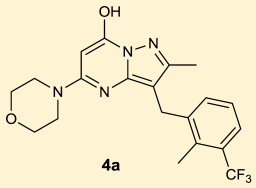
Supporting Information

ABSTRACT: A series of novel [3*a*,4]dihydropyrazolo[1,5*a*]-pyrimidines were identified, which were highly potent and selective inhibitors of PI3K*β*. The template afforded the opportunity to develop novel SAR for both the hinge-binding (R_3) and back-pocket (R_4) substitutents. While cellular potency was relatively modest due to high protein binding, the series displayed low clearance in rat, mouse, and monkey.



PI3Kα pIC_{50} = 6.5
PI3Kβ pIC_{50} = 9.4
PI3Kδ pIC_{50} = 7.6
PI3Kγ pIC_{50} = 6.5
Cl (mouse) = 3.7 ml/min/kg
DNAUC (mouse p.o.) = 2321 ng•hr/ml/mg/kg
F = 51%

KEYWORDS: *PI3K-β inhibitor, PTEN-null, phosphatidylinositol-3-kinase, pyrazolopyrimidine, structure−activity relationship*

The phosphatidylinositol 3-kinases (PI3Ks) are members of a larger family of lipid kinases that phosphorylate the inositol moiety of membrane-associated phosphatidyl inositols.[1] PI3Ks catalyze the conversion of phosphatidylinositol-4,5-diphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3), which allows recruitment of proteins containing the PIP_3-binding pleckstrin homology (PH) domain to the plasma membrane. The PI3K class I family comprises four isoforms (*α*, *β*, *δ*, and *γ*), with the *α* and *β* isoforms most widely expressed. Each is a heterodimer consisting of an isoform-specific p110 catalytic subunit and a regulatory subunit.

PI3Ks are an important mediator of receptor tyrosine kinase (RTK) and G-protein-coupled receptor (GPCR) signal transduction and are involved in the regulation of cellular metabolism, survival, and proliferation.[2] Aberrant activation of the PI3K pathway is strongly associated with malignant transformation, through either overexpression of PI3K*α*, mutations that lead to constitutive activity of PI3K*α*, or loss of function of the tumor suppressor PTEN (phosphatase and tensin homologue), which opposes PI3K function by converting PIP_3 to PIP_2.[3−9] While the PI3K isoforms share the same substrate specificity, they have distinct biochemical roles and cellular functions.[10] In particular, there is considerable evidence that in PTEN-null tumors, malignant transformation is primarily driven by the PI3K*β* isoform.[11−15] The development of PI3K*β*-selective inhibitors is therefore an attractive target for the treatment of PTEN-null tumors.

We have previously disclosed imidazopyrimidones (**1**) and triazolopyrimidones (**2**), as shown in Figure 1, which are potent and selective inhibitors of PI3K*β*.[16,17] However, the compounds were not suitable for in vivo studies due to high clearance in rat and mouse. Both **1** and **2** contain an embedded benzylamine moiety, a potential site for metabolism, which might contribute to the high clearance. We therefore designed a pyrazolo-pyrimidine scaffold, in which the benzyl group is attached to a carbon atom rather than a nitrogen, in an attempt to improve clearance.

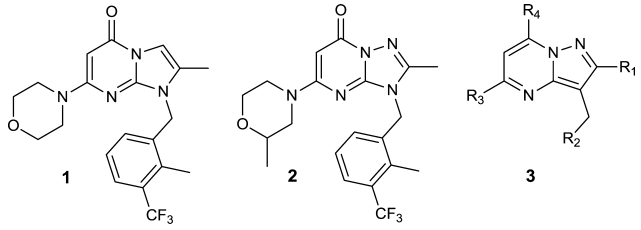


Figure 1. Imidazopyrimidone (**1**), triazolopyrimidone (**2**), and pyrazolopyrimidine (**3**) PI3K*β* inhibitors.

Pyrazolopyrimidines were generally prepared according to previously reported methods[18−20] as shown in Scheme 1. An *α*-cyanocarbonyl compound **I** is alkylated[21] with a benzyl bromide **II** and then condensed with hydrazine to afford the key aminopyrazole intermediate **IV**. The aminopyrazole is then condensed with a *β*-carbonyl-containing ester **V** to afford the 7-hydroxypyrazolopyrimidines **VI**. Where **V** is diethyl malonate, the resulting dihydroxypyrimidine (**VI**, R_3 = OH) is chlorinated with $POCl_3$, and the chlorines are selectively displaced with hydroxide and then morpholine to afford the final products **VII**. To introduce the R_4 substituent, **VII** is again chlorinated with $POCl_3$, and chlorine is either displaced with nucleophiles (methoxide or amines) or carbonylated, and the resulting

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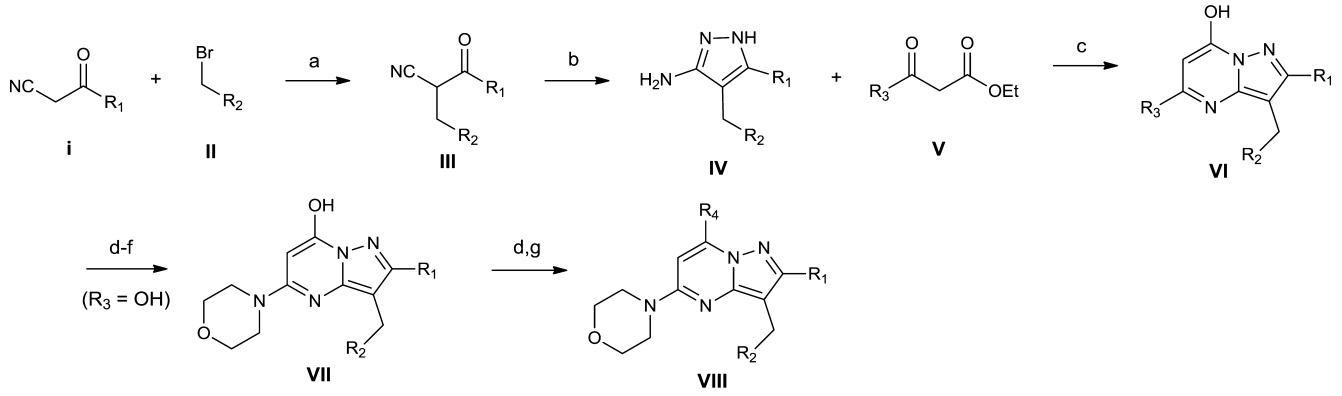
Scheme 1. Synthesis of Pyrazolopyrimidines[a]

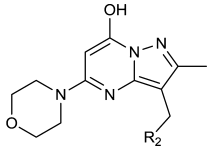


[a]Reagents and conditions: (a) K_2CO_3, THF, reflux. (b) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux. (c) NaOEt, EtOH, reflux or HOAc, 140°, microwave. (d) $POCl_3$, *N*,*N*-diethylaniline, 110°. (e) 1 N NaOH, THF. (f) Morpholine, EtOH, 140°, microwave. (g) RNH_2, MeOH, 135° or NaOMe, MeOH, 0−40°, or $Pd(OAc)_2$, dppf, CO, Et_3N, DMF/THF, 70°.

functional groups are elaborated according to standard methodology.

To evaluate the pyrazolopyrimidine core, we prepared two 2-methyl-5-morpholine-7-hydroxy analogues with different benzyl substituents at position 3. The pyrazolopyrimidine core introduces the possibility of three tautomeric forms, two of which display an NH moiety, which is not the case in either the imidazopyrimidone (**1**) or the triazolopyrimidone (**2**) cores. We were gratified to see that despite these potentially significant structural differences, both pyrazolopyrimidines retained the high PI3Kβ potency and excellent isoform selectivity observed with the other inhibitor series, as shown in Table 1. Because the SAR at this benzyl position had

Table 1. SAR of 3-Benzyl 7-Hydroxypyrazolopyrimidinines[a]



		PI3K pIC_{50}[22]			
compd	R_2	α	β	δ	γ
4a	2-Me-3-CF_3-phenyl	6.5	9.4	7.6	6.5
4b	1-naphthyl	6.5	9.0	7.6	6.2

[a]pIC_{50} values are the mean of a minimum of two determinations.

previously been well-established,[16] we were able to focus our further efforts mainly on the 2-methyl-3-trifluoromethyl benzyl analogues while we sought to better define the much less well-established hinge-binding and back-pocket SAR.

A docking pose of **4a** in a PI3Kβ homology model[16] is shown in Figure 2. The morpholine oxygen provides the key hydrogen-bonding interaction with the hinge residue Val[854]. The pyrimidine hydroxyl interacts with the back-pocket Tyr[839] via a bridging water molecule. A key feature is the interaction of the benzylic side chain with the flexible P-loop, which caps the ATP binding pocket. A conformational change induced by binding of the inhibitor creates a small hydrophobic pocket lined by Met[779] and Trp[787], which is ideally suited to accommodate a 2,3-disubstituted phenyl ring. This is consistent with the observed SAR of substituted benzyl moieties and is hypothesized to be a major contributor to the β-isoform

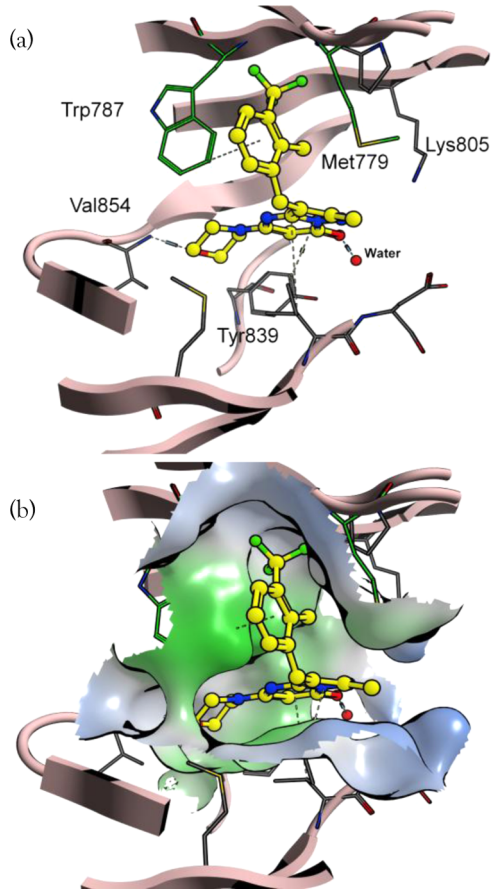


Figure 2. Homology model of **4a** docked into PI3Kβ. (a) Ribbon diagram showing key residues and interactions. (b) Surface showing hydrophobic pocket induced by the 2,3-disubstituted benzyl group.

selectivity. With this model in mind, we designed analogues to explore the SAR for the other substituents on the pyrazolopyrimidine core.

We next examined the hinge-binding morpholine moiety. We were particularly interested in analogues, which modulated the basicity of the morpholine either by replacing the nitrogen or by converting it to an amide. The tetrahydropyranyl and pyridyl analogues **5a** and **5e** were prepared from the corresponding β-keto esters, and the morphlin-3-one analogue **5b** was prepared

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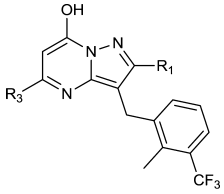
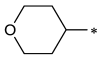
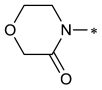
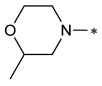
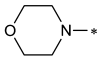
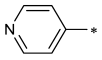
via a Buchwald amidation[23] of the 5-chloro-7-hydroxypyrazolopyrimidone intermediate.

The enzymatic activity of these analogues is shown in Table 2. Although the morpholine nitrogen is not involved in any specific interactions with the enzyme, its substitution by carbon (**5a**) causes a 10-fold drop in activity. The piperidone analogue **5b** drops nearly 1000-fold in activity, possibly due to an intramolecular hydrogen bond between the carbonyl and the NH of the pyrimidone tautomer, which could cause the morpholinone ring to adopt an unfavorable conformation. The 2-methylmorpholine substitution had provided equipotent analogues in the imidazopyrimidone series[17] but gave a less potent compound in this series, and resolution of the two enantiomers was not attempted.

Table 2. Enzymatic Activity of 5a−e

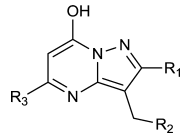


Cmpd	R3	R1	PI3K pIC$_{50}$ α	β	δ	γ
5a		Me	< 5.8	8.4	6.9	5.6
5b		Me	6.6	6.5	6.8	5.5
5c		Me	6.7	8.6	7.8	6.4
5d		H	6.5	8.3	7.7	6.3
5e		H	6.3	8.6	7.0	6.5

We then examined the effect of substitution at the 2-position of the pyrazolopyridine moiety. According to the homology model, there are no close contacts to the protein in this region, so we expected that a variety of substitutions might be tolerated. Enzymatic activity of the 2-substituted pyrazolopyrimidines is shown in Table 3. The presence of a 2-position substitutent improves activity about 10-fold (**5d** vs **4a**). Small, aliphatic substituents like Me, Et, or CF_3 are all well tolerated and provide similar activity. There was a 10−100-fold reduction in activity with the polar hydroxyl or amino substituents. Interestingly, the loss in activity with the hydroxyl analogue **6f** could be overcome by methylation of the oxygen (**6g**), suggesting that the poor activity of **6f** and **6h** is less due to changes in the electronics of the pyrazolopyrimidine core and more likely due to introduction of hydrogen bond donors at that position.

The tautomeric nature of the pyrazolopyrimidine template permits substitution at the 7-position, something not possible with the imidazopyrimidone and triazolopyrimidone templates. This allowed us to prepare variously substituted analogues to evaluate the requirement for a hydrogen-bonding interaction with Tyr^{839}, either directly or via a bound water molecule.

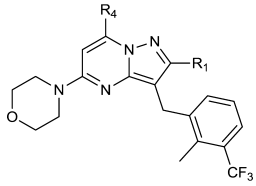
Table 3. Enzymatic Activity of 2-Substituted Analogues



compd	R3[a]	R2[b]	R1	PI3K pIC$_{50}$ α	β	δ	γ
5d	A	X	H	6.5	8.3	7.7	6.3
4a	A	X	Me	6.5	9.4	7.6	6.5
6a	B	Y	Et	6.8	9.2	7.7	6.4
6b	C	Y	Et	<5.8	7.9	<6.4	<5.1
6c	A	Y	CF_3	6.7	9.4	8.2	6.6
6d	B	Y	CF_3	6.2	8.6	7.1	6.4
6e	C	Y	CF_3	5.6	8.2	7.0	<5.2
6f	C	X	OH	<5.7	7.2	<6.2	<5.3
6g	B	Y	OMe	6.8	8.9	7.6	6.3
6h	C	Y	NH_2	5.8	8.1	6.2	<5.4

[a]A = morpholinyl, B = 4-pyridyl, and C = 4-tetrahydropyranyl. [b]X = 2-Me-3-CF_3-phenyl, and Y = 2,3-dichlorophenyl.

Table 4. Enzymatic Activity of 7-Substituted Analogues



compd	R4	R1	PI3K pIC$_{50}$ α	β	δ	γ
4a	OH	Me	6.5	9.4	7.6	6.5
7a	OMe	H	<5.3	6.0	<6.2	<5.3
7b	Cl	H	<5.3	6.5	<6.2	<5.3
7c[a]	H	Me	<5.8	6.1	<6.3	5.7
7d	CO_2H	Me	5.8	8.3	7.4	<5.2
7e	$CONH_2$	Me	<5.2	7.6	<6.2	<5.2
7f	CN	Me	<6.0	<5.9	<6.6	<5.5
7g	NH_2	Me	<5.7	7.4	6.8	<5.8
7h	NHMe	Me	<5.8	<5.5	<6.2	<5.2
7i[a]	NHOH	Me	<5.8	7.4	6.6	<5.2
7j[a]	$NHCH_2CH_2OH$	Me	<5.8	5.9	<6.2	5.3
7k	$NHCOCH_3$	Me	<5.7	<5.9	<6.2	<5.3
7l	$NHCONH_2$	Me	<5.2	7.7	<6.2	<5.2

[a]5-THP analogue.

The enzymatic activity of the various 7-substituted analogues is shown in Table 4. While the original 7-hydroxy (**4a**) provided the highest activity, a wide variety of substitutions could be tolerated to varying degrees. In particular, the 7-carboxy analogue **7d** retained considerable activity, while the amino, hydroxyl-amine, carboxamide, and urea analogues **7g**, **7i**, **7e**, and **7l** also displayed modest potency. Interestingly, both the unsubstituted analogue **7c** and the chloro analogue **7b** retained measurable activity, indicating that a hydrogen-bonding interaction at that position is not absolutely essential for activity.

The compounds were also evaluated for cellular activity in two human breast cancer cell lines: a PTEN-null cell line, MDA-MB-468, and a PTEN wild-type cell line, HCC1954. The MDA-MB-468 cells are expected to be sensitive to PI3Kβ

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inhibition, while the HCC1954 cells should be much less sensitive and serve as a control cell line. Inhibition of proliferation was measured in an anchorage-independent (soft agar) growth assay,[16] and functional activity was determined by measuring inhibition of the PI3K-dependent phosphorylation of AKT at Ser[473].[16,17]

The cellular activity of selected compounds is shown in Table 5. Both the functional and the antiproliferative activities

Table 5. Cellular Activity of Selected Pyrazolopyrimidines[a]

	pEC_{50}			
	MDA-MB-468		HCC1954	
compd	growth	pAKT	growth	pAKT
4a	5.3	5.8	4.8	<4.8
4b	5.4	6.0	4.5	<4.5
5a	4.8	5.0	4.6	<4.5
5d	5.5	6.0	4.8	<4.5
5e	4.8	5.5	<4.5	<4.5
6a	ND	4.7	ND	<4.5
6c	<4.5	5.3	<4.5	<4.5
6g	4.6	4.9	<4.5	<4.5
6h	5.0	5.7	<4.5	<4.5
7d	5.5	6.0	<4.5	<4.5
7e	5.7	<4.5	<4.5	<4.5
7g	4.9	6.0	4.9	<4.5
7l	5.9	<4.5	4.9	<4.5

[a]ND, not done.

were unexpectedly poor for this entire class of compounds. Despite a wide variety of structural changes, there did not seem to be any SAR for cellular activity. Both polar (**6g**,**h**) and nonpolar substituents (**6a**,**c**) at position 2 of the pyrazolopyridine moiety gave similar activities. Substitution of the hinge-binding morpholine with either tetrahydropyran (**5a**) or pyridine (**5e**) did not improve cell potency, and replacement of the 7-hydroxy group with polar (**7e**,**l**), acidic (**7d**), or basic substituents (**7g**) likewise had little effect. The poor cellular activity of the pyrazolopyrimidines is in sharp contrast to the structurally similar imidazopyrimidone (**1**) and triazolopyrimidone (**2**) inhibitors, where compounds of similar enzymatic potency demonstrated nanomolar potency in both cellular assays.

To investigate the possible reasons for this lack of cellular potency, we selected one of the most potent analogues, **4a**, for further studies. It has reasonably good aqueous solubility (134 uM) and demonstrated excellent cellular permeability in MDCK cells (753 nM/s). In a high-throughput HPLC-based protein binding assay,[24] the estimated protein binding was indistinguishable from that of representative imidazopyrimidones and triazolopyrimidones that displayed excellent cellular activity. However, when we evaluated protein binding by equilibrium dialysis, we found that **4a** was extremely highly protein bound, 99.9% or greater in rat, mouse, and human serum. The very small free fraction of drug would be consistent with both the poor cellular activity and the lack of apparent SAR. To test the hypothesis that high protein binding was responsible for the poor cellular activity, compounds were evaluated in the phospho-AKT functional assay in MDA-MB-468 cells in the presence and absence of serum.[25] The results are shown in Table 6. We were delighted to see that all of the compounds tested demonstrated significantly improved cellular

Table 6. Cellular Activity in MDA-MB-468 Cells in the Presence and Absence of Serum

		pAKT pEC_{50}	
compd	PI3Kβ pIC_{50}	+ serum	− serum
4a	9.4	5.8	7.5
5d	8.3	6.0	7.2
5e	8.6	5.5	7.6
6g	8.9	4.9	6.8
8[23]	9.2	7.8	8.2

activity when assayed in the absence of serum. Compound **8** is a potent PI3Kβ inhibitor,[26] which has significantly lower protein binding by equilibrium dialysis (rat, 74.6%; mouse, 88.2%; and human, 86.3%) and is included as a control to demonstrate that the presence or absence of serum has very little effect on the cellular activity of a compound that is not highly protein bound.

Compound **4a** was also evaluated for pharmacokinetic properties, and we were pleased to see that it exhibited low clearance in rat, mouse, and monkey and good oral bioavailability and exposure in mouse, as shown in Table 7.[27]

Table 7. DMPK Profile of 4a

species	Cl (mL/min/kg)	DNAUC (po)[a] (ng h/mL/mg/kg)	% *F*
rat	6.5	ND	ND
mouse	3.7	2321	51
monkey	0.33	ND	ND

[a]1% DMSO, 10% encapsin, pH 4.5; ND, not determined.

This improvement in clearance over the imidazopyrimidone and triazolopyrimidone series is undoubtedly due in large part to the high protein binding, and we were therefore unable to determine the contribution of the *N*-benzyl group to in vivo clearance. While high protein binding does not necessarily correlate with poor in vivo efficacy,[28] we desired a compound with an unambiguously higher free fraction for in vivo target validation studies, and **4a** was not pursued further.

In summary, we have identified novel pyrazolopyrimidines that are highly potent and selective inhibitors of PI3Kβ. This template allowed facile substitution at both the 5- (hinge-binding) and the 7- (back-pocket) positions, and we were able to demonstrate considerable tolerance to substitution at the 7-position, which is thought to make a key hydrogen bond interaction. Because the SAR has generally been conserved among related series, this may afford the opportunity to fine-tune physicochemical properties in other inhibitor series without sacrificing enzymatic potency. The compounds exhibit only modest cellular potency under standard conditions, which we have shown to be due to high protein binding. Compound **4a** displays low clearance and high oral exposure in mouse, but the high protein binding precluded further development.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures and characterization for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): we have been requested to add the following disclosure:The authors are all employees of GlaxoSmithKline and have a financial interest in GlaxoSmithKline.

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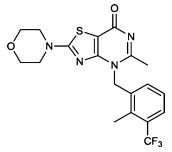
(24) Protein binding was estimated by HPLC analysis using commercially available immobilized human serum albumin or α-acid glycoprotein columns eluted with a gradient of 50 mM NH_4OAc, pH 7.4, and isopropanol.

(25) Cells were plated and grown overnight in normal serum-containing medium (10% FBS) as described previously.[17] The medium was then exchanged for serum-free medium, and the cells were incubated with or without compound for 30 min and assayed as usual.



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