

Discovery of an *in Vivo* Tool to Establish Proof-of-Concept for MAP4K4-Based Antidiabetic Treatment

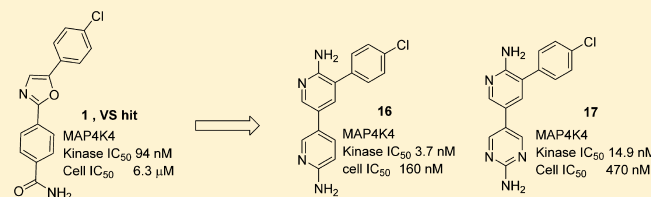
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Supporting Information

ABSTRACT: Recent studies in adipose tissue, pancreas, muscle, and macrophages suggest that MAP4K4, a serine/threonine protein kinase may be a viable target for antidiabetic drugs. As part of the evaluation of MAP4K4 as a novel antidiabetic target, a tool compound, **16** (PF-6260933) and a lead **17** possessing excellent kinome selectivity and suitable properties were delivered to establish proof of concept *in vivo*. The medicinal chemistry effort that led to the discovery of these lead compounds is described herein together with *in vivo* pharmacokinetic properties and activity in a model of insulin resistance.

KEYWORDS: MAP4K4, HGK, ZC1, kinase inhibitor, MAP4K4 inhibitor, P-loop, PF-6260933



Mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4), also called HGK or ZC1, is a serine/threonine protein kinase that belongs to the mammalian STE20/MAP4K family.¹ MAP4K4 is overexpressed in human cancers and cancer cell lines and appears to play an important role in cell transformation, invasiveness, adhesion, and migration.² More recently, studies in adipose tissue, pancreas, muscle, and macrophages suggest that MAP4K4 could also be a potential target for antidiabetic drugs.^{3,4} For example, suppression of the expression of MAP4K4 using small interfering RNA in human skeletal muscle cells from both healthy subjects and type II diabetic patients prevented TNF- α induced insulin resistance of glucose uptake.⁵ Furthermore, a recent genetic analysis suggests that common genetic variants in the MAP4K4 locus are associated with type II diabetes underlying pathomechanisms, insulin resistance, and β -cell failure.⁶ As part of the evaluation of MAP4K4 as a novel antidiabetic target, it was desirable to deliver a tool compound with greater kinome selectivity than the existing chemical matter (unpublished results) and reasonable properties to establish proof of concept in a mouse diabetes model. Herein we describe the medicinal chemistry that led to the discovery of such a compound. Since our work was conducted, alternative series of MAP4K4 inhibitors for oncology and neurodegenerative indications have recently been described.^{7–9}

A virtual screening effort of the internal Pfizer file using an in-house crystal structure of the kinase domain of MAP4K4, identified compound **1** as a potent MAP4K4 inhibitor (Table 1). It was selective in a standard kinase panel and upon cocrystallization the complex between **1** and MAP4K4 revealed that the P-loop adopts a rare folded conformation state (Figure 1).¹⁰ The P-loop, which normally exists in an extended conformation, is a stretch of nine amino acids that in MAP4K4 is delimited by residues Val31 to Val39. The binding site adopts a tunnel-like shape that provides a significant hydrophobic enclosure for the inhibitor. There are potential π - π interactions between the MAP4K4 P-loop Tyr36 residue and the three aromatic rings of compound **1**. The primary amide is the only group that escapes the hydrophobic tunnel, being partially exposed to solvent. The structure suggests that it forms a hydrogen bond with Asp115.

Compound **1** had low molecular weight (MW = 299) and a measured LogD of 3.6. It was a weak inhibitor of MAP4K4 (Table 1) but with good ligand efficiency (LE = 0.48). The kinase activity is measured using a FRET-based assay that utilizes catalytic domain of human recombinant MAP4K4. The cell assay is designed to identify compounds inhibiting

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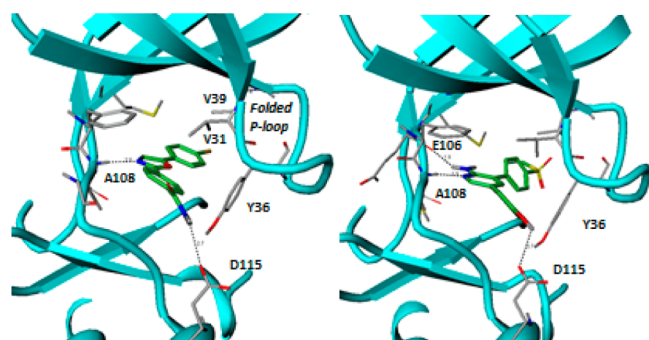


Figure 1. Crystal structure of the complex between MAP4K4 and (a) compound 1 (in green) (PDB code: 4ZP5) and (b) compound 13 (in green) (PDB code: 5DI1).

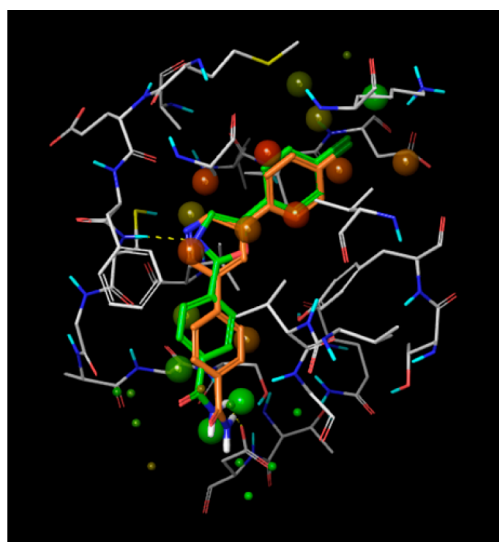


Figure 2. Hydration sites obtained using WaterMap simulations. The crystal structure of the complex between MAP4K4 and compound 1 was used. The compound is omitted in the simulations (compounds 1 and 2 were added to the figure above for comparison). The most favorable and unfavorable water molecules with respect to a water molecule with bulk-like properties are colored green and red, respectively.

MAP4K4's phosphorylation of serine/threonine residues on traf2 (see [Supporting Information](#) for full details). In an attempt to improve the enzyme and cell potencies of 1, several heterocyclic alternatives to the oxazole hinge binding group were evaluated by the MM-GB/SA scoring procedure.¹¹ The MM-GB/SA method involves simulations of a ligand in its bound and unbound states and computes its free energy of binding from the energy differences between these two states. The energy terms include intramolecular and intermolecular interactions, solvation energy, and conformational entropy. The most promising analogues from this analysis were synthesized and evaluated for MAP4K4 inhibitory activity ([Table 1](#)). The best scoring compounds, 1–4, were the most potent in the enzyme assay, whereas heterocycles 5–7, which had higher scores (and hence predicted to be less potent), displayed weaker potencies. The pyridine analogue 2 was found to be the most potent inhibitor in the enzyme assay but displayed weak inhibition in the cell-based assay. After the identification of pyridine as a competent hinge binder, the WaterMap methodology¹² was employed to characterize unstable water

Table 1. MM-GB/SA Score, Enzyme and Cell Potencies, and Permeability Data of Analogues of Compound 1

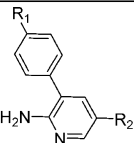
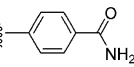
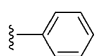
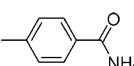
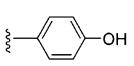
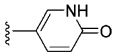
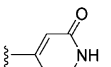
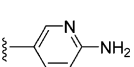
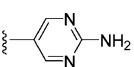
		Score ^a	Enzyme IC ₅₀ (nM) ^b	Cell IC ₅₀ (μM) ^b	P _{app} ^d
1		0.9	94±14	6.3±2.4	ND
2		0.1	46±13	7.5 ^c	8.7 ^c
3		0.0	248±63	>30	ND
4		0.4	270 ^c	>30	2.9
5		3.6	1509±51	ND	15.6
6		3.6	861 ^c	>30 ^c	ND
7		3.9	1722±482	>30 ^c	4.4
8		ND	45±12	0.60±0.16	8.7
9		ND	48±9	6.2±1.6	3.5

^aMM-GB/SA relative score in kcal/mol. The best scoring analogue according to the MM-GB/SA method is used as reference (0.0 kcal/mol). ^bArithmetic mean and standard error of ≥3 measurements (unless noted otherwise). ^cLess than three independent replicates. ^dPassive permeability (P_{app} in 10^{−6} cm/s) measured using low-efflux MDCKII cells.¹³

molecules that could lead to incremental increase in potency when liberated to the bulk upon ligand binding. The hydration sites obtained from a simulation using the crystal structure of the complex between MAP4K4 and 1 are illustrated in [Figure 2](#); the compound is omitted in the simulation (compounds 1 and 2 are included in the figure for comparison). The overlap between the map and compounds 1 and 2 suggests that they replace the favorable waters (colored green) with the primary amide and displace most of the unfavorable waters (colored red), except for three moderately unstable waters that sit in a deep pocket close to the methionine gatekeeper residue.

This map inspired the design of analogues 8 and 9 ([Table 1](#)). The methoxy and cyclopropoxy groups were expected to lead to gains in potency due to partial or full displacement of one or two of the hydration sites highlighted in [Figure 2](#). These

Table 2. Enzyme and Cell Potencies and Permeability Data of Aminopyridines

		MW	eLogD ^a	Enzyme IC ₅₀ (nM) ^b	Cell IC ₅₀ (μM) ^b	P _{app} ^d
10	R ₁ = Cl; R ₂ = 	324	3.6	13±4	0.36±0.24	11.9
11	R ₁ = Cl; R ₂ = 	281	4.4	84 ^c	ND	ND
12	R ₁ = SO ₂ Me; R ₂ = 	367	1.5	64±26	4.2 ^c	6.7
13	R ₁ = SO ₂ Me; R ₂ = 	340	2.4	21±9	2.9 ^c	17.2
14	R ₁ = Cl; R ₂ = 	298	2.0	96 ^c	2.9±1.3	ND
15	R ₁ = Cl; R ₂ = 	298	2.7	20±2	0.59±0.09	15.7
16	R ₁ = Cl; R ₂ = 	297	3.3	3.7±0.14	0.16±0.02	7.1
17	R ₁ = Cl; R ₂ = 	298	2.8	14.9±1.7	0.47±0.08	22

^aeLogD = measured logD at pH 7.4 [calculated logD (ACD Laboratories program v12) in italics]. ^bArithmetic mean and standard error of ≥ 3 measurements (unless noted otherwise). ^cLess than three independent replicates. ^dPassive permeability (P_{app} in 10⁻⁶ cm/s) measured using Low-Efflux MDCKII cells as described.¹³

modifications unfortunately did not result in improved enzyme potency when compared to compound **2**. It is possible that the gain resulting from displacing the unstable waters is offset by an effect that opposes binding, such as a greater desolvation penalty for the ether oxygen or that the WaterMap estimates were not accurate.

Despite reasonable enzyme potency for some compounds (**1–4**, **8–9**), they all have weak or no activity in a cell assay. As expected, enzyme potency is necessary but not sufficient to ensure cell activity. Impaired membrane permeability, differences in protein constructs between enzyme and cell assays (kinase domain vs full length), and competition with cellular ATP concentrations may be some of the determining factors in this enzyme-whole cell potency disconnect. Passive permeability data, obtained using the MDCKII-LE cell line, which displays low expression of active transporters,¹³ indicated that most of the compounds have moderate permeability (Table 1) suggesting that it is not an impeding factor. Measured PgP efflux ratios for many of these compounds showed no evidence

of efflux. To assess the impact of physiological ATP concentrations on compound activity, **1**, **3**, and **9** were tested in the enzyme assay using [ATP] = 1 mM instead of [ATP] = K_m (~10 μM for MAP4K4). The respective IC₅₀ values under these conditions were >10 μM for **1** and **3** and 9.8 μM for **9**, which are similar to their cell activities. It is plausible that this chemotype relies heavily on the single hinge contact with the protein for activity and, therefore, is less capable of competing with ATP, which establishes two hinge contacts. To test this hypothesis, an amino group was introduced to the C(2) position of the pyridine ring in **2** to provide compound **10** (Table 2). This was accompanied by a modest 4-fold gain in enzyme potency and led to a ~30-fold gain in cell activity. The H → NH₂ change led to a drop of 0.5 units of logD but not much of a change in permeability despite the increased hydrogen bond donors. The efflux ratios were similar (MDR BA/AB efflux ratio of 1.2 for **2** vs 1.4 for **10**) and the kinetic solubility was improved (<1.1 μM @ pH 6.5 for **2** vs 299 μM for **10**). Several analogues of **10** that preserved the amino-

pyridine motif as the potential hinge-binder, but with alterations of the substituents on the two phenyl rings, were prepared (Table 2) in an effort to optimize overall properties directed toward identification of an *in vivo* tool compound.

Compound **11**, lacking the amide group, shows loss of kinase potency accentuating the importance of the interactions between the primary amide group of compound **10** and Asp115 for achieving potency. Exchanging the Cl substituent in **10** for a SO₂Me group (**12**) resulted in some loss of potency. Replacement of the amide of **12** by a hydroxy group in **13** resulted in similar potency in the enzyme and cellular assays despite the improved passive permeability. Crystallization of the complex between **13** and MAP4K4 revealed the same folded conformation state for the P-loop as the one adopted in the complex with **1** (Figure 1).¹⁰ The crystal structure illustrates the double hinge contact with the aminopyridine ring. It also displays a direct hydrogen bond between the phenol OH and Asp115, reinforcing the role of this interaction in MAP4K4 potency. The crystal structure also shows that the two flanking aromatic rings are not in plane relative to the central aromatic ring, thus mitigating ADME concerns of an otherwise planar triaromatic scaffold. To alleviate metabolic issues of a phenolic group, the pyridone analogue **14** was made, which led to a loss in both the enzyme and cell potencies. The reversed pyridone (**15**) regained some of the potency lost in **14** presumably due to the interactions with Asp115.

To optimize the hydrogen bond with Asp115, compound **16** was prepared, as modeling indicated that the NH₂ group of the newly introduced aminopyridine group interacts with Asp115 in a similar fashion as the phenol OH in **13**. Compound **16** (PF-6260933) was very potent against the enzyme with a very good ligand efficiency (LE = 0.57) and was the most potent analogue in the cell assay.

The kinome selectivity of **16** was assessed at a dose of 1 μ M in a panel of kinases (Figure 3). Bolstered by the promising

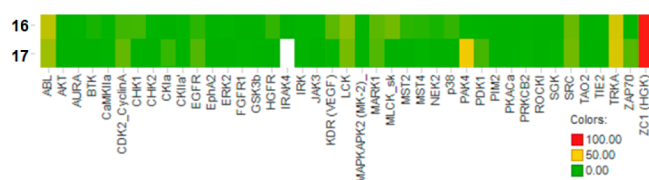


Figure 3. Heat map obtained from an Invitrogen selectivity panel (% inhibition of kinases tested @1 μ M).

selectivity, this compound was advanced to an ActivX screen, which allows for profiling protein and lipid kinase interactions in cell lysates.¹⁴ Remarkably, the initial *in vitro* kinome selectivity profile translated to cell lysates; with **16** demonstrating very good selectivity against more than 150 native kinases that could be quantified in human peripheral blood mononuclear cells; with only MAP4K4 (ZC1, HGK) and closely related GSK family members (ZC2, TNIK and ZC3, MINK) showing >70% inhibition at 1 μ M (see Supporting Information). The intrafamily lack of selectivity with an ATP-site inhibitor was expected as the ATP-site similarity is >95% between the three kinases (MAP4K4, TNIK, and MINK). Indeed, compound **16** showed a kinase activity IC₅₀ (tested at K_m) of 15 and 8 nM for TNIK and MINK, respectively.

Compound **16** demonstrated good physicochemical properties as well as moderate human liver microsomal (HLM) clearance (Table 3). Compound **16** had good solubility at both

Table 3. Physicochemical/ADME Properties for **16/17**

properties	16	17
MW/eLogD/TPSA ^a	296/3.3/78	297/2.8/91
HLM Cl _u (mL/min/kg) ^b	89	13
solubility, pH 1.2, mg/mL	13.0	3.96
solubility, pH 6.5, mg/mL	5.8	0.002
MDR BA/AB ratio ^c	2.7	1.1
drug–drug interactions (DDI)	>30 μ M ^d	>30 μ M ^e
CYP inhibition IC ₅₀	3 μ M (1A2)	
TDI ^f % inhibition (dose)	54% (30 μ M) 61% (60 μ M)	8% (30 μ M) 24% (60 μ M)
hERG IC ₅₀ ^g	1.8 μ M	>10 μ M
THLE IC ₅₀ ^h	>119 μ M	>300 μ M
AMES ⁱ	Positive	Negative

^aeLogD = measured logD at pH 7.4. ^bFree clearance calculated using measured protein binding in human liver microsomes (HLM). ^cMDR BA/AB efflux ratio using MDCK cell line transfected with human MDR1. ^d<15% Inhibition at 3 μ M for isoforms (3A4, 2D6, 2C8, and 2C9). ^e<15% Inhibition at 3 μ M for isoforms (3A4, 2D6, 1A2, 2C8, and 2C9). ^fChange in midazolam metabolism after incubation with compound. ^gMeasurement of potassium current in human embryonic kidney 293 (HEK293) cells stably transfected with the human ether-a-go-go-related gene (hERG) channel. ^hTransformed human liver epithelial cell assay (THLE). ⁱActivity in a bacterial mutagenesis assay.¹⁸

stomach and intestinal pH¹⁵ and a reasonably clean DDI profile. It also showed cytotoxicity (THLE assay) only at levels above 119 μ M. Thus, **16** was progressed to pharmacokinetic (PK) evaluation in the mouse. Following oral dosing (10 mg/kg), it displayed a plasma exposure that provided free drug concentrations above the cell IC₅₀ value for about 4–6 h after administration (Figure 4). Based on this profile, compound **16** was selected as a fit-for-purpose tool for *in vivo* pharmacological characterization.

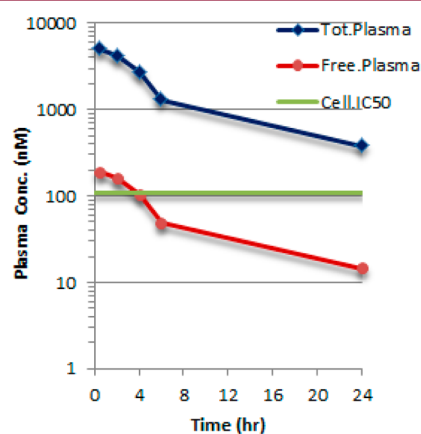


Figure 4. Total and free plasma concentration of **16** after PO dosing (10 mg/kg) to mice. Mouse plasma protein fraction unbound (*f*_u = 0.038).

To recapitulate the *in vivo* effects demonstrated by MAP4K4-siRNA,⁴ compound **16** was dosed orally (15 mg/kg) in a mouse model of LPS-induced TNF α levels in blood (compounds were dosed 30 min before an intravenous injection of LPS). Compound **16** showed >90% inhibition (similar to the effect of rosiglitazone) of LPS-induced plasma TNF α levels at 1.5 h with a corresponding plasma

concentration of 439 ng/mL (unbound levels of 56.2 nM, which was lower than the cellular IC₅₀).

In order to gain further understanding of the *in vivo* effect of a MAP4K4 inhibitor, compound **16** and rosiglitazone, a thiazolidinone-based antidiabetic drug, were tested in the *ob/ob* rodent model of type II diabetes.^{19,20} Animals were treated with vehicle (*n* = 12), rosiglitazone (*n* = 12) at 1 mg/kg, p.o., bid, or **16** (*n* = 12) at 10 mg/kg, p.o., bid for 4 weeks. Compound **16** (10 mg/kg, bid) provided significant improvement in fasting hyperglycemia (44% reduction in blood glucose, Figure 5), which was comparable to the effect observed with rosiglitazone and also consistent with the profile observed with MAP4K4-siRNA knockdown.⁵

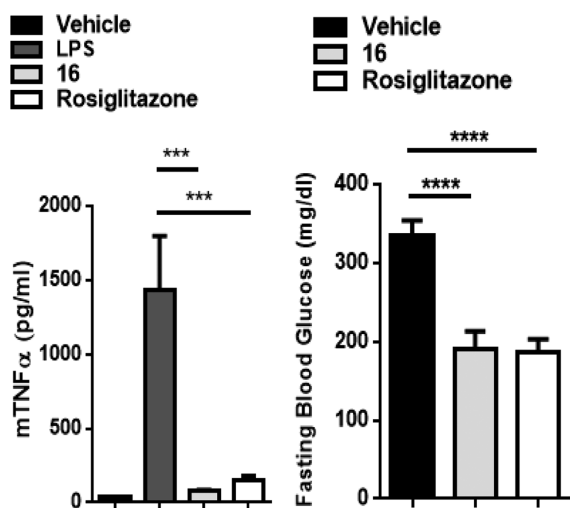


Figure 5. (a) Effects of **16** on LPS-induced TNFα levels in CS7-BL/6J mice). (b) Effects of **16** on glycemic control in *ob/ob* mice.

Despite its kinase selectivity and *in vivo* activity, compound **16** had liabilities that prevented its advancement beyond its utility as a biological tool compound. Compound **16** displayed inhibition in the hERG assay, nonkinase pharmacological promiscuity (Cerep), and demonstrated time-dependent inhibition (TDI)¹⁶ of CYP3A4, which led to drug accumulation and toxicity when dosed beyond 10 mg/kg in mice. Toxicity limited dosing to 10 mg/kg bid in chronic pharmacodynamic models, though this was sufficient to provide ~24 h coverage of cellular IC₅₀ levels. In addition, compound **16** had a mutagenic risk demonstrated by the AMES assay.¹⁸

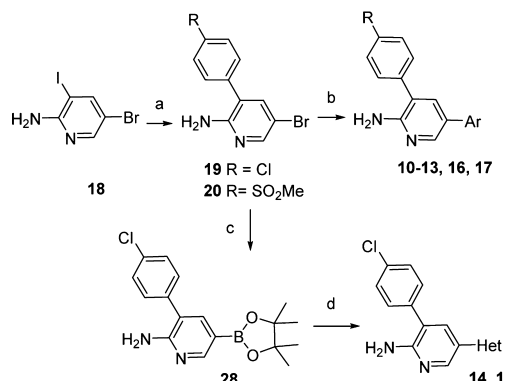
In order to understand the underlying causes of TDI, compound **16** was subjected to incubation with human liver microsomes. These studies showed that oxidation of the distal aminopyridine ring leads to hydroxylation of the ring followed by metabolism to a putative pyridoquinone-imine species that was trapped with glutathione (Supporting Information). Thus, a covalent adduct of the pyridoquinone-imine intermediate with CYP3A4 could result in the observed TDI and may also be contributing to the observed mutagenicity.

Various design strategies to attenuate the observed TDI were explored.²¹ Addition of substituents *ortho* to the terminal amine (F, CN, CF₃) or incorporation of additional nitrogen atoms to the aminopyridine core provided no improvement. However, conversion of the aminopyridyl tail to the corresponding 2-aminopyrimidine (**17**) retained potency with a good ligand efficiency (LE = 0.53) and kinase selectivity profile (Figure 3), while eliminating the TDI risk (Table 3). Gratifyingly,

compound **17** was negative in the AMES assay. Commensurate with the reduced lipophilicity, **17** demonstrated a more desirable physicochemical, metabolic (Table 3), and off-target profile (Supporting Information). Compound **17** thus offered a lead compound for further development of a MAP4K4 inhibitor with an improved overall profile.

Compounds **1**–**9** were synthesized according to modified literature procedures (Supporting Information). Scheme 1

Scheme 1. Synthesis of Pyridines **10**–**17**^a



^aConditions: (a) 4-ClPhB(OH)₂ (**21**) or (4-SO₂Me)PhB(OH)₂ (**22**), Pd(dppf)Cl₂, Na₂CO₃, dioxane, H₂O, 110 °C or KOH, PhCH₃, EtOH, H₂O, 140 °C; (b) (4-CONH₂)PhB(OH)₂ (**23**) or PhB(OH)₂ (**24**), (4-OH)PhB(OH)₂ (**25**), or (pyridin-2-NH₂)-B₂Pin₂, (**26**), or (pyrimidin-2-NH₂)Bpin (**27**); catalyst: Ph(AMPhos)₂Cl₂, Pd(dppf)Cl₂, or Pd(Ph₃P)₄; base: Na₂CO₃, K₂CO₃, Cs₂CO₃, or KOH; solvent: EtOH, THF, PhCH₃, or dioxane, H₂O, heat; (c) B₂Pin₂ (**26**), Pd(dppf)Cl₂, KOAc, dioxane, 60 °C; (d) 5-bromopyridin-2-ol (**28**) or 4-chloropyridin-2-ol (**29**), Pd(dppf)Cl₂, Na₂CO₃, dioxane, H₂O, 110 °C.

illustrates the synthesis of the analogues having 3,5-disubstituted-pyridine (or 2-aminopyridine) as the hinge binding group. Commercially available halopyridine **18** underwent sequential Suzuki couplings with a variety of substituted phenylboronic acids or pinacol esters to provide compounds **10**–**13**, **16**, and **17**. Compounds **14** and **15** were obtained from bromide **19** via Pd-catalyzed borylation to provide a boronate ester **28** that was then coupled with the appropriate halogenated pyridones.

In summary, we describe two 2-aminopyridine leads **16** (PF-6260933)²² and **17** that are potent and selective MAP4K4 inhibitors. Compound **16** had suitable PK properties in mouse to be used as a tool in an *in vivo* model of diabetes. Compound **17** showed improved overall drug-like properties compared to **16** and thus offered a lead compound for advanced preclinical development. Further studies with these leads will be the subject of future publications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.5b00215.

Description of synthesis procedures and identification of compounds, experimental protocols for the *in vitro* and *in vivo* assays, ActivX results for compound **16** and metabolite ID information, and Cerep promiscuity profiles of compounds **16** and **17** (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MW, molecular weight; TPSA, topological polar surface area; HLM, human liver microsomes; LPS, lipopolysaccharide; dpfp, 1,1'-Bis(diphenylphosphino)ferrocene; B₂Pin₂, Bis(pinacolato)-diboron; hERG, human ether-a-go-go-related gene; PK, pharmacokinetic; TDI, time-dependent inhibition; MM-GB/SA, molecular mechanics with generalized born/surface area solvation; ND, not determined

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