Fragment-based identification and optimization of a class of potent pyrrolo[2,1-f][1,2,4]triazine MAP4K4 inhibitors

Lan Wang^a, Mark Stanley^a, Jason W. Boggs^b, Terry D. Crawford^a, Brandon J. Bravo^c, Anthony M. Giannetti^c, Seth F. Harris^d, Steven R. Magnuson^a, Jim Nonomiya^c, Stephen Schmidt^c, Ping Wu^d, Weilan Ye^e, Stephen E. Gould^e, Lesley J. Murray^b, Chudi O. Ndubaku^{a*}, Huifen Chen^{a*}

^aDepartment of Discovery Chemistry, ^bDepartment of Drug Metabolism and Pharmacokinetics, ^cDepartment of Biochemical and Cellular Pharmacology, ^dDepartment of Structural Biology, ^eDepartment of Molecular Oncology, Genentech, Inc., 1 DNA Way, South San Francisco, California 94080, United States

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ABSTRACT

MAP4K4 has been shown to regulate key cellular processes that are tied to disease pathogenesis. In an effort to generate small molecule MAP4K4 inhibitors, a fragment-based screen was carried out and a pyrrolotriazine fragment with excellent ligand efficiency was identified. Further modification of this fragment guided by X-ray crystal structures and molecular modeling led to the discovery of a series of promising compounds with good structural diversity and physicochemical properties. These compounds exhibited single digit nanomolar potency and compounds **35** and **44** achieved good *in vivo* exposure.

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Mitogen Activated Protein Kinase Kinase Kinase Kinase 4 (MAP4K4), also known as hepatocyte progenitor kinase-like/germinal center kinase-like kinase (HGK),¹ is a member of the Sterile 20 (Ste20) group of kinases,² which includes the closely-related enzymes TNIK and MINK1.^{3,4} Of particular interest, MAP4K4 has been linked to a number of key cellular processes including cell migration, adhesion, invasion and neuronal degeneration.^{5,6} The exact mechanism by which MAP4K4 functions in these processes has yet to be fully elucidated. Therefore, further understanding of the role of this kinase using selective small molecule inhibitors could prove informative as to the potential of MAP4K4's therapeutic applications. Towards this aim, we began to investigate whether such an inhibitor could be derived from a fragment-based lead discovery (FBLD) effort as a strategic starting point.⁷

A screen of 2361 fragments (molecular weight <350 Da) at 100 μ M as singletons was carried out using Surface Plasmon Resonance (SPR)⁸ to measure binding to recombinant MAP4K4 kinase domain, which identified 225 confirmed hits after 6-point K_D determination. The enzyme affinities of the hits had K_D values from 10–2010 μ M and ligand efficiencies (LE) from 0.30–0.67 (Figure 1).^{9,10}

Several fragment hits displayed excellent MAP4K4 SPR affinities and ligand efficiencies, and were followed up by medicinal chemistry efforts. We recently reported the optimization of fragment derived 4-amino-pyridopyrimidine series where a highly selective and ligand efficient lead compound was identified.¹¹ Herein we describe the optimization of a different fragment from the same SPR fragment screen.

Figure 1. Workflow of SPR fragment screening and hit follow up.

* Corresponding author. E-mail: ndubaku.chudi@gene.com.

* Corresponding author. E-mail: chen.huifen@gene.com.

Figure 2. A) The chemical structure of compound **1**. B) A docking model of **1** in the ATP binding site of a MAP4K4 structure. The structure is viewed from the opening of the ATP binding site with the N-terminal lobe on top and C-terminal lobe at the bottom. The kinase hinge is on the left side while the P-loop is on the right side. Intermolecular hydrogen bonds (including two non-classical C-H type) between fragment **1** and the kinase hinge are shown as green dashed lines.

A pyrrolotriazine hit **1** (Figure 2A) with SPR K_D = 88 μ M was of particular interest due to its excellent ligand efficiency of 0.56. First of all, this fragment was a known substructure in a number of kinase inhibitors including the VEGFR-2 inhibitor brivanib,¹² yet, to the best of our knowledge, it has not been reported as an inhibitor of MAP4K4. Evaluation of the binding modes of several inhibitors with the pyrrolotriazine substructure in other kinases (as reported for p38 α ^{13,14} and insulin-like growth factor (IGF) receptor¹⁵) suggested that compound **1** may bind in a different location within the ATP-binding pocket of MAP4K4 based on its K_D and LE, providing an opportunity to achieve selectivity against these other kinases.

Initial attempts to obtain a high-resolution crystal structure of compound **1** in complex with MAP4K4 were not successful. Therefore, in the absence of a crystal structure we relied on a docking model¹⁶ of this fragment hit bound to MAP4K4 kinase domain (Figure 2B). The docking model suggested that compound **1** binds to the adenine pocket of the ATP binding site with three possible intermolecular hydrogen bonds (including the non-classical C-H type) to the hinge region: one from the C5-hydrogen of the pyrrole ring to the carbonyl oxygen of Cys108, one from the N2 nitrogen of the triazine ring to the amide NH of Cys108, and another from the C3-hydrogen of the triazine ring to the carbonyl oxygen of Glu106. The NH₂ group is situated in the center of the ATP site and provides a vector to explore the regions near the catalytic Lys54 and phosphate binding region.

Building off this model, we decided to explore the region next to the primary amine group as the first round of hit expansion efforts. The corresponding compounds **3** to **9** were prepared from the commercially available material 4-chloropyrrolotriazine **2** using reactions shown in Scheme 1.

Scheme 1. Reagents and conditions: (i) Cs_2CO_3 , DMF, 150 $^\circ\text{C}$, 0.5 h; (ii) cat. 4 N HCl/dioxane, 150 $^\circ\text{C}$, 0.5 h, 58%.

Table 1. MAP4K4 SPR binding affinity of primary amine substitutions and ether replacement

Cmpd	Structure	MAP4K4 SPR K_D (μ M) ^a	MAP4K4 SPR LE ^b
1		88	0.56
3		155	0.48
4		125	0.41
5		17	0.44
6		48	0.37
7		8.7	0.43
8		510	0.30
9		693	0.27

^aAll SPR results are reported as the arithmetic mean of at least 2 separate runs. For details on the SPR assay, see Supplementary Information.

^bLE = $-RT\ln(\text{SPR } K_D)/N_{\text{heavy atoms}}$

Addition of simple alkyl groups to the amine moiety such as methyl (**3**) or branched isopropyl (**4**) led to reduced SPR binding affinity and ligand efficiency (Table 1). We reasoned from these results that the area of the protein in close proximity to this region of the molecule had a very shallow cavity, and would require a planar substituent for shape complementarity and possibly a hydrogen bond acceptor to pick up additional interactions with the catalytic Lys54 residue. Consequently, incorporation of a pyrazole or 3-pyridyl led to compounds **5** and **6**, each having improved SPR K_D (17 μ M for **5**, and 48 μ M for **6**). However there is a notable drop in the LE for both of these compounds, which is known to happen during fragment hit expansion.¹⁷ To test the effect of the position of H-bond acceptor, we also prepared the 4-pyridyl analog (**7**). Gratifyingly, we saw nearly 6-fold improvement in the potency of **7** in comparison to **6**, which also translated into better ligand efficiency (0.43 for **7** versus 0.37 for **6**). We also investigated additional mono-substituted (data not shown) and di-substituted (**8**) analogs. Compound **8** with a morpholine moiety lost approximately 60-fold in potency compared to **7**. We hypothesized that the potency loss in **8** was driven by the non-planarity of the morpholine ring. To test our hypothesis, we synthesized a more flexible ether analog (**9**) of compound **6** where the 3-pyridyl ring is likely to be out of plane.¹⁸ Compound **9** lost 14- and 80-fold in potency relative to compounds **6** and **7**, respectively, validating our hypothesis that the region tolerates only flat and rigid systems.

During the course of optimizations, an X-ray structure of the 3-pyridyl compound **6** in complex with MAP4K4 was solved (PDB code: 4U43) (Figure 3). The overall protein structure is very similar to the recently published MAP4K4 structures with the exception of its P-loop conformation.^{11,19,20} The P-loop in this structure adopts an extended conformation and does not fold in as described previously. A survey of the overlay with a folded P-loop structure (Figure S1) suggests that compound **6** could be accommodated into the folded P-loop conformation despite not being able to form favorable interactions with the P-loop Tyr36 side chain. This P-loop conformation change could be series dependent and might have impacts on kinase selectivity. The

binding mode of **6** is identical to the predicted docking pose for the original fragment hit **1**. It is noteworthy from the structure that the nitrogen on the 3-pyridyl ring is not able to form a hydrogen bond with Lys54 due to poor hydrogen bond angles. On the contrary, the 4-pyridine nitrogen position would allow for better polar interaction with Lys54, as indicated by the affinity improvement going from compound **6** to **7**.

Figure 3. X-ray crystal structure of compound **6** (cyan) in complex with MAP4K4, solved at 2.18 Å resolution (PDB code: 4U43). Intermolecular hydrogen bond interactions (including the non-classical C-H type) are shown as green dashed lines.

The substitution of a primary amine at the right-hand side improved the potency of fragment hit **1** from K_D of 87 μ M to 8.7 μ M (Table 1). At this point, we started to test compounds in a biochemical activity assay and compound **7** showed an IC_{50} of 1.6 μ M (SPR K_D : 8.7 μ M). We began exploration around the left-hand side pyrrole area to further improve the potency while keeping the calculated logD (clogD) less than 3.5. A wide variety of substitutions including an ester, amide, hydroxymethyl, phenyl and heterocycles were examined to identify the best substituent with good potency and stability. The compounds described subsequently in Table 2 were generally prepared by Pd-catalyzed coupling reactions of **10** or, alternatively, by transformations of **12** shown in Scheme 2.

Scheme 2. Reagents and conditions: (i) 4 N HCl/dioxane, 150 °C; (ii) RB(OH)₂, Pd(dppf)Cl₂, Cs₂CO₃, CH₃CN, H₂O, 100 °C; (iii) Zn(CN)₂, Pd(PPh₃)₄, DMF, 100 °C; (iv) NaN₃, Et₃N, DMSO/DMAc, 110 °C; (v) LiOH, MeOH; (vi) RNH₂, HATU, DIPEA, DMF; (vii) LiAlH₄, 1,4-dioxane; (viii) CH₃Li, THF.

Table 2. SAR on the left hand side expansions of pyrrolotriazine

Cmpd	R	MAP4K4 LC3K IC ₅₀ (μM) ^a	MAP4K4 LC3K LE ^b	Kinetic Solubility (μM)	cLogD	LM CL _h (H/M) (ml/min/kg) ^c
7	H	1.6	0.43	ND	1.6	ND
11	Br	0.15	0.55	ND	2.6	ND
13		0.07	0.47	1	2.2	19 / 76
14		0.71	0.49	18	1.3	15 / 72
16		0.008	0.51	1	3.5	18 / 66
17		0.16	0.52	53	0.8	11 / 79
18		0.84	0.42	171	1.8	8 / 82
19		0.30	0.47	7	0.4	9 / 79
20		0.64	0.40	28	1.3	14 / 76
21		0.13	0.43	1	0.9	16 / 79
22		0.18	0.33	186	0.3	8 / 73
23		0.01	0.52	14	2.6	14 / 56
24		0.07	0.40	13	2.0	3 / 60
25		0.01	0.53	1	1.8	16 / 70

^aAll biochemical results are reported as the arithmetic mean of at least 2 separate runs. For details on the LC3K assay, see Supplementary Information.

ND: Not determined.

^bLE = $-RT \ln(\text{IC}_{50}) / N_{\text{heavy atoms}}$

^cLM CL_h (H/M): Predicted human and mouse hepatic clearance using liver microsomes.

Based on the crystal structure of compound **6** (Figure 3), there is a partially hydrophobic pocket adjacent to the pyrrole ring formed by the side chains of Phe107, Cys108 and Val31 (not shown) where additional substitutions can be introduced to improve potency. Beyond this cavity, MAP4K4 opens up to solvent providing opportunities to add polar and solubilizing groups to improve solubility and pharmacokinetics profiles. We began by evaluating a simple bromo substitution (**11**), a versatile intermediate for further substitutions, and saw dramatic improvement in potency (from IC₅₀ of 1.6 for H to 0.15 μM for Br) and ligand efficiency from 0.43 to 0.55 (Table 2). Due to the lipophilic nature of this substituent, the potency increase may be driven by desolvation effects and van der Waals interactions. This initial success suggested that substitutions in this region could improve potency. Cyano substitution (**14**) resulted in significant loss of potency but an ethyl ester substitution (**13**)

improved potency by 2-fold compared to the bromo substitution (**11**). The phenyl substituted analog **16** was approximately 20-fold more potent than **11**, and was the first compound in this series to achieve single digit nanomolar potency. Despite the excellent potency, compound **16** had poor solubility and in vitro stability, which was attributed to high cLogD. We tried other more polar substitutions with the aim of improving solubility and stability. All the alcohol (compounds **17** and **18**) and amide (compounds **19** to **22**) substitutions with the exception of **21** resulted in potency loss in comparison to the bromo substitution (**11**), mainly due to the flatness of the pocket and its relatively hydrophobic nature. However, these substitutions did improve solubility significantly and also improved in vitro stability in human liver microsomes. We tried several five-membered heterocycles, and found that furan (**23**) and 4-pyrazole (**25**) analogs achieved equivalent potency to the phenyl analog (**16**), but the tetrazole analog (**24**) showed lesser potency. The furan motif is a structural alert that has the potential risk of metabolic activation and cross-reaction,²¹ and thus was not pursued further. Based on the SAR information and synthetic tractability, the phenyl (**16**) and pyrazole (**25**) analogs served as good templates for further optimization.

While the potency of phenyl analog **16** is remarkable, the physical properties of the molecule began to diverge outside of ideal drug-like space, especially with respect to lipophilicity. Compound **16** also had poor solubility, which could lead to poor absorption and pharmacokinetic issues. Therefore we focused the optimization of **16** on reducing lipophilicity by installing polar substitutions or introducing heteroatoms on the phenyl ring (Table 3). Compounds **26-41** in Table 3 were prepared by the similar Pd-catalyzed coupling reactions used in Scheme 2.

Figure 4. Crystal structure of compound **16** (magenta) in complex with MAP4K4, solved at 2.43 Å resolution (PDB code: 4U44). Intermolecular hydrogen bond interactions (including non-classical C-H type) with the hinge are shown as green dashed lines. Other potentially favorable intermolecular electrostatic interactions are shown in yellow dashed lines.

A crystal structure of compound **16** in complex with MAP4K4 was determined (Figure 4). Similar to the crystal structure with compound **6**, the P-loop adopts an extended conformation. It confirmed that the phenyl ring binds in a relatively flat and partially hydrophobic region with the para position pointing towards solvent. Based on the structure, the ortho position could allow for small substitutions. The fluoro-substituted analog (**26**) was well tolerated, while both methyl (**27**) and Cl-substitutions (**28**) led to potency loss (Table 3). One possible reason for the observed potency loss with larger groups is that the phenyl ring had to rotate out of plane significantly and would not fit well into a rather flat pocket. Conversely the meta substitutions were better tolerated due to slightly larger space with the hydroxyl methyl substitution (**31**) having the best potency and overall properties. However, **31** still lacked good solubility and in vitro

stability. We then turned to the para position where the protein/ligand structure predicted greater flexibility in the types of groups that could be placed here. Therefore a wide variety of polar groups were investigated (**32** to **39**) and all showed excellent potency and reasonable metabolic stability. Although all of the para-substituted analogs that were made (other than the para-F analog **32**) had lower clogD, they still showed poor

solubility with the exception of compound **34** that bears a basic amine. We then tried to introduce nitrogen atoms to the ring and both compound **40** and **41** showed excellent potency and good stability, yet only **40** that again bears a basic amine showed reasonable solubility. This prompted us to begin evaluating the front pocket pyrazoles as an alternative sub-series to improve the solubility of the compounds.

Table 3. Modifications on the left hand side phenyl ring to improve potency and solubility

Cmpd	X	R ¹	R ²	R ³	MAP4K4 LC3K IC ₅₀ (nM) ^a	MAP4K4 LC3K LE ^b	Kinetic Solubility (μM)	cLogD	LM CL _h (H/M) (ml/min/kg) ^c
16	CH	H	H	H	8	0.51	1	3.5	18 / 66
26	CH	F	H	H	11	0.47	1	3.6	ND
27	CH	CH ₃	H	H	19	0.46	1	3.9	15 / 71
28	CH	Cl	H	H	37	0.44	1	4.1	ND
29	CH	H	CH ₃	H	7	0.49	1	3.9	11 / 71
30	CH	H	Cl	H	23	0.46	1	4.2	15 / 56
31	CH	H	CH ₂ OH	H	3	0.49	1	2.7	16 / 78
32	CH	H	H	F	10	0.48	1	3.7	14 / 70
33	CH	H	H	CH ₂ OH	6	0.47	1	2.7	14 / 78
34	CH	H	H	CH ₂ N(CH ₃) ₂	1	0.47	82	1.9	10 / 77
35	CH	H	H	SO ₂ CH ₃	4	0.44	1	2.2	14 / 62
36	CH	H	H	SO ₂ CH ₂ CH ₃	2	0.44	1	2.8	13 / 52
37	CH	H	H	SO ₂ NHCH ₃	2	0.45	1	2.2	11 / 52
38	CH	H	H		1	0.43	1	3.0	11 / 48
39	CH	H	H		2	0.43	1	3.4	12 / 23
40	N	H	H		2	0.41	52	2.3	14 / 60
41	N	H	H		2	0.43	1	2.6	16 / 56

^aAll biochemical results are reported as the arithmetic mean of at least 2 separate runs. For details on the LC3K assay, see Supplementary Information.

ND: Not determined.

^bLE = $-RT\ln(\text{IC}_{50})/N_{\text{heavy atoms}}$.

^cLM CL_h (H/M): Predicted human and mouse hepatic clearance using liver microsomes.

A crystal structure of compound **25** in complex with MAP4K4 was determined and the binding site region is shown in Figure 5. As predicted from modeling, the pyrazole binds to the relatively flat, partially hydrophobic front pocket region with the pyrazole 1-NH and N2 atoms facing solvent. The 1-NH of pyrazole provides a good handle to explore substitutions to address the aforementioned issues related to solubility and metabolic clearance without adversely affecting potency (Table 4). Compounds **42** to **46** were made analogously to the previously described Suzuki coupling reactions (Scheme 3). Substitution with morpholino ethyl at N1 (**42**) resulted in dramatic improvement in solubility while maintaining potency. However, metabolic clearance remained high in liver microsomes despite the increase in polarity presumably due to a shift of the site of P450 oxidation to the morpholine ring. A metabolite ID experiment was not carried out to confirm this hypothesis. We next explored alcohol substitutions, **43** and **44**, and saw marginally improved potency and significant improvement in solubility. The clearance in human liver microsomes was improved slightly, but not in mouse liver microsomes. Finally by investigating amides (**45** and **46**) we achieved a balance of solubility and metabolic stability. The potency for these compounds was very similar to the unsubstituted pyrazole **25**. Overall the strategy to go to substituted 4-pyrazoles was fruitful and yielded compounds with significantly improved solubility and in some cases also better in vitro metabolic stability.

Scheme 3. Suzuki coupling conditions: Pd(dppf)Cl₂, Cs₂CO₃, CH₃CN, H₂O, 100 °C.

Figure 5. Crystal structure of compound **25** (green) in complex with MAP4K4, solved at 2.58 Å resolution (PDB code: 4U45). Intermolecular hydrogen bond interactions (including non-classical C-H type) with the hinge are shown as green dashed lines. Other possible favorable electrostatic interactions are shown in yellow dashed lines.

Based on the available data that were gathered thus far, three compounds (**35**, **40**, and **44**) were selected for evaluation in mouse PK studies (Table 5). Both compounds **35** and **44** were stable in vivo while compound **40** was cleared more rapidly. The clearances were over-predicted by the in vitro liver microsome assay. The PK profile of **35** and **44** were deemed acceptable for further evaluations in vivo. To probe their broader kinase selectivity profiles, compounds **35** and **44** were profiled against a 63-kinase selectivity panel from Invitrogen using 1 μM test compound concentrations (Table 5). The results were

confounding since the less soluble compound **35** exhibited reasonable selectivity profile (>75% inhibition of only 6/63 kinases) while the highly soluble compound **44** was much less selective (>75% inhibition of 16/63 kinases).²² This suggested that compound **35** could be precipitating out of solution in the selectivity assays and the good selectivity profile could be due to lower actual concentration than the 1 μM test concentration. A number of other compounds were also tested in the kinase selectivity panel. However, none exhibited a clean profile to warrant further in vivo evaluation.

Table 4. SAR on the 1-N substitutions of the left hand side 4-pyrazole

Cmpd	R	MAP4K4 LC3K IC ₅₀ (nM) ^a	MAP4K4 LC3K LE ^b	Kinetic Solubility (μM)	cLogD	LM CL _h (H/M) (ml/min/kg) ^c
25	H	8	0.53	1	1.8	16 / 70
42		6	0.39	108	1.5	14 / 71
43		3	0.48	19	1.3	13 / 67
44		5	0.44	106	2.0	10 / 67
45		10	0.42	3	1.1	9 / 54
46		8	0.40	100	1.6	7 / 59

^aAll biochemical results are reported as the arithmetic mean of at least 2 separate runs. For details on the LC3K assay, see Supplementary Information.

^bLE = -RTln(IC₅₀)/N_{heavy atoms}.

^cLM CL_h (H/M): Predicted human and mouse hepatic clearance using liver microsomes.

Table 5. Summary of PK parameters and kinase selectivity profiles of lead compounds

Compound	35	40	44
MAP4K4 LC3K IC ₅₀ (nM) ^a	4	2	5
Kinetic solubility (μM)	<1	53	106
Mouse i.v. CL _P (ml/min/kg), 1 mg/kg dose	25	78	22
V _{ss} (L/kg)	1	15	2
F%, 25 mg/kg dose	72	12	ND
# of kinases out of 63 inhibited >75% @ 1 μM	6	ND	16

^aAll biochemical results are reported as the arithmetic mean of at least 2 separate runs. For details on the LC3K assay, see Supplementary Information.

ND: Not determined.

The lack of kinase selectivity for this series could be related to the extended P-loop conformation. Based on an observation we and others have published on, a folded P-loop conformation in MAP4K4 may contribute to better kinase selectivity of bound ligands.^{11,19} This suggests that a way to improve the kinase selectivity of this series is to design changes to induce a folded P-loop conformation.

In summary, we have applied a fragment-based discovery approach to identify small molecule MAP4K4 inhibitors. Starting from a micromolar pyrrolotriazine fragment hit, we developed a series of MAP4K4 inhibitors with single digit nanomolar IC₅₀ by utilizing crystal structures and molecular modeling. We also used calculated properties to guide the optimization of solubility and in vitro stability. The compounds not only have excellent biochemical potencies, but also maintained excellent ligand efficiencies. Several compounds also demonstrated favorable in vivo bioavailability in mouse. One issue with the series is the moderate kinase selectivity. To further progress the compounds so they can be useful as in vivo tool compounds, additional optimization of kinase selectivity is required.

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- Four distinct binding modes were observed in the top ten docking poses ranked by Glide docking score. Two of the binding modes were eliminated based on the SPR data on another close-in pyrrolotriazine fragment in the fragment library. The other two poses are related by transposition of the three point hinge interactions shown in Figure 2B. The pose that was selected for use had more favorable intermolecular interactions with the hinge that is more consistent with its SPR K_D and LE.
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- Kinase selectivity data generated at Invitrogen® at 1 μ M test concentration with inhibition >75% for **35**: Cdk1 (81%); Flt3 (92%), KDR (94%), KHS1 (83%), MAP4K4 (101%), Mink1 (100%); for **44**: Aurora A (87%), CDK1 (98%), FLT3 (97%), GSK3 β (84%), KDR (98%), KHS1 (96%), MAP4K4 (104%), MARK1 (84%), MKNK1 (87%), Mink1 (103%), PDGFR α (75%), PLM1 (76%), RSK1 (78%), SYK (87%), TSSK1 (91%), TYK2 (96%).