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Letter

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Discovery of a Type III Inhibitor of LIM Kinase 2 That Binds in a DFG-Out Conformation

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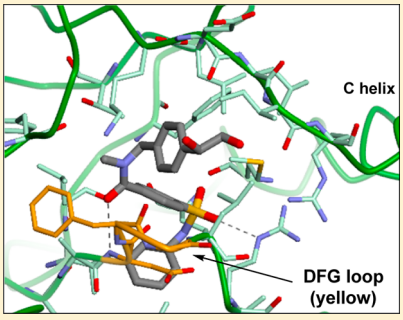
Supporting Information

ABSTRACT: The first allosteric, type III inhibitor of LIM-kinase 2 (LIMK2) is reported. A series of molecules that feature both an *N*-phenylsulfonamide and tertiary amide were not only very potent at LIMK2 but also were extremely selective against a panel of other kinases. Enzymatic kinetic studies showed these molecules to be noncompetitive with ATP, suggesting allosteric inhibition. X-ray crystallography confirmed that these sulfonamides are a rare example of a type III kinase inhibitor that binds away from the highly conserved hinge region and instead resides in the hydrophobic pocket formed in the DFG-out conformation of the kinase, thus accounting for the high level of selectivity observed.



KEYWORDS: *LIM kinase 2, DFG-out, type III kinase inhibitor, allosteric, selective, noncompetitive*

Protein kinases continue to be of interest in the pharmaceutical industry because of their roles in phosphorylation cascades, which control many cellular processes within the cell. Deregulation of protein kinase activity has been implicated in a variety of therapeutic areas, including cancer, metabolic disorders, and immunology.[1] Inhibitors of kinases have been pursued for many years resulting in a number of approved small molecule drugs.[2] As a better understanding of kinase inhibitors has been developed, five different classes of inhibitors have been identified, in addition to covalent inhibitors. Type I inhibitors bind in the ATP-binding pocket and compete with the adenine moiety of ATP for the essential hydrogen bond interactions in the "hinge region". Type II inhibitors were defined with the discovery of imatinib.[3] These inhibitors not only occupy the ATP-binding pocket but also extend into a hydrophobic, allosteric pocket that is formed when the Asp-Phe-Gly (DFG) residues of the activation loop fold out ("DFG-out") from their normal position and stabilize an inactive conformation of the kinase.[4−7] Types III−V inhibitors have been defined more recently. Type III inhibitors bind in the catalytic domain of the kinase near the ATP-binding pocket but do not interact with the hinge region and include molecules that bind in the hydrophobic pocket formed in the DFG-out confirmation.[7−10] Type 4 inhibitors bind in a truly allosteric site away from the catalytic domain of the kinase.[11,12] Lastly, type V inhibitors are bivalent or bisubstrate compounds.[13,14] The residues in the catalytic domain of kinases are highly conserved, especially in the ATP-binding pocket, making selectivity particularly difficult to achieve with type I and some type II inhibitors. Thus, identification of allosteric inhibitors that do not occupy the hinge region of the ATP-binding pocket would provide molecules with a greater level of specificity for their kinase target and minimize off-target pharmacology.

LIM-kinases (LIMK) 1 and 2 have been implicated as key regulators of the dynamics of actin polymerization in the cytoskeleton due to their role in the phosphorylation and subsequent deactivation of cofilin, a protein that depolymerizes the actin filaments.[15] LIM-kinases have been investigated for their role in a variety of therapeutic indications, including cancer[16] and, as we previously reported, open-angle glaucoma.[17] In an effort to identify an alternative chemical series to inhibit LIMK2, high-throughput screening identified sulfonamide **1** as a low micromolar inhibitor. The low molecular weight and modular structure that would be amenable to a rapid structure−activity relationship (SAR) made it an attractive candidate for further investigation.

Our initial lead optimization efforts focused on the sulfonamide side. Reversal of the *S*-thiophenylsulfonamide linkage of **1** to the *N*-phenylsulfamoyl linker of **2** resulted in an 800-fold increase in potency (Figure 1). With this low nanomolar inhibitor in hand, further in vitro characterization was done to establish its selectivity profile against a panel of representative kinases from the seven kinase families at low millimolar physiological ATP concentration,[18,19] while the LIMK2 biochemical assay was run at a slightly lower

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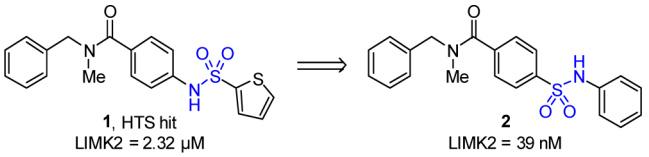


Figure 1. Lead identification of arylsulfonamide series.

concentration of ATP.[20] As ATP has a relatively weak affinity for LIMK2 (K_m = 85 μM) compared to other kinases, overall selectivity of compound **2** will likely increase at the higher physiological ATP concentrations. As shown in Chart 1, sulfonamide **2** was surprisingly selective for LIMK2 (39 nM) in this screen and even had almost a 100-fold level of selectivity against the closely related LIMK1 (3.2 μM).

Chart 1. Selected Kinase Panel (μM)[a]

TKL family		*STE family*		*TK family*	
LIMK2	**0.039**	MST1	>100	Abl	5.6
LIMK1	3.2	MST2	>100	Src	>100
IRAK4	>100	MAP4K4	>100	Syc	>100
Raf1	>100	MAP4K2	>100	Lck	10.9
AGC family		MAP4K5	>100	Fgr	>100
Rock2	>100	*CMGC family*		Flt1	>100
PKCα	>100	CDK1	>100	Jak3	>100
PKA	>100	CDK2	>100	Jak2	>100
PDK1	>100	GSK3β	>100	*CAMK family*	
		ERK2	>100	CHK2	>100

[a]LIMK2 assays conducted in the presence of 300 μM ATP. Other assays were conducted in the presence of 1 mM ATP.

The spectacular level of selectivity of sulfonamide **2** led us to examine the nature of the inhibition of this compound. This level of selectivity is rare among kinase inhibitors, and thus, it was proposed that this selectivity could be explained if sulfonamide **2** was binding in an allosteric manner away from the more conserved hinge region of LIMK2 and thus would not bind competitively against ATP. To determine whether or not sulfonamide **2** competes with ATP for binding to LIMK2, kinetic experiments varying both ATP levels and inhibitor concentrations were performed using **2** and a known type I LIMK2 inhibitor.[21] As shown in Figure 2a, the ATP Michaelis–

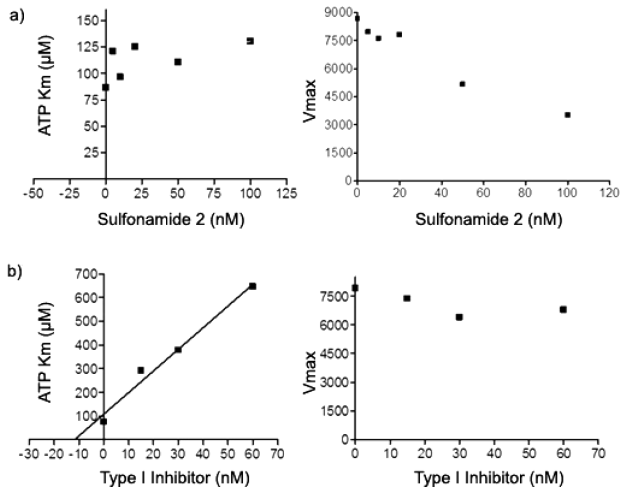


Figure 2. (a) Enzyme kinetics of sulfonamide **2**. (b) Enzyme kinetics of a known ATP competitive LIMK2 inhibitor. See text for more detailed description.

Menten constant (K_m) is unaffected and V_{max} decreases as the concentration of sulfonamide **2** increases. In contrast, as shown in Figure 2b, titration of the known ATP competitor of LIMK2 results in a linear increase in the ATP K_m but no change in V_{max}.[22] A Lineweaver–Burk plot (Figure 3) was completed to

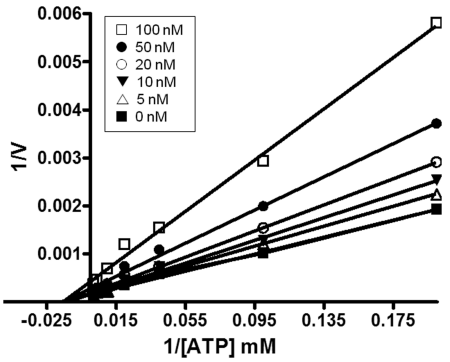
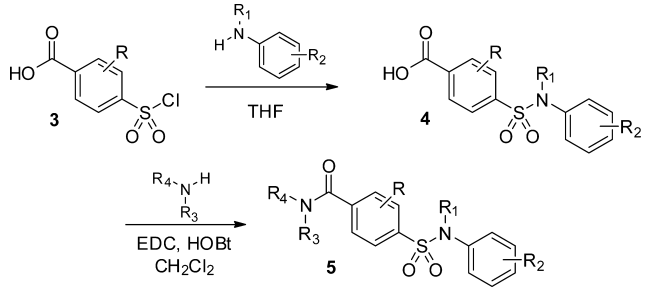


Figure 3. Lineweaver–Burk plot of the inverse reaction rate against the inverse concentration of ATP, where each line represents a different concentration of sulfonamide **2**.

further characterize the type of enzyme inhibition. The plots for six different concentrations of inhibitor **2** intersect on the *X*-axis but have different slopes and points of intersection on the *Y*-axis. This suggests that **2** is functioning as a noncompetitive inhibitor LIMK2, rather than an uncompetitive (lines never intersect), competitive (lines intersect on the *Y*-axis), or mixed inhibitor (lines intersect somewhere in between the *X*- and *Y*-axes).[23,24]

The excitement over this chemical series of selective, allosteric inhibitors prompted a rapid expansion of the SARs to increase the potency at LIMK2. The synthetic strategy, outlined in Scheme 1, was straightforward. Substituted anilines

Scheme 1. General Procedure for Sulfonamides



were reacted with commercially available 4-(chlorosulfonyl)-benzoic acid **3** to form *N*-aryl sulfonamides **4**. These sulfonamides were then reacted with substituted amines in the presence of 1-ethyl-3-(3- dimethyl-aminopropyl) carbodiimide (EDCI) and *N*-hydroxybenzotriazole (HOBt) to provide functionalized amides **5**.

SAR studies began with the *N*-phenyl of the sulfonamide. Replacements of the phenyl group with heteroaryl, alkyl, or cycloalkyl groups were detrimental to potency (not shown). Changes to the aryl group of the sulfonamide are shown in Table 1. In general, only minor substitutions to the phenyl ring were permitted. Addition of groups at the ortho position (**6**–**8**) and para position (**9**–**11**) led to a decrease in LIMK2 activity. Small groups such as fluoro (**12**) and methyl (**14**) installed at the meta position did provide a slight improvement in activity,

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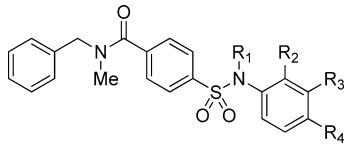
Table 1. SAR of the Aryl Sulfonamide



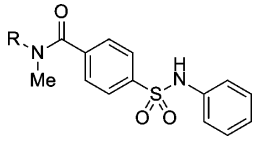
cmpd	R_1	R_2	R_3	R_4	LIMK2 IC_{50} (μM)[a]
2	H	H	H	H	0.039
6	H	F	H	H	0.052
7	H	CH_3	H	H	0.12
8	H	OCH_3	H	H	1.0
9	H	H	H	F	0.081
10	H	H	H	CH_3	3.2
11	H	H	H	OCH_3	24.1
12	H	H	F	H	0.022
13	H	H	CI	H	0.051
14	H	H	CH_3	H	0.022
15	H	H	OCH_3	H	0.54
16	H	H	*t*-Bu	H	>27
17	H	H	NMe_2	H	>27
18	CH_3	H	H	H	0.095

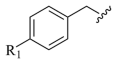
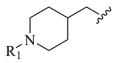
[a]IC_{50} values are averages of 4−8 indepedent IC_{50} measurements.

but as the size continued to increase, potency abated (**16** and **17**). Capping of the amide with a methyl group (**18**) also provided a less active compound.

Unlike the limited SAR exhibited in Table 1, the tertiary amide on the left side of the molecule had a greater scope (Table 2). While the amide itself was irreplaceable, the benzyl group could be elaborated or replaced with a saturated ring system. In particular, the para position on the benzyl group could tolerate a wide variety of substitutions. Installation of neutral groups, as shown in ether **20** and carboxylic acid **21**,

Table 2. SAR of the Benzyl Group of the Amide



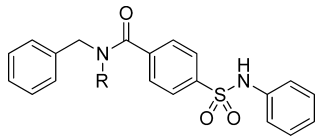
cmpd	R	R_1	LIMK2 IC_{50} (μM)[a]
2		H	0.039
19		Et	0.053
20		$O(CH_2)_2OMe$	0.034
21		$(CH_2)_2CO_2H$	0.019
22			0.092
23		4-piperidine	1.64
24		C(O)Me	0.90
25		CO_2t-Bu	0.036
26		H	>27
27		Me	>27
28		*i*-Pr	>27
29			0.11
30			0.12

[a]IC_{50} values are averages of 4−8 indepedent IC_{50} measurements.

were equipotent or 2-fold more potent, respectively, than the parent compound **2**. However, pendant basic amines, such as piperidine **23**, led to a marked decrease in potency. Replacement of the benzyl group with a saturated piperdine moiety retained submicromolar potency as long as the nitrogen remained electron deficient (**24** and **25**), but when the amine was rendered basic by simple *N*-alkyl substitution (**27** and **28**), compounds lost all activity at LIMK2. Lastly, substitution at the methylene position on the benzyl was investigated. Small groups like methyl were tolerated (enantiomers **29** and **30**), but larger groups lost potency (data not shown).

SAR at the *N*-methyl position of the benzyl amide of **2** was investigated (Table 3). Similar to the benzyl position, the scope

Table 3. *N*-Substitution SAR of the Benzyl Amide



cmpd	R	LIMK2 IC_{50} (μM)[a]
2	Me	0.0039
31	Et	0.003
32	$(CH_2)_2OH$	0.019
33	$(CH_2)_2CN$	0.052
34	$(CH_2)_2NMe_2$	0.16
35	$(CH_2)_2NH_2$	1.2
36	$(CH_2)_3NH_2$	1.5

[a]IC_{50} values are averages of 4−8 indepedent IC_{50} measurements.

of this position allowed for a variety of elongated, neutral moieties to be incorporated while retaining potency at LIMK2 (**31**−**33**), but basic amines were less potent (**34**−**36**). The simple exchange of *N*-methyl for *N*-ethyl (**31**) added a 10-fold increase in potency, providing the most potent compound synthesized in this series at 3 nM.

Finally, we hypothesized that a 1,2- or 1,3-disubstituted heterocycle could be a viable isosteric replacement for the amide. 1,2-Disubstitution patterns were found to be preferred over 1,3-disubstitution (data not shown). Various 1,2-disubstituted heterocycles are shown in Table 4. The most potent isosteres kept a hydrogen acceptor that mapped onto the oxygen of the amide carbonyl. Imidazole **39** was the most potent of this series, while pyridine **40** and pyrazine **41** also retained LIMK2 potency. However, none of these compounds were more potent than the simple *N*-ethyl-*N*-benzylamide **31**.

Because of its good aqueous solubility and the decent potency, compound **22** was selected to be cocrystallized with LIMK2. The X-ray structure was obtained and confirmed the type III binding mode of these sulfonamide inhibitors. As shown in Figure 4, ligand **22** is exclusively binding in the hydrophobic pocket formed when the DFG residues of the activation loop are in the DFG-out conformation. The carbonyl of the amide participates in an interaction with the backbone N−H of D469 of the DFG residues in the activation loop, thus explaining the observation that a hydrogen bond acceptor was critical at this position. Meanwhile, the sulfonamide carbonyl further anchors the ligand by forming a hydrogen bond with the R474 residue adjacent to the DFG motif on the activation loop. The nonpolar phenyl group of the sulfonamide contributes an additional hydrophobic interaction by occupying the newly formed hydrophobic cleft. Occupation of this small

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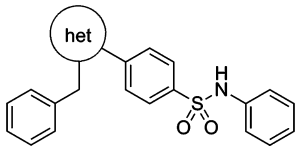
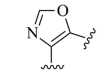
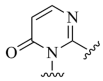
Table 4. Heteroaryl Isosteres for the Amide Group



cmpd	heteroaryl	LIMK2 IC_{50} (μM)[a]
37		1.53
38		>27
39		0.022
40		0.10
41		0.090

[a]IC_{50} values are averages of 4−8 indepedent IC_{50} measurements.

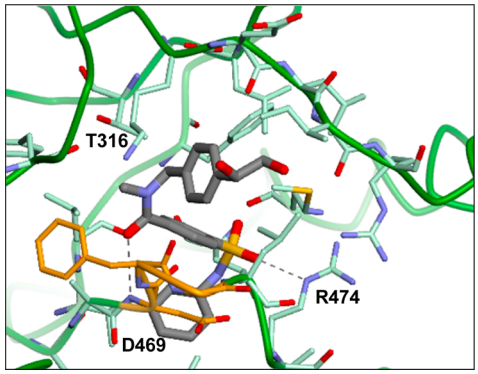


Figure 4. X-ray crystal structure with ligand **22**. The ligand is shaded gray, while the DFG residues are shaded yellow. Key hydrogen bonds are represented as dotted gray lines.

hydrophobic pocket can account for the stringent SAR observed at this position (see Table 1). Meanwhile, the *N*-benzylamide moiety is pointed out to the solvent front and thus explains the diversity that was tolerated at this position (see Table 2). As can be seen, the inhibitor **22** does not extend past the gatekeeper residue T316 and therefore has no interaction with the hinge region, which is located in the area to the left of the structure depicted in Figure 4.

In summary, we have discovered and characterized a novel series of sulfonamides, which are potent and extremely selective inhibitors of LIMK2. A series of kinetic experiments showed these inhibitors to be non-ATP competitive. X-ray crystallography further demonstrated that these molecules were binding as type III kinase inhibitors that bind in the DFG-out orientation. The highly selective and potent nature of this series gives it the potential to expand the mechanistic understanding and application toward future therapies targeting LIMK2.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization of compounds in this letter, detailed information about the biological activity assays, and crystallographic information. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Coordinates for the LIMK2-**22** structure have been deposited in the Protein Data Bank with accession code 4TPT.

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

LIMK2, LIM Kinase 2; ATP, adenosine triphosphate; K_m, Michaelis−Menten constant; V_{max}, maximum rate achieved at saturating substrate concentration; SAR, structure−activity relationship; IC_{50}, half maximal inhibitory concentration; THF, tetrahydrofuran; EDCI, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide; HOBt, *N*-hydroxybenzotriazole

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(20) Increased concentrations of cold ATP in the LIMK2 assay reduced the signal from hot ATP, resulting in unacceptable assay performance. Because of the non-ATP competitive nature of these compounds, this is not expected to alter the observed levels of selectivity.
(21) A known type I LIMK2 inhibitor used as a reference was 1-(5-methyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)-*N*-(3-phenoxyphenyl) piperidine-4-carboxamide and is described in ref 17.
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