Center Probe Report
Probe reports are generated for each compound that meets or surpasses the MLSCN requirements for a new probe. *Please provide the following specific information:*

Counter-Screening Project Title: Development of Ruthenium-based TrkA Inhibitor
Assay Submitter & Institution: Scott L. Diamond and Eric Meggers
University of Pennsylvania

Project Title: Fluorescence polarization assay for PLK1 Inhibitors
Grant Number: 1 X01 MH077611-01
Screening Center Name: Penn Center for Molecular Discovery
Principal Investigator of Screening Center: Scott L. Diamond, Ph.D.
Assay Submitter & Institution: Sharlow, Elizabeth (University of Pittsburgh)

PubChem Bioassay Identifier (AID): n/a
Probe PubChem Substance Identifier (SID): SID 49649811

Please provide the following descriptions:
Specific Aim:
To design potent, specific, and novel Ruthenium-based small-molecule inhibitors of the protein kinase TrkA.

Significance/Rationale:
The protein kinases [1], responsible for the phosphorylation of tyrosine, threonine, and serine residues in other proteins, are among the most extensively studied gene families [2-3]. Protein kinases are critical components of cellular signal transduction cascades. They are directly involved in many diseases, including cancer and inflammation, and have become one of the most important target classes for drug development [4-5]. The approval of imatinib (Gleevec) for chronic myeloid leukemia (CML), and gefitinib (Iressa) and erlotinib (Tarceva) for non-small cell lung cancer (NSCLC) has provided proof-of-principle that small molecule kinase inhibitors can be effective drugs. Over 30 kinase inhibitors are currently in clinical development, and many more are in preclinical studies. The vast majority of these compounds target the kinase ATP site, and because all of the more than 500 protein kinases identified in the human genome have an ATP site [1], there is great potential for cross-reactivity. Being able to selectively target a particular kinase then is invaluable in understanding its specific binding site, which is critical for the design of anti-cancer drugs that have minimal non-specific interactions.

TrkA (Tropomyosin-Related Kinase A) is the kinase receptor for a class of proteins called neutrophins, and in tandem they are responsible for the differentiation and survival of the sensory neurons. Upon binding with a neutrophin, the Trk family of kinases autophosphorylates and phosphorylates members of the MAPK pathway. Mutations of this gene have been shown to lead to the insensitivity to pain, a lack of functionality of the sweat glands, retarded mental development, and the onset of several forms of cancer [9-11, 18].

Assay Implementation and Screening
PubChem Primary Assay Description:
Protein kinases function by phoshorylating protein substrates. They undergo bi-bi reactions, and one of the substrates, adenosine triphosphate (ATP), is universal among all kinases. Any assay that utilizes radiolabeled ATP as a substrate can therefore be used to screen against any kinase to achieve a very high level of flexibility. The screens used for kinase inhibitors reported here consisted of end-point assays monitoring the transfer of the radiolabeled phosphate from the

ATP substrate to the protein substrate of each kinase. The phosphorylated substrate was then captured by cellulose phosphate paper and analyzed, a common and effective methodology used for radioisoptope-based kinase assays [12]. This assay protocol was used in both in-house screens and outsourced profiling assays. **As an outgrowth of the PLK-1 program at the University of Pittsburgh and exchange with the Pittsburgh Center of Ruthenium-coordinated compounds synthesized within the PCMD, a potent selective hit against TrkA was identified by the PCMD and prioritized for Probe development.**

Center Summary of the Primary Screen:
It was first discovered in the early 1990s that staurosporine, a naturally-occurring compound isolated from a strain of streptomyces bacteria, is a strong kinase inhibitor because it acts as an ATP-mimic due to the indolocarbazole moiety [19]. Because ATP is the universal substrate for all kinases, it was thought that staurosporine may serve as a potent weapon against kinases. The Meggers group devised a synthetic scheme making use of the staurosporine head group; by combining it with the heavy metal ruthenium, which can form six, stable coordination bonds with good rigidity, multiple ligands could be added in a simple manner, effectively allowing unique chemical groups to be built around this "heat-seeking missile" for ATP-binding pockets. For these inert, organometallic protein kinase inhibitors, the metal center plays only a structural role in organizing the pendant ligands in three-dimensional space. These scaffolds tend to be easier to synthesize than purely organic scaffolds, and.they also form structures/geometries that may not be achievable by a purely organic scaffold. To this end, the group has developed highly potent and selective inhibitors for the kinases GSK3 (glycogen synthase kinase-3, a serine/threonine kinase) and Pim1 (Proviral Integration Site for Moloney murine leukemia virus-1, a serine/threonine kinase) using staurosporine as a lead structure [5]. Over the course of preparing these inhibitors, a number of modified scaffolds were generated. With this collection of compounds in hand, a screening campaign against a panel of kinases was carried out to identify lead structures for kinases other than GSK3 and Pim1.

A library containing 58 of these organometallic compounds, along with staurosporine (as a control), were profiled for an IC_{50} determination against a panel of 50 kinases spanning the kinome. The purpose of the profiling was to try and elucidate chemical information about how these compounds interacted with other kinases in an attempt to synthesize a potent, specific inhibitor molecule against a kinase outside of Pim1 and GSK3. The IC50-kinase profiling was performed on a nanoliter microarray format. Final concentrations were: 1 µM ATP, 10 µM protein substrate, nanomolar enzyme, and a 10 point, 3-fold dilution of inhibitor concentrations spanning from 10 µM down to 0.5 nM. The screen identified correctly staurosporine as a potent inhibitor against most of the kinases profiled (about 80% were hit strongly), and also correctly identified a known nonspecifity displayed by the ruthenium compounds in general against Pim-1 and GSK-3 [5]. Most of the compounds displayed no activity against the 50 kinases. For the screen, we labeled compounds that had IC_{50} values of 1 nM or less as potent.

We were able to identify 4 kinases were inhibited by at least one of the organometallic probe library members: TrkA (Tropomyosin-Related Kinase A), SGK1 (Serum/Glucocorticoid-Regulated Kinase, HIPK2 (Homeo-Domain Interacting Protein Kinase-2), and MELK (Maternal Embryonic Leucine-zipper Kinase) (Fig 1). Of these, we focused on an inhibitor of TrkA, HB637, which clearly displayed selectivity over other similarly structured compounds without showing much change in its nonspecific interaction against Pim-1 and GSK-3 by simply replacing a pyridine structure on the compound to an isoquinoline. Steps were taken to try and exploit this.

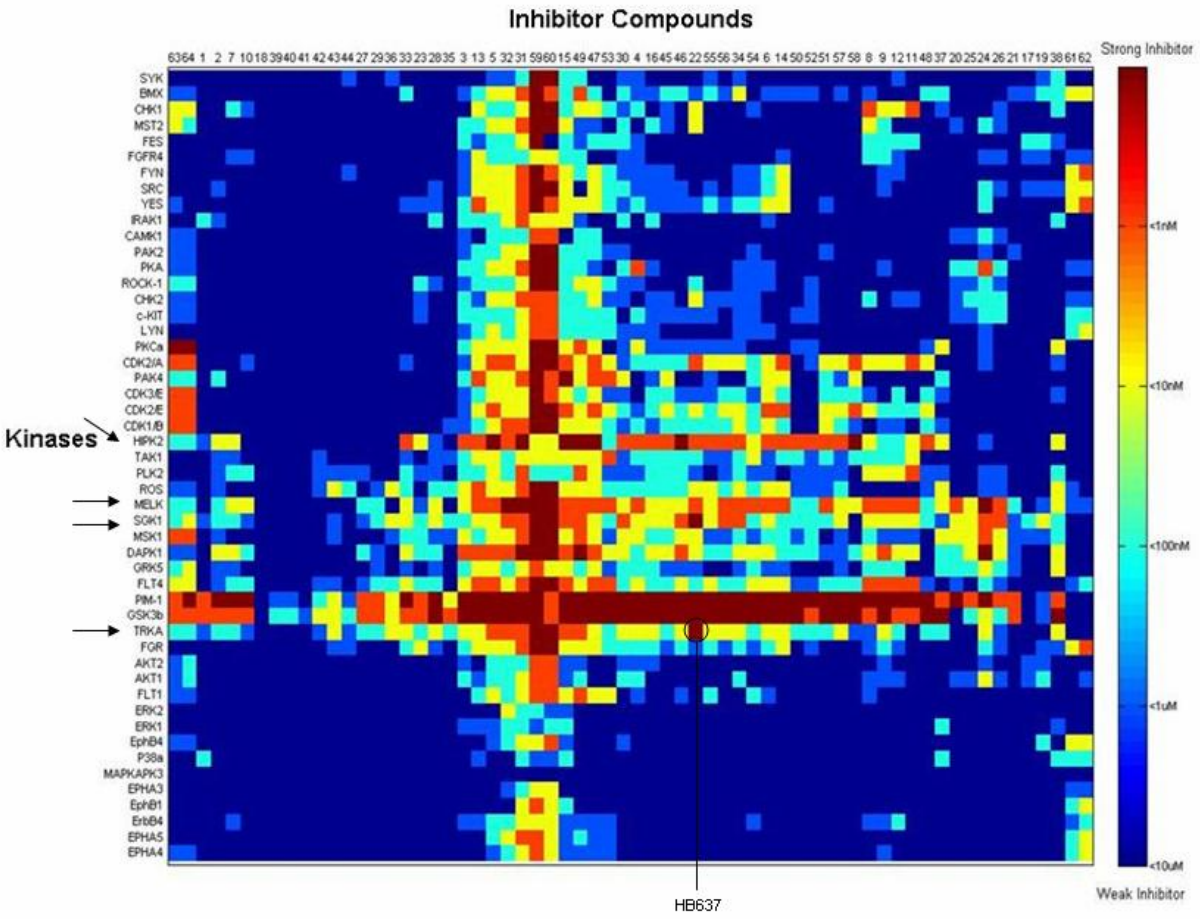
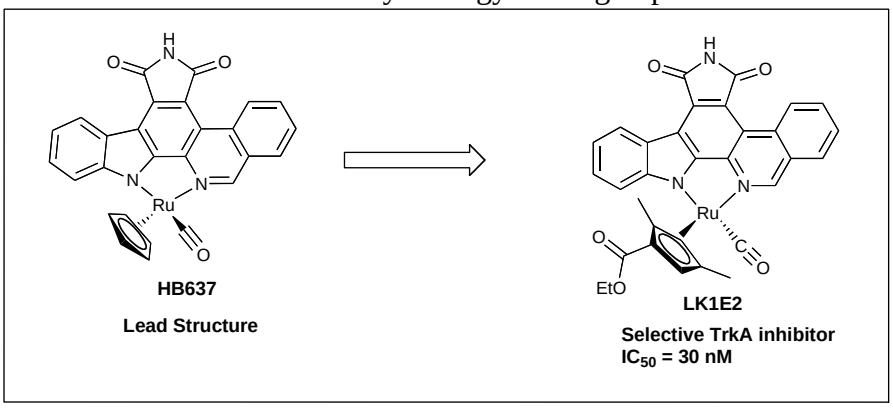


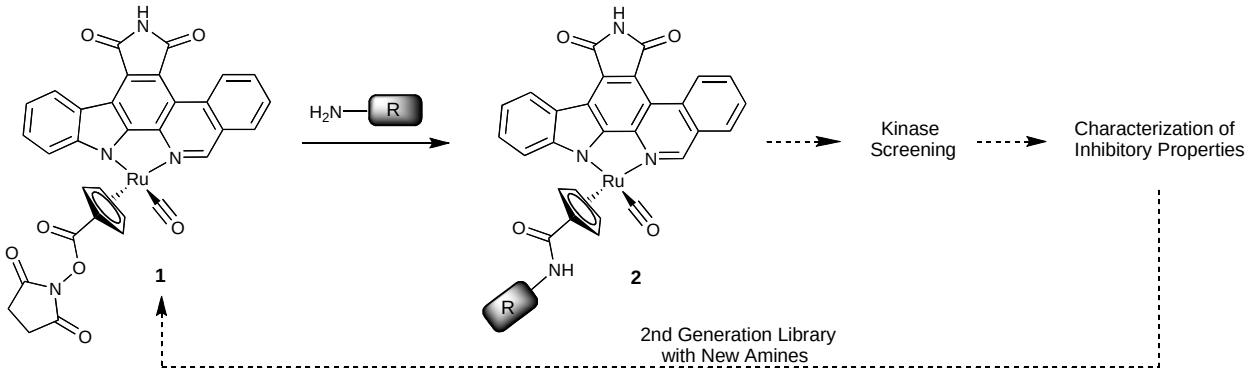
Figure 1. Clustered heatmap displaying potency of hits from the primary IC_{50} profile screen. The arrows indicate 4 of the kinases of interest, and the lead structure HB637 is shown.

Probe Optimization

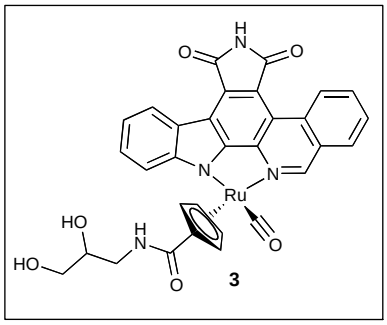
Description of secondary screens used to optimize probe structure:
Center comments on chemistry strategy leading to probe identification:

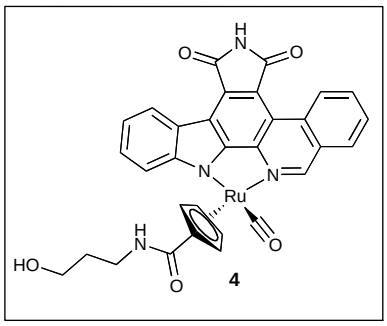


The Meggers group has shown that substituents at the cyclopentadienyl moiety can have dramatic effects on affinity and selectivity [13-14]. This strategy can be applied to the above lead structure in order to gain greater potency and selectivity. Using recently developed methodology of the group, the derivitization of the cyclopentadienyl fragment was performed in a library approach in order to rapidly identify the optimal substituent. This strategy is outlined in Scheme 1.



Scheme 1. Strategy for the rapid formation of an amide library and subsequent screening.

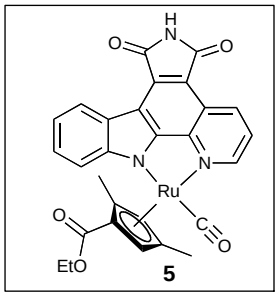




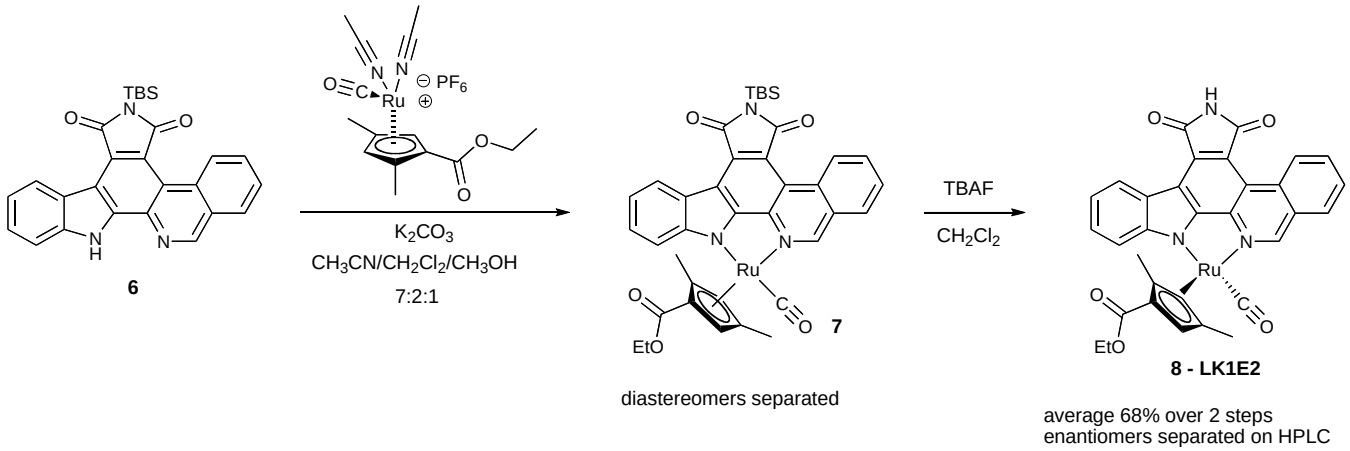
This strategy was applied using a selection of 44 primary amines that were chosen based on structural diversity. In a 96-well plate, a stock solution of **1** was distributed among 44 wells followed by the addition of a stock solution of an individual amine to each of these wells. The reactions were incubated overnight at room temperature. Reaction completion could be verified via TLC analysis of random wells. The wells were then diluted and screened against TrkA at 20 nM. These screening results showed an interesting trend. All of the hits, which displayed greater than 60% inhibition, had a similar substructure: $H_2N(CH_2)_{2\text{-}3}OH$ where the ethyl or propyl chain was substituted. The best, **3**, showed 75% inhibition at 20 nM.

With this hit in mind, a tertiary library was prepared from 25 amines that all had the requisite substructure of $H_2N(CH_2)_{2\text{-}3}OH$. This library was prepared utilizing the same strategy outlined in Scheme 1. Screening this library at 10 nM revealed

simple ethanolamine (compound **4**) as the best hit with 93% inhibition of enzyme activity. This compound is also active against the kinases GSK3 (92% inhibiton at 10 nM) and Pim1 (96% inhibiton at 10 nM).



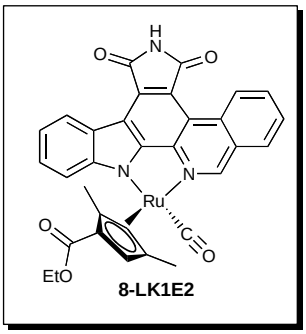
Based on a combination of high potency and selectivity, compound **5** was optimized further. To further explore the chemical space of the TrkA active site, the Cp ring of compound **5** was combined with the isoquinoline head group of **HB637** as outlined in Scheme 2. This compound (**8**) was resolved using chrial HPLC providing enantiopure material. The most potent stereoisomer, LK1E2, is a 30 nM inhibitor for TrkA. Selectivity against Pim1 and GSK3 is somewhat improved as **8-LK1E2** has an IC_{50} of 100 nM for Pim-1 and 150 nM IC_{50} against GSK3.



Scheme 2. Synthesis of third generation TrkA inhibitor.

As the second library of compounds indicated, the addition of an ethanolamine tail at the Cp moiety provided a high degree of potency against TrkA. Addition of this tail to compound **8** in place of the ethylester does in fact provide a high degree of potency, showing 75% inhibition at 10 nM. However, this compound is much more potent against Pim1, which shows 88% inhibition at 10 nM. Thus, compound **8-LK1E2** offers the best combination of potency and selectivity.

Center summary of probe properties and recommendations for the scientific use of probe as research tool:



Compound **8-LK1E2** is a highly potent inhibitor for the kinase TrkA with an IC_{50} of 30 nM. While this is not the most potent inhibitor for TrkA, it offers a high degree of selectivity in that it only inhibits Pim1 and GSK3 with similar potency (see screening data). The Meggers group has previously developed highly potent and selective inhibitors for Pim1 and GSK3 (see Appendix, compounds **9** and **10**, currently for sale from Calbiochem), which can be used in combination with **8-LK1E2** as a control. As an ATP-competitive inhibitor, compound **8-LK1E2** can be used as an effective tool for the study of TrkA. The availability of the kinase profiling data is an important attribute of this probe as such a thorough screening campaign has not been reported for any compound in the literature

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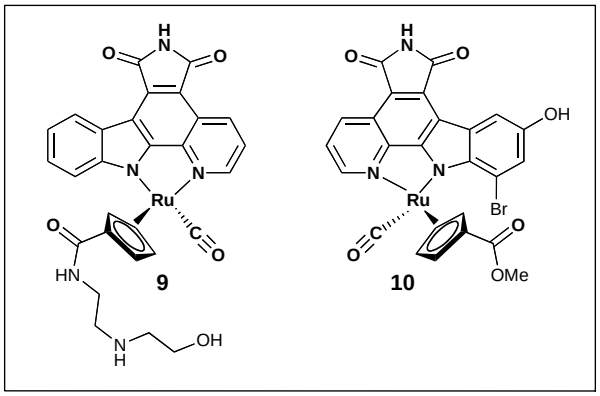
Table 1. Properties of Compound 8-LK1E2

MW	630.61
Formula	$C_{32}H_{23}N_3O_5Ru$
TrkA IC_{50}	30 nM
GSK3 IC_{50}	150 nM
Pim1 IC_{50}	100 nM

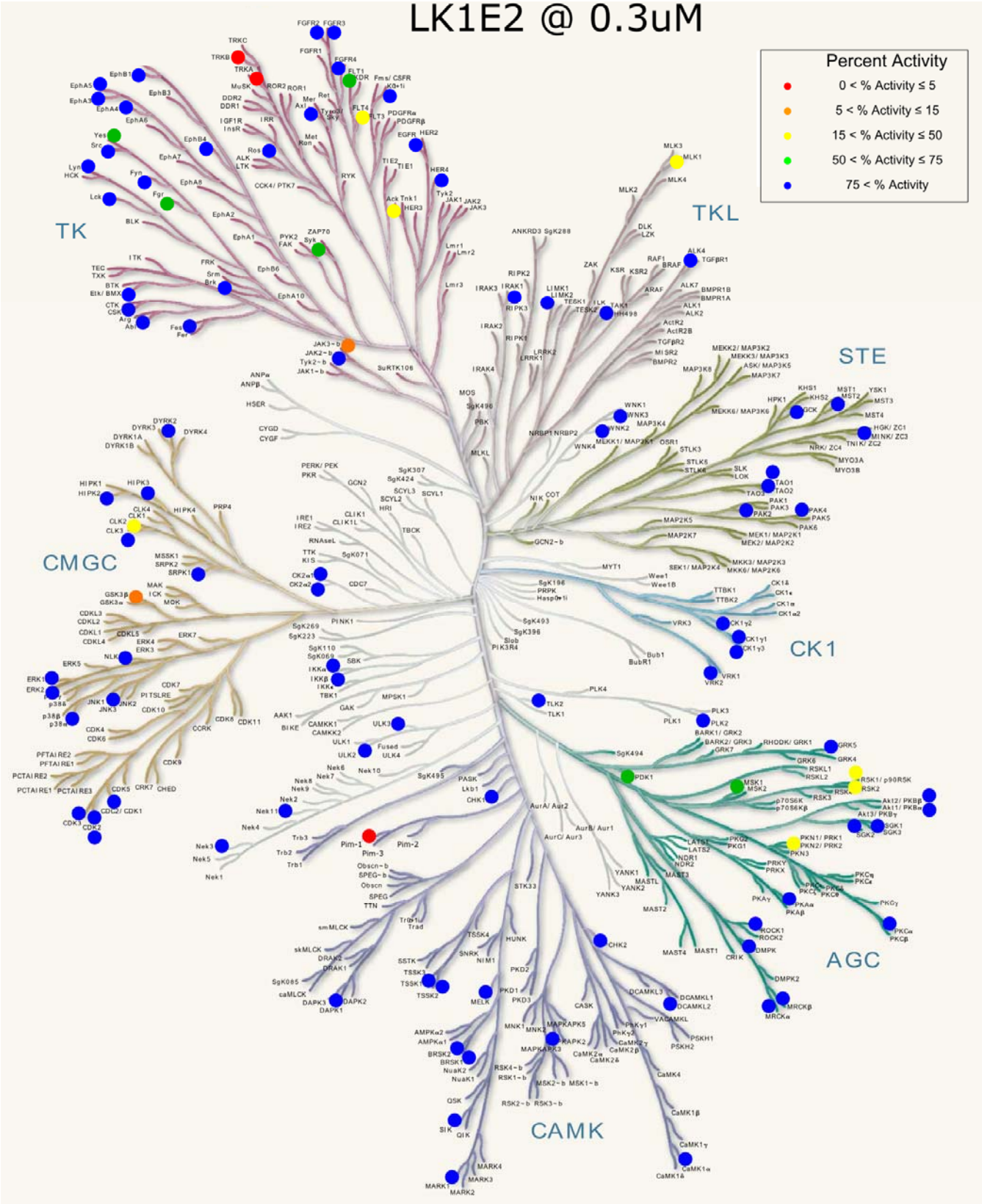
Appendices

Comparative data on probe, similar compound structures and prior probes



Our currently reported IC_{50} value of 30 nM for our optimized LK1E2 compound is not as potent as some previously reported inhibitors of TrkA which have IC50 values in the 6-17 nM range [6-7, 16-18]; however, with the high level of crosstalk that is known to be exhibited by kinase inhibitors, including late stage, high-profile, marketed medical compounds [8], the selectivity of LK1E2 is superior. Our profiling also included a wider range of included kinases. In an activity profile performed with the LK1E2 compound conducted through a proprietary radioactive, cellulosephosphate paper-based assay from Millipore to determine the percent inhibition of the compounds, it was shown that it strongly inhibited the intended target TrkA with good potency; the only other kinases to show a strong interaction were Pim-1 and GSK-3.

Figure 2. A map of the human kinome indicating which kinases were inhibited strongly by the LK1E2 compound in a percent activity profile assay.



	8-LK1E2	
Abl(h)	98	ABL
ACK1(h)	47	ACK
ALK4(h)	108	ALK4
Axl(h)	94	AXL
Bmx(h)	84	BMX
BRK(h)	107	BRK
BrSK1(h)	90	BRSK1
BrSK2(h)	85	BRSK2
CaMKI(h)	107	CaMK1a
CDK1/cyclinB(h)	106	CDK1
CDK2/cyclinA(h)	85	CDK2
CDK2/cyclinE(h)	89	CDK2
CDK3/cyclinE(h)	105	CDK3
CHK1(h)	102	CHK1
CHK2(h)	92	CHK2
CK1γ1(h)	99	CK1g1
CK1γ2(h)	97	CK1g2
CK1γ3(h)	104	CK1g3
CK2(h)	106	CK2a1
CK2α2(h)	105	CK2a2
CLK2(h)	17	CLK2
CLK3(h)	117	CLK3
cKit(h)	103	KIT
CSK(h)	116	CSK
cSRC(h)	83	SRC
DAPK1(h)	106	DAPK1
DCAMKL2(h)	113	DCAMKL2
DMPK(h)	110	DMPK1
DYRK2(h)	106	DYRK2
EGFR(h)	91	EGFR
EphA3(h)	101	EphA3
EphA4(h)	106	EphA4
EphA5(h)	105	EphA5
EphB1(h)	131	EphB1
EphB4(h)	145	EphB4
ErbB4(h)	99	HER4/ErbB4
Fes(h)	105	FES
FGFR2(h)	99	FGFR2
FGFR3(h)	77	FGFR3
FGFR4(h)	118	FGFR4
Fgr(h)	62	FGR
Flt1(h)	58	FLT1
Flt4(h)	47	FLT4
Fyn(h)	101	FYN
GCK(h)	115	GCK
GRK5(h)	88	GPRK5
GSK3β(h)	9	GSK3B
HIPK2(h)	104	HIPK2
HIPK3(h)	114	HIPK3
IKKα(h)	110	IKKa
IKKβ(h)	101	IKKb
IRAK1(h)	107	IRAK1
JAK2(h)	95	JAK2

	8-LK1E2	
JAK3(h)	14	JAK3
JNK3(h)	92	JNK3
Lck(h)	87	LCK
LIMK1(h)	101	LIMK1
Lyn(h)	95	LYN
MAPK1(h)	107	Erk1
MAPK2(h)	116	Erk2
MAPKAP-K3(h)	107	MAPKAPK3
MARK1(h)	93	MARK1
MELK(h)	113	MELK
MINK(h)	117	ZC3/MINK
MLK1(h)	47	MLK1
MRCKα(h)	90	MRCKa
MRCKβ(h)	110	MRCKb
MSK1(h)	67	MSK1
MST2(h)	114	MST2
NEK3(h)	101	NEK3
NEK11(h)	117	NEK11
NLK(h)	116	NLK
PAK2(h)	109	PAK2
PAK4(h)	104	PAK4
PDK1(h)	55	PDK1
Pim-1(h)	3	PIM1
PKA(h)	93	PKACa
PKBα(h)	104	PKBa
PKBβ(h)	97	PKBb
PKCα(h)	88	PKCa
PRK2(h)	30	PKN2
ROCK-I(h)	108	ROCK1
Ros(h)	123	ROS
Rsk1(h)	20	RSK1
Rsk2(h)	23	RSK2
SAPK2a(h)	123	SAPK2a
SGK(h)	98	SGK
SGK2(h)	101	SGK2
SIK(h)	98	SIK
Snk(h)	103	PLK2
SRPK1(h)	104	SRPK1
Syk(h)	64	SYK
TAK1(h)	105	TAK1
TAO1(h)	98	TAO1
TAO2(h)	98	TAO2
TLK2(h)	108	TLK2
TrkA(h)	5	TRKA
TrkB(h)	5	TRKB
TSSK1(h)	101	TSSK1
TSSK2(h)	107	TSSK2
ULK2(h)	109	ULK2
ULK3(h)	105	ULK3
WNK2(h)	119	Wnk2
WNK3(h)	120	Wnk3
VRK2(h)	122	VRK2
Yes(h)	64	YES

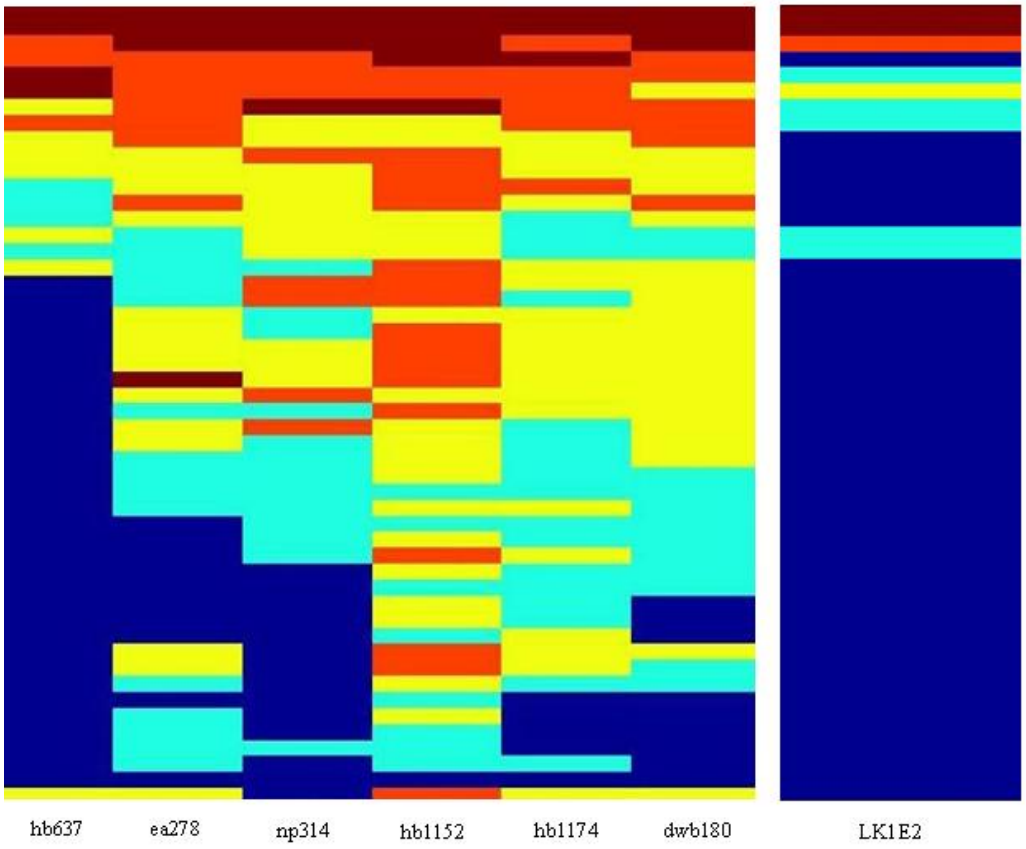


Figure 3. Improved specificity of our final TrkA inhibitor against 50 kinases in a percent activity profile compared to the 6 compounds that showed strong activity against TrkA from the primary IC_{50} profiling screen.

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