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A Potent and Selective Quinoxalinone-Based STK33 Inhibitor Does Not Show Synthetic Lethality in KRAS-Dependent Cells

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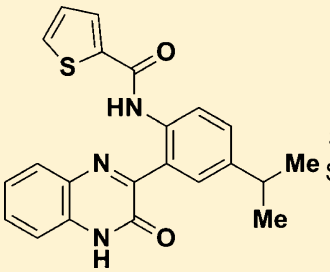
Supporting Information

ABSTRACT: The KRAS oncogene is found in up to 30% of all human tumors. In 2009, RNAi experiments revealed that lowering mRNA levels of a transcript encoding the serine/threonine kinase STK33 was selectively toxic to KRAS-dependent cancer cell lines, suggesting that small-molecule inhibitors of STK33 might selectively target KRAS-dependent cancers. To test this hypothesis, we initiated a high-throughput screen using compounds in the Molecular Libraries Small Molecule Repository (MLSMR). Several hits were identified, and one of these, a quinoxalinone derivative, was optimized. Extensive SAR studies were performed and led to the chemical probe ML281 that showed low nanomolar inhibition of purified recombinant STK33 and a distinct selectivity profile as compared to other STK33 inhibitors that were reported in the course of these studies. Even at the highest concentration tested (10 μM), ML281 had no effect on the viability of KRAS-dependent cancer cells. These results are consistent with other recent reports using small-molecule STK33 inhibitors. Small molecules having different chemical structures and kinase-selectivity profiles are needed to fully understand the role of STK33 in KRAS-dependent cancers. In this regard, ML281 is a valuable addition to small-molecule probes of STK33.



ML281
STK33 IC_{50} = 0.014 μM
Selectivity vs PKA: 700-fold
Selectivity vs AurB: 550-fold

KEYWORDS: *STK33 inhibitor, KRAS synthetic lethality, MLPCN probe*

The rat sarcoma (RAS) oncogene was discovered as a genetic element of the Harvey and Kirsten rat sarcoma (KRAS) viruses capable of immortalizing mammalian cells.[1−3] Subsequently, RAS protooncogenes were identified in normal cells. Mutated RAS oncogenes (i.e., HRAS, NRAS, and KRAS) are found in 30% of all human cancers. KRAS mutations are found in >90% of pancreatic cancers, 50% of colon cancers, and 25% of lung adenocarcinomas.[4] RAS proteins are members of a family of guanine-nucleotide-binding proteins with intrinsic GTPase activity that are often found associated with the plasma membrane as a consequence of posttranslational modifications. In the GTP-bound form, RAS proteins are active and serve as nucleotide switches converting extracellular cues (e.g., from growth factors) into intracellular signals. Missense mutations within the RAS gene result in oncoproteins with reduced GTPase and therefore overactive in signaling. Tumor cells harboring RAS mutations are often dependent upon RAS protein constitutive activity for survival—a defining feature of "oncogene addiction". Consequently, KRAS is an exceptional albeit challenging target for targeted cancer therapies. Direct targeting of KRAS function with small molecules has not yet proven achievable; therefore, alternative approaches of selectively targeting KRAS-dependent tumors have been explored. One of these involves the targeting of a nononcogene codependency evident exclusively in the context of KRAS dependency.[5,6]

In 2009, RNAi experiments revealed that lowering mRNA levels of a transcript encoding the serine/threonine kinase STK33 was selectively toxic to KRAS-dependent cancer cell lines, suggesting that small-molecule inhibitors of STK33 might selectively target KRAS-dependent cancers.[7] Although its function in normal biology is not fully understood, STK33 was found to be critical for the survival of KRAS-dependent hematopoietic cancer cell lines (acute myeloid leukemia, multiple myeloma, and T cell acute lymphoblastic leukemia) and epithelial cancer cell lines (colon, breast, pancreatic, and lung cancer). Using mutations in the ATP-binding loop, the kinase activity of STK33 was inferred to be required for the survival of KRAS-dependent cancer cell lines. These results raised the possibility that small-molecule inhibitors of STK33 may lead to the selective killing of KRAS cancers.

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After we initiated our studies, small-molecule inhibitors of STK33 were published by two groups (**1** and **2**, Figure 1).[8]

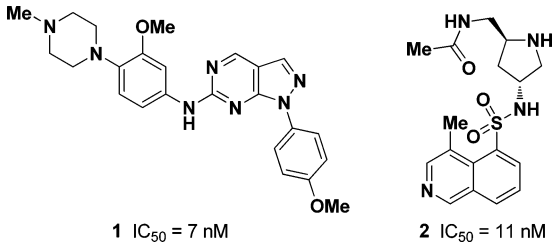


Figure 1. Known STK33 inhibitors.

Both of these compounds show excellent in vitro inhibition of STK33 but do not show selective killing of KRAS-dependent cancer cells. Kinase profiling experiments revealed that compounds **1** and **2** also inhibited a number of other kinases in addition to STK33. For example, **1** is 2-fold selective for Aurora-B (AurB) versus STK33, and **2** is only ~5-fold selective for STK33 versus both AurB and PKA. This lack of selectivity could render difficult the correlation between the STK33 activity and the observed phenotype and thus could make it difficult to understand the role of STK33 in KRAS-dependent and -independent cell lines.

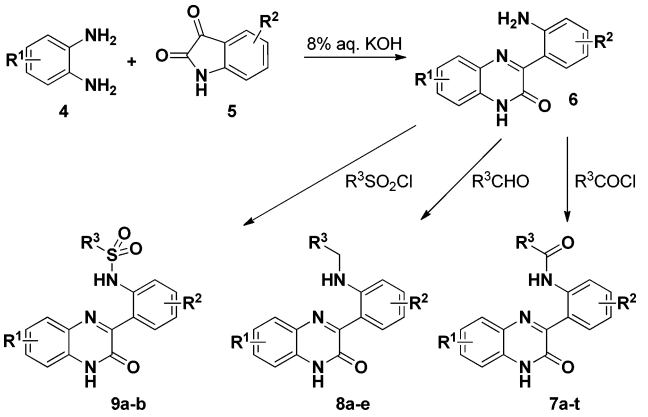
A small molecule that demonstrates exclusive selectivity for STK33 would be ideal, but this may not be achievable. Hence, a collection of small molecules that demonstrates different kinase selectivities could be useful for interpreting results from genomic cancer cell-line profiling. On the basis of the kinase profiling results of **1** and **2**, we chose PKA and AurB as counter screens in an effort to develop a selective STK33 inhibitor that would have a significantly different kinase profile. In addition, PKA and STK33 belong to a structurally related class of protein kinases.[10] Hence, PKA inhibition serves as a measure of selectivity against kinases closely related to STK33. AurB falls into the atypical structural class and hence was expected to report on broader kinase selectivity.

Our studies began with a high-throughput screening to find STK33 inhibitors. Screening of 321811 compounds from the MLSMR library in 1536-well plate format led to the identification of 13 hits with submicromolar IC_{50} values for STK33. On the basis of synthetic tractability and selectivity against PKA and AurB, we selected quinoxalinone **3** for further development (Figure 2).

Quinoxalinone **3** exhibited selectivities against PKA and AurB greater than 35-fold and was considered to be a good starting point for SAR study. The synthetic strategy for accessing analogues of **3** is described in Scheme 1. Benzene-1,2-

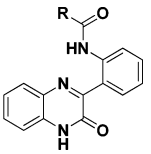
Figure 2. HTS hit compound **3**.

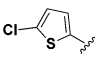
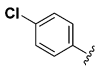
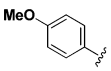
Scheme 1. Synthetic Strategy To Explore SAR on 3



diamine derivatives **4** were first condensed with isatin derivatives **5** to deliver intermediate compounds **6**. These intermediates were then used as common building blocks to prepare a series of amide analogues **7a−t** (through amide bond coupling), amine analogues **8a−e** (through reductive amination reactions), and sulfonamide analogues **9a,b** (through coupling reactions with sulfonyl chlorides). SAR on the northern portion of **3** is presented in Tables 1 and 2.

Table 1. SAR at the Northern Position: Amides[a]



Compound	R	STK33 IC_{50} (μM)	PKA IC_{50} (μM)	PKA Fold selectivity
7a		0.28	> 10	> 35
7b		0.17	ND	-
7c		0.38	ND	-
7d		0.53	ND	-
7e		0.076	> 10	> 130
7f		0.58	> 10	> 15
7g		0.15	> 10	> 65
7h		0.30	ND	-
7i		0.98	> 10	> 10
7j		> 10	> 10	-

[a]ND, not determined.

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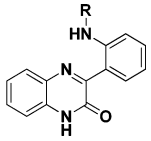
Table 2. SAR at the Northern Position: Amines and Sulfonamides[a]



Compound	R	STK33 IC_{50} (μM)	PKA IC_{50} (μM)	PKA Fold selectivity
8a		> 10	> 10	-
8b		> 10	> 10	-
8c		1.6	ND	-
8d		> 10	ND	-
8e		> 10	ND	-
9a		> 10	ND	-
9b		7.3	ND	-

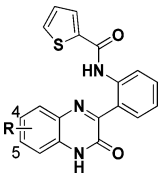
[a]ND, not determined.

To replace the thiophene ring present in the hit compound, a large number of amide analogues were synthesized (Table 1). Replacing the thiophene ring with a phenyl group (**7b**) led to a slight increase in activity. Using deactivated thiophene such as 5-chlorothiophene (**7c**) or thiazole (**7d**) led to a slight decrease in activity. Replacement of the thiophene ring by a 2-pyridyl (**7e**) led to a 4-fold increase in activity, but the compound was found to be chemically unstable as a DMSO solution (LCMS follow-up over a few days showed decomposition). The 3-pyridyl analogue (**7f**) was more stable, but it was less potent than the hit compound. The 4-pyridyl analogue was also synthesized but could not be obtained in sufficient purity, probably because of its chemical instability. Substituted benzoic acid derivatives were then used in the amide bond coupling reaction. The 4-fluorophenyl analogue (**7g**) led to a 2-fold increase in potency. However, similar to what was observed with the 2-pyridyl analogue, the 4-fluorophenyl analogue was found to be unstable. Introduction of a chloro (**7h**) or methoxy substituent (**7i**) led to decreases in activity, independent of their position on the phenyl ring. Using aliphatic groups in place of the thiophene ring led to inactive compounds; the cyclohexyl analogue (**7j**) is shown as a representative.

Amine and sulfonamide analogues were then investigated using reductive amination and coupling reactions with sulfonyl chloride, respectively (Table 2). In most cases, the compounds were found to be inactive against STK33. Remarkably, in the amine series, the thiophene (**8a**), phenyl (**8b**), 2-pyridyl analogue (**8c**), and 4-fluorophenyl analogues (**8d**) were completely inactive against STK33, showing that the carbonyl group present in the corresponding amide analogues is critical for activity.

Next, the influence of substituents on the southern phenyl ring was investigated (Table 3) and was limited to symmetric

Table 3. SAR at the Southern Phenyl Ring

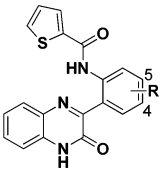


compd	R	STK33 IC_{50} (μM)	PKA IC_{50} (μM)	PKA fold selectivity
7a	H	0.28	>10	>35
7k	4,5-diCl	4.0	4.8	1.1
7l	4,5-diF	1.6	>10	>5
7m	4,5-diMe	1.6	>10	>5

diamines to avoid regioselectivity issues during the condensation reaction with isatin. The use of 4,5-dichloro- (**7k**), 4,5-difluoro- (**7l**), or 4,5-dimethyl substituents (**7m**) led to a decrease in potency and selectivity versus PKA.

Next, we investigated the SAR on the eastern phenyl ring (Table 4). In this case, the synthetic strategy relied on

Table 4. SAR on the Eastern Phenyl Ring



compd	R	STK33 IC_{50} (μM)	PKA IC_{50} (μM)	PKA fold selectivity
7n	4-Cl	0.16	>10	>60
7o	4-F	0.41	>10	>20
7p	4-OMe	0.25	7.5	30
7q	4-OCF_3	0.11	>10	>90
7r (ML281)	4-*i*-Pr	0.014	>10	>700
7s	5-Cl	0.27	>10	>35
7t	5-F	0.078	>10	>130

amidation of diversely substituted isatins with thiophene-2-carbonyl chloride, followed by a condensation of the obtained intermediate with benzene-1,2-diamine (see the Supporting Information). Introduction of a 4-chloro substituent (**7n**) led to a 2-fold increase in activity. Introduction of a 4-fluoro-substituent (**7o**) led to a slight decrease in potency. The use of a 4-methoxy (**7p**) or a 4-trifluoromethoxy substituent (**7q**) led to an increase in potency. The use of a bulkier, electron-donating 4-isopropyl substituent (**7r**, ML281) provided a 20-fold increase in activity against STK33. With an IC_{50} of 14 nM, ML281 showed greater than 700-fold selectivity over PKA. Introducing electron-withdrawing groups at the 5-position (**7s** and **7t**) also led to an increase in activity, and the 5-fluoro analogue **7t** showed an IC_{50} value against STK33 of 78 nM.

ML281 showed a solubility of 5.8 μM in PBS, high plasma protein binding (99.6% in human and 99.9% in mouse), and variable plasma stability (80.3% in human and 10.0% in mouse).

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Throughout this study, AurB was also used as a counter screen, and the results for selected quinoxalinone analogs are presented in Table 5. The most potent STK33 inhibitors were

Table 5. Selectivity versus AurB for Selected Analogues

compd	R	STK33 IC_{50} (μM)	fold selectivity	
			PKA	AurB
7a	H	0.28	>35	>35
7o	4-F	0.41	>20	>20
7q	4-OCF_3	0.11	>90	>90
7t	5-F	0.078	>130	>130
7r (ML281)	4-*i*-Pr	0.014	>700	550
1		0.007	28	0.4
2		0.011	5	5

found to be mostly inactive against AurB. ML281 showed a 550-fold selectivity over AurB and greater than 700-fold selectivity over PKA. We also resynthesized **1** (see the Supporting Information) and compared it with ML281 and **2** (Table 5). Compound **1** shows an IC_{50} of 7 nM against STK33 and 28- and 0.4-fold selectivities over PKA and AurB, respectively. Similarly, the fasudil analogue **2** shows an IC_{50} of 11 nM against STK33 and 5-fold selectivities over PKA and AurB. Hence, ML281 does not inhibit kinases that are strongly inhibited by **1** and **2** and will constitute a valuable complement tool to better correlate STK33 activity to phenotype in cells.

ML281 was then profiled against a panel of 83 kinases chosen for diversity and toxicity (Figure 3). ML281 was found

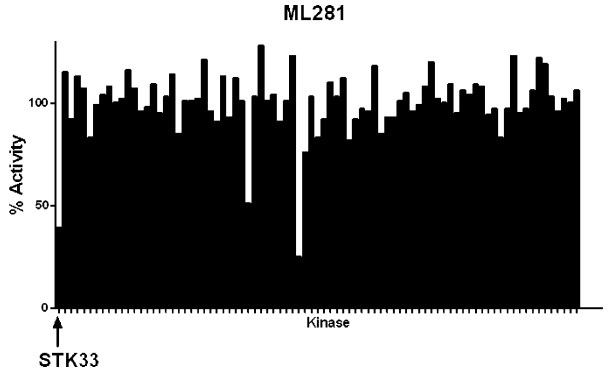

Figure 3. Profiling of ML281 (1 μM) against a panel of 83 kinases.

to be extremely selective and inhibits only two kinases other than STK33 in the panel tested at a level of 25% or more: FLT3 (a proto-oncogene) and KDR (VEGF R2 associated with vascularization). Full data for the 83 kinases tested are provided in the Supporting Information.

ML281 was finally profiled in cellular assays to test the hypothesis of STK33 synthetic lethality in KRAS-dependent cell lines. Figure 4 shows the relative viability of two KRAS-dependent (NOMO-1 and SKM-1) and two KRAS-independent (THP-1 and U937) AML-derived cell lines. ML281 does not appear to significantly alter viability of any of the tested cell lines at concentrations of up to 10 μM. This experiment has been repeated with more than 20 KRAS-dependent and KRAS-independent cell lines, and no significant correlation between KRAS dependency and cell viability was found (see the Supporting Information). Whether ML281 is not inhibiting STK33 in cells (due to off-target effects or high plasma protein binding) or whether STK33 inhibition is not synthetic lethal to

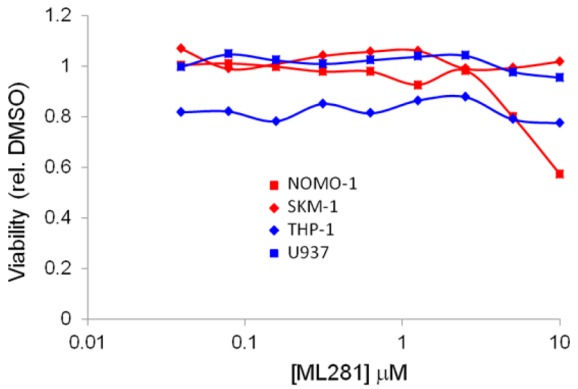

Figure 4. Effect of ML281 on KRAS-dependent (red) and KRAS-independent (blue) cell viability.

KRAS-dependent cell line remains to be determined. In this context, biomarkers of STK33 activity should greatly help the cancer research community to answer that question.

The small-molecule probe ML281 is a nanomolar inhibitor of STK33 and is in a novel chemical class as compared to the other recently reported inhibitors. Although the potency of ML281 for in vitro STK33 inhibition is not significantly improved as compared to **1** and **2**, it exhibits greater selectivity against PKA and AurB (as well as excellent selectivity when profiled against 83 protein kinases) and provides a new chemical class and selectivity profile of STK33 inhibitors. This new probe may be useful to elucidate the cellular functions of STK33. Compounds **1** and **2** have proven unsuccessful in selectively killing KRAS-dependent cancer cell lines. Our own results with ML281 shed more light on the biology of STK33 and further suggest that the synthetic lethality with KRAS oncogenes cannot be recapitulated with selective STK33 small-molecule inhibitors. However, the development of STK33 biomarkers is strongly desired to understand the role of STK33 in cells and to correlate inhibitors activity to cell fate better. ML281 has been registered with NIH Molecular Libraries Program and is available upon request.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, assay protocols, confirmatory biological testing, and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MLPCN, Molecular Libraries Probe Production Centers Network; CAMK, calcium calmodulin-dependent protein kinase

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