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Discovery and characterization of BAY-6035, a novel benzodiazepine-based SMYD3 inhibitor

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INTRODUCTION

- SMYD3 (SET and MYND domain-containing protein 3) is a protein lysine methyltransferase, which was initially described as a H3K4 methyltransferase involved in transcriptional regulation.[1]
- SMYD3 has recently been reported to methylate and regulate several cancer-relevant non-histone proteins such as mitogen-activated protein kinase kinase kinase 2 (MAP3K2),[2] vascular endothelial growth factor receptor 1 (VEGFR1),[3] RAC-alpha serine/threonine-protein kinase (AKT1),[4] and the human epidermal growth factor receptor 2 (HER2)[5] (**Figure 1**).
- In addition, the overexpression of SMYD3 has been linked to poor prognosis in certain cancers, supporting its possible oncogenic role for SMYD3 and making it a potential target for anticancer drug development.[6,7]
- Here, we report the discovery of a novel, potent and selective SMYD3 inhibitor BAY-6035.

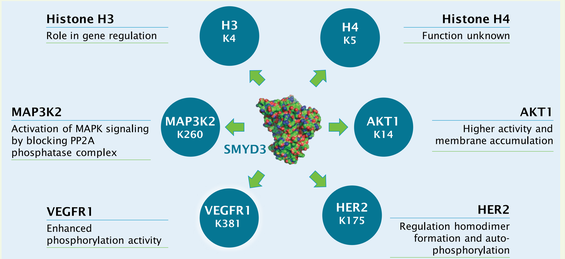

Figure 1. Reported methylation substrates of the SMYD3 methyltransferase. SMYD3 is described as a protein methyltransferase regulating the transcription of oncogenes and signaling pathways frequently dysregulated in cancer. AKT1, RAC-alpha serine/threonine-protein kinase; HER2, human epidermal growth factor receptor 2; MAP3K2, mitogen-activated protein kinase kinase kinase 2; PP2A, protein phosphatase 2; VEGFR1, vascular endothelial growth factor receptor 1.

METHODS

- Thermal shift assay, ultra-high-throughput screening (TSA uHTS) was used to identify compounds that induce a positive shift (stabilization of the protein) at the SMYD3 protein melting point (Tm). Scans were measured from 25 °C to 80 °C at a scanning rate of 0.1 °C/s.
- Catalytic inhibition of SMYD3 was confirmed by scintillation proximity assay (SPA). S-Adenosyl-L-methionine (SAM) was used as the methyl donor, and MEK kinase 2 (MEKK2)-derived peptide employed as the substrate of SMYD3.
- The binding of SMYD3 inhibitors was confirmed by isothermal titration calorimetry (ITC). In addition, surface plasmon resonance (SPR) measurements were done to confirm binding. Kinetic curve fittings were done with a 1:1 binding model. The experiments were performed in the presence of SAM.
- The structure of SMYD3 was determined by using X-ray crystallography, as described previously[7] with slight modifications.
- The on-target activity of SMYD3 inhibitors was assessed by cellular MAP3K2 methylation assay. HeLa cells were transfected with HA-tagged MAP3K2 and untagged SMYD3. MAP3K2 methylation was assessed by Western blot using specific in-house generated K260me3 antibody.

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RESULTS

Ultra-high-throughput screening (uHTS) and identification of a benzodiazepine cluster as potential SMYD3 inhibitor

- A core library of ~410,000 small molecules was screened to identify compounds that bind to and stabilize SMYD3 in a TSA assay.
- More than 3,900 confirmed hits which bind to SMYD3 at a compound concentration of 120 µM were identified.
- With a reduced compound concentration of 100 µM and selectivity testing against SMYD1 and SMYD2, 1,238 confirmed hits which bind specifically to SMYD3 (**Figure 2**) were identified.
- A benzodiazepine cluster was selected for further optimization based on the dose-response analysis and structural attractiveness (**Table 1**).
- Inhibition of the catalytic SMYD3 activity was in the 10-40 µM potency range as determined by SPA (**Table 1**).

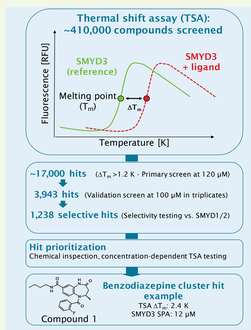


Figure 2. Thermal shift assay screening. Screening cascade resulted in 1,238 primary hits. A benzodiazepine cluster was selected for further optimization. RFU, relative fluorescence units; SPA, scintillation proximity assay.

Table 1. Structure-activity relationship (SAR) of the initial benzodiazepine hit cluster.

			First SAR of the benzodiazepine hit cluster derived from the primary screening results									
Cpd	R1	R2	Concentration dependent SMYD3 TSA ΔTm [K]							SMYD1 TSA ΔTm [K]	SMYD2 TSA ΔTm [K]	SMYD3 SPA IC_{50} [M]
1			0.7	1.3	2.0	2.8	2.5	3.4	4.1	0.0	0.0	1.2E-05
2			0.4	1.0	1.4	1.3	2.1	2.1	3.0	-0.2	0.2	1.8E-05
3			0.2	0.8	1.2	1.5	1.8	2.1	3.3	-0.2	0.2	2.4E-05
4			0.2	0.6	0.9	1.4	1.7	1.6	2.3	0.2	0.2	2.2E-05
5			0.0	0.2	0.8	1.0	1.6	1.2	2.1	-0.1	0.1	2.7E-05
6			0.0	0.7	0.6	0.9	1.4	1.0	1.4	0.0	0.2	n.d.
7			0.2	0.7	0.8	0.8	1.2	0.9	1.8	-0.2	0.0	1.8E-05

First structure-activity relationship (SAR) for the benzodiazepine cluster was derived from primary screening. The catalytic inhibition of SMYD3 was confirmed using a scintillation proximity assay. n.d., not determined; SAR, structure-activity relationship; TSA, thermal shift assay.

Benzodiazepine-based SMYD3 inhibitors are peptide substrate-competitive

- Direct binding of the inhibitor series into the catalytic pocket of SMYD3 was confirmed by X-ray crystallography (**Figure 3**).
- The co-crystal structure revealed binding to the substrate binding site and occupation of the hydrophobic channel for lysine binding (**Figure 3**).
- The SMYD3 inhibitors within the benzodiazepine cluster were found to exploit a thus far unprecedented hydrogen bond pattern (**Figure 3**).

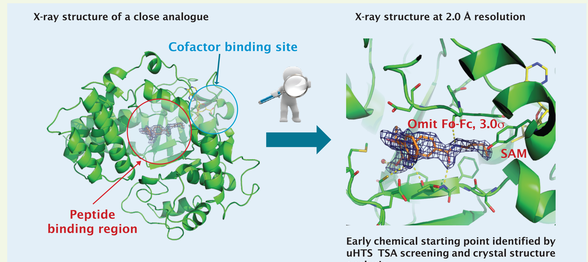


Figure 3. Crystal structure of the benzodiazepine-based SMYD3 inhibitor binding. SAM; S-Adenosyl-L-methionine; TSA, thermal shift assay; uHTS, ultra-high-throughput screening.

BAY-6035 is a novel selective tool for the exploration of SMYD3 inhibition

- BAY-6035 demonstrated potent SMYD3 inhibition with an IC_{50} of 88 nM (**Figure 4A**) and an equilibrium dissociation constant (K_d) of 100 nM as determined by ITC (**Figure 4B**) or with SPR of (**Figure 4C**).
- BAY-6035 showed highly selective inhibition of SMYD3 with no activity on other methyltransferases (**Figure 4D**) or kinases (data not shown).
- The X-ray structure of BAY-6035 binding is presented in **Figure 4E**.

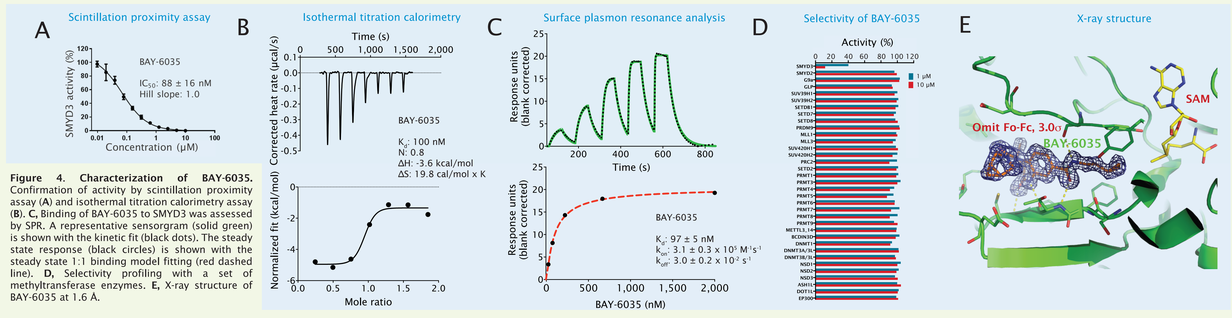


Figure 4. Characterization of BAY-6035. Confirmation of activity by scintillation proximity assay (**A**) and isothermal titration calorimetry assay (**B**). **C,** Binding of BAY-6035 to SMYD3 was assessed by SPR. A representative sensorgram (solid green) is shown with the kinetic fit (black dots). The steady state response (black circles) is shown with the steady state 1:1 binding model fitting (red dashed line). **D,** Selectivity profiling with a set of methyltransferase enzymes. **E,** X-ray structure of BAY-6035 at 1.6 Å.

BAY-6035 inhibits cellular methylation of MAP3K2 by SMYD3

- BAY-6035 inhibited the cellular methylation of MAP3K2 by SMYD3 dose-dependently with an IC_{50} of 183 ± 13 nM in MAP3K2 and SMYD3-transfected HeLa cells (**Figure 5**).

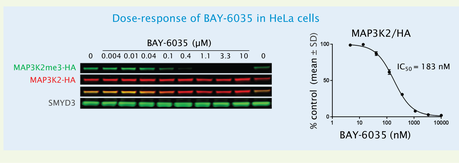


Figure 5. BAY-6035 potently inhibits cellular methylation of MAP3K2 in HeLa cells. HeLa cells were transfected with SMYD3 and HA-MAP3K2 and treated with the indicated compounds. MAP3K2 methylation was assessed by Western blot using a K260me3-specific antibody. The methylated MAP3K2 signal was normalized to total MAP3K2. MAP3K2, mitogen-activated protein kinase kinase kinase 2; MAP3K2me3, tri-methylated MAP3K2.

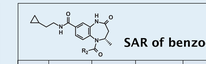
Significant improvement of SMYD3 inhibition by X-ray-guided optimization

- X-ray data of the catalytic pocket of SMYD3 in complex with a compound allowed a better understanding of the binding mode (**Figure 3**).
- Combination of the best "moiety" to benzodiazepine derivative resulted in the identification of BAY-6035 (**Table 2**).

Table 2. Structure-activity relationship (SAR) development of the benzodiazepine cluster leading to BAY-6035.

SAR of benzodiazepine ureas

Cpd	R2	Stereo-center	SMYD3 TSA ΔTm [K]	SMYD3 SPA IC_{50} [M]	logD (pH 7.5)	Kinetic solubility (pH 6.5) [mg/L]
1		(rac)	5.3	1.9E-6	2.1	95
2		(s) (r)	10.9 8.5	2.4E-8 1.9E-7	1.4	319
3		(s)	10.2	5.3E-8	1.4	> 413
4		(s)	9.9	1.7E-7	1.5	> 455
5		(s)	8.7	2.5E-7	1.4	294
6		(s)	7.2	7.9E-7	1.4	330
7		(s)	7.2	2.5E-7	1.4	374
8		(s)	7.5	3.9E-7	1.3	252
9		(s)	7.2	4.8E-7	1.3	257



SAR of benzodiazepine ureas

Cpd	R2	Stereo-center	SMYD3 TSA ΔTm [K]	SMYD3 SPA IC_{50} [M]	logD (pH 7.5)	Kinetic solubility (pH 6.5) [mg/L]
10		(s)	8.8	4.5E-7	1.7	> 427
11		(s)	6.1	1.1E-6	1.5	254
12		(s)	2.8	4.7E-6	2.4	345
13		(s)	5.5	9.7E-7	2.2	312
14		(s)	5.7	1.3E-6	2.2	-
15		(s)	7.3	7.5E-7	2.0	294
16		(s)	6.1	2.3E-6	2.1	231
17		(s) (r)	9.9	8.8E-8 2.5E-6	1.9	363

Compound (s)-17: BAY-6035

CONCLUSIONS

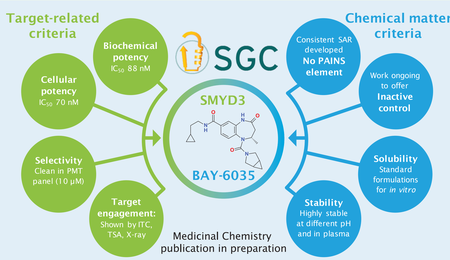


Figure 6. The SMYD3 inhibitor BAY-6035 fulfils the probe criteria. ITC, isothermal titration calorimetry; PAINS, pan-assay interference compounds; PMT, protein methyltransferases; SAR, structure-activity relationship; TSA, thermal shift assay.

- BAY-6035 was identified as a highly potent and selective SMYD3 inhibitor by the optimization of a primary benzodiazepine hit cluster.
- The binding geometry of BAY-6035 to its substrate pocket in SMYD3 was unambiguously characterized by X-ray crystallography.
- BAY-6035 specifically inhibited cellular methylation of MAP3K2 by SMYD3.
- In conclusion, BAY-6035 is a novel, selective and potent SMYD3 inhibitor probe and will foster a better understanding of the biological role of SMYD3 in cancer (**Figure 6**).

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