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Published in:
European Journal of Medicinal Chemistry

DOI:
10.1016/j.ejmech.2017.05.015

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Wapenaar, H., van den Bosch, T., Leus, N. G. J., van der Wouden, P. E., Eleftheriadis, N., Hermans, J., Hailu, G. S., Rotili, D., Mai, A., Domling, A., Bischoff, R., Haisma, H. J., & Dekker, F. J. (2017). The relevance of K-i calculation for bi-substrate enzymes illustrated by kinetic evaluation of a novel lysine (K) acetyltransferase 8 inhibitor. *European Journal of Medicinal Chemistry*, *136*, 480-486. https://doi.org/10.1016/j.ejmech.2017.05.015

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Download date: 22-04-2025

European Journal of Medicinal Chemistry 136 (2017) 480–486

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

The relevance of K_i calculation for bi-substrate enzymes illustrated by kinetic evaluation of a novel lysine (K) acetyltransferase 8 inhibitor

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ARTICLE INFO

Article history:
Received 17 February 2017
Received in revised form
11 April 2017
Accepted 4 May 2017
Available online 5 May 2017

Keywords:
Histone acetyltransferases
KAT8
Fragment screening
Histone acetylation
Inhibitor
Enzyme kinetics

ABSTRACT

Histone acetyltransferases (HATs) are important mediators of epigenetic post-translational modifications of histones that play important roles in health and disease. A disturbance of these modifications can result in disease states, such as cancer or inflammatory diseases. Inhibitors of HATs (HATi) such as lysine (K) acetyltransferase 8 (KAT8), could be used to study the epigenetic processes in diseases related to these enzymes or to investigate HATs as therapeutic targets. However, the development of HATi is challenged by the difficulties in kinetic characterization of HAT enzymes and their inhibitors to enable calculation of a reproducible inhibitory potency. In this study, a fragment screening approach was used, enabling identification of 4-amino-1-naphthol, which potently inhibited KAT8. The inhibitor was investigated for enzyme inhibition using kinetic and calorimetric binding studies. This allowed for calculation of the K_i values for both the free enzyme as well as the acetylated intermediate. Importantly, it revealed a striking difference in binding affinity between the acetylated enzyme and the free enzyme, which could not be revealed by the IC_{50} value. This shows that kinetic characterization of inhibitors and calculation of K_i values is crucial for determining the binding constants of HAT inhibitors. We anticipate that more comprehensive characterization of enzyme inhibition, as described here, is needed to advance the field of HAT inhibitors.

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1. Introduction

Epigenetics is a field of study in which novel therapeutic targets are found for various diseases, such as inflammatory diseases and cancer [1]. It is defined as the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered transcriptional activity [2]. Epigenetic processes include post-translational modifications of histones such as lysine acetylations, which play an important role in the regulation of gene transcription by controlling the chromatin structure of DNA [3]. Lysine acetylations are installed by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs). These enzymes balance lysine acetylation, resulting in a controlled expression of genes. A disturbance of this balance can result in disease states, such as cancer or inflammatory diseases [4]. Therefore, restoring the balance between HAT and HDAC activity using small molecule HAT inhibitors (HATi) could be a therapeutic strategy for several diseases.

Development of HATi is an important challenge that has been addressed with limited success so far. Important drawbacks of

Abbreviations: HAT, histone acetyltransferase; HATi, histone acetyltransferase inhibitor; KAT8, Lysine (K) acetyltransferase 8; Ac-CoA, acetyl coenzyme A.

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http://dx.doi.org/10.1016/j.ejmech.2017.05.015
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current HATi include intrinsic chemical reactivity, instability, low potency, lack of selectivity as well as the limited kinetic characterization of the inhibitors [5]. Kinetic characterization of inhibitors in an early stage of lead identification is essential. Since enzyme activity assays are often used for the discovery of novel inhibitors, the 50% inhibitory concentration (IC_{50}) is a common measure for the potency of inhibitors. However, the IC_{50} depends on the conditions used in the assay, such as the concentration of the enzyme substrates and their respective K_m values. This value is therefore not reproducible unless exactly the same assay conditions are used. The inhibitory potency (K_i) value is a potency value independent of the assay conditions and is therefore much more representative as potency of the inhibitor [6]. Calculation of the K_i value from the IC_{50} is crucial for the comparison of the potency with known inhibitors or between different assays and for the determination of selectivity. In case of competitive inhibitors of enzymes converting only one substrate, the K_i can be calculated using methods like the Cheng-Prusoff equation, Dixon plot or using double reciprocal plots [7–9]. However, since HATs use a cofactor, acetyl coenzyme A (Ac-CoA), for the acetylation of the lysine, they are bisubstrate enzymes: they convert two substrates into two products. In this case, it is not possible to use standard methods for calculation of the K_i and more elaborate kinetic evaluations are necessary [10]. It is therefore important to investigate the kinetic behavior of HAT inhibitors toward determination of reproducible inhibitory constants.

The HATs are a disparate group of enzymes from which most isoenzymes can be assigned to five main families based on primary structure homology. Three families that have been studied extensively are the GNAT (GCN5-related *N*-acetyltransferase) family, the p300/CBP (p300/CREB binding protein) family and the MYST (acronym for MOZ, Ybf2, Sas2, and Tip60) family [11]. In this study, we focused on Lysine (K) acetyltransferase 8 (KAT8), a member of the MYST HAT family. KAT8 (also: males absent on the first, MOF, or MYST 1) is part of the male-specific-lethal (MSL) complex, which specifically acetylates histone H4 lysine 16 [12]. KAT8 has additionally been shown to form different complexes containing WD repeat domain 5 (WDR5, MSL1v1 or NSL complex) and MLL, broadening the substrate specificity to histone H4 lysines 5 and 8 and non-histone targets, such as lysine 120 on the tumor-suppressor protein p53 [13–15]. As part of these complexes, KAT8 has been shown to play a role in stem cell pluripotency, cell proliferation and DNA damage response [16]. Using KAT8 conditional deletion and a small molecule inhibitor for MYST family HATs in cell lines and a mouse model, it was recently shown to be important for sustaining MLL-AF9-driven leukemia and was suggested as a potential therapeutic target for MLL-rearranged leukemia [17]. Therefore, small molecule KAT8 inhibitors could facilitate investigation of its function in disease or may be used as potential therapeutic agents.

Currently, one class of HATi have been described to inhibit KAT8, which are anacardic acid and a number of its derivatives [18]. These inhibitors were shown to inhibit KAT8 by interacting with an acetylated form of the enzyme, required the binding of Ac-CoA and competed with the lysine substrate. Calculation of the K_i values revealed that although the determined IC_{50} values were above 200 μM, the K_i values were in the range of 37–64 μM. This shows that the K_i value can differ significantly from tested IC_{50} values and that this is dependent on the mechanism of inhibition of the inhibitors. Therefore, we aimed at discovering novel, structurally unrelated KAT8 inhibitors and determining their kinetic profile.

Using a fragment screening approach, 4-amino-1-naphthol (compound **13**) was identified as a potent KAT8 inhibitor. The mechanism of KAT8 inhibition and structure-activity relationship (SAR) were investigated. Enzyme kinetic measurements as well as calorimetric binding studies suggested a reversible inhibition mode and a direct interaction with KAT8 for compound **13**. Kinetic studies allowed calculation of the K_i value for both the free enzyme form of KAT8 (K_{i1} = 2.6 μM), and the acetylated form (K_{i2} = 0.017 μM), which indicated very high potency for the acetylated enzyme intermediate. Taken together, our approach to link fragment screening with enzyme kinetic analysis demonstrated large affinity differences for the different enzyme species involved in catalysis, which is not obvious from the IC_{50} values. We anticipate that unravelling the inhibitory potencies of inhibitors for individual enzyme species is key to inhibitor discovery for this type of enzymes.

2. Results and discussion

2.1. Fragment screening

Towards discovery of a novel inhibitor of KAT8, an in-house library of fragments with a broad range of structures and low molecular weight (MW < 250 Da), was screened for inhibition of the KAT8 HAT. An assay based on fluorescence-detection of CoA was used to screen all fragments. Currently known KAT8 inhibitors showed an IC_{50} of higher than 200 μM under the same assay conditions [18]. Taking this potency as a reference, a concentration of 200 μM was chosen for screening the fragments. A control was included for potential fluorescence quenching by the fragments to identify and rule out any hits directly interfering with the assay. Structures containing maleimide or thiol moieties were excluded due to reactivity with the assay product or fluorophore. The resulting hits (**1** and **10**) and a small number of similar fragments were tested for their 50% inhibitory concentration (IC_{50}) (Table 1, Fig. S1).

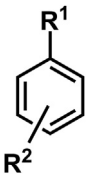
4- fluoro phenyl hydrazine (**1**) showed an IC_{50} of 310 μM. Several phenyl hydrazines similar to **1** were investigated. 4- methoxy phenyl hydrazine (**2**) lost activity compared to **1**. Other substitution patterns like hydrogen or chlorine were not active (**3**–**5**). To investigate the hydrazine moiety further, 4- fluoroaniline or benzylamine (**6**, **7**) were tested. These were not active, and neither were two other aniline compounds (**8**, **9**), showing that the inhibition was specific for the hydrazine moiety. Taken together, no phenyl hydrazine structures were found to be more potent inhibitors than **1**. Therefore, this structural entity was not further investigated.

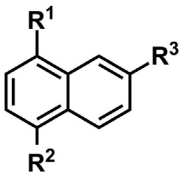
The hit 1-aminonaphthalene (**10**), showed an IC_{50} of 180 μM; the most potent hit from the screening. Testing similar structures showed that 1-nitro naphthalene (**11**) or a sulphonic acid substituted aminonaphthalene (**12**) were not active. The hydroxyl substituted fragment, 4-amino-1-naphthol (**13**), showed an excellent IC_{50} of 9.7 ± 3.0 μM. Strikingly, **9**, which is very similar to **13**, was not active, showing that the naphthalene moiety is also important for KAT8 activity. Therefore, compound **13** was investigated in more detail.

2.2. Structure-activity relationship

A SAR study was done around the structure of **13** to investigate the importance of the different parts of the scaffold. Derivatives of **13** were synthesized and tested for their inhibitory potency on KAT8 (Table 2, Fig. S2). The naphthalene ring was replaced by a isoquinoline (**14**). This moiety has a slightly different logP and is weakly basic, but it did not significantly influence the activity. To investigate the importance of the free amine on R^1 position, the amine was included in the ring (**16**) or a carbon or carboxyl spacer was introduced (**17, 18**). These compounds did not show inhibitory activity on KAT8. Additionally, the amine was replaced by a nitro (**19**). This compounds had a reduced potency compared to **13**, but

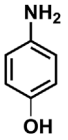
Table 1
IC_{50} values of the fragment screening hits and a small number of related structures for KAT8.

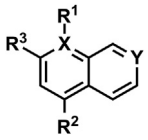


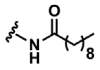
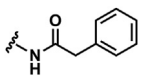


Compound	R^1	R^2	R^3	IC_{50} (μM)
1	$-NHNH_3^+ Cl^-$	4 - F	–	310
2	$-NHNH_3^+ Cl^-$	4 - OCH3	–	680
3	$-NHNH_2$	H	–	>1000
4	$-NHNH_3^+ Cl^-$	H	–	>1000
5	$-NHNH_3^+ Cl^-$	2, 4 - Cl	–	>1000
6	$-NH_2$	4- F	–	>1000
7	$-CH_2NH_2$	4-F	–	>1000
8	$-NH_2$	4- Cl	–	>1000
9	$-NH_2$	4- OH	–	>1000
10	$-NH_2$	H	H	181
11	$-NO_2$	H	H	>1000
12	$-NH_2$	H	SO_3^-	940
13	$-NH_2$	OH	H	9.7 ± 3.0

Table 2
IC_{50} values of compound **13** derivatives and the selectivity for KAT2B and KAT3B of compounds **13**, **9** and the derivatives showing activity on KAT8. Data presented are IC_{50} values.





Compound	R^1	R^2	R^3	X	Y	IC_{50} KAT8 (μM)	IC_{50} KAT2B (μM)	IC_{50} KAT3B (μM)
13	NH_2	OH	H	C	C	9.7 ± 3.0	3.6 ± 1.1	1.4 ± 0.1
9						>500	>500	>500
14	NH_2	OH	H	C	N	7.6 ± 0.8	7.1 ± 0.8	1.1 ± 0.3
15	NH_2	CN	H	C	C	>500	n.d.	n.d.
16	–	OH	H	N	C	>500	n.d.	n.d.
17	CH_2NH_2	OH	H	C	C	>500	n.d.	n.d.
18	$CONH_2$	OH	H	C	C	>500	n.d.	n.d.
19	NO_2	OH	H	C	C	141 ± 43	221 ± 115	106 ± 22
20	NH_2	OH	OMe	C	C	177 ± 22	200 ± 28	52 ± 28
21	NH_2	CH_2OH	H	C	C	>500	n.d.	n.d.
22	NH_2	OMe	H	C	C	89 ± 11	>500	95 ± 60
23	NH_2	OAc	H	C	C	>500	n.d.	n.d.
24	NH_2	OBn	H	C	C	>500	n.d.	n.d.
25	NMe_2	OH	H	C	C	23 ± 4	4.2 ± 1.9	3.7 ± 2.1
26	NHAc	OH	H	C	C	>500	n.d.	n.d.
27		OH	H	C	C	>500	n.d.	n.d.
28		OH	H	C	C	250 ± 96	160 ± 25	60 ± 22
29		OH	H	C	C	365 ± 110	>500	250 ± 137
30	$NHSO_2Me$	OH	H	C	C	66 ± 12	3.8 ± 2.8	3.0 ± 1.9
31	$NHSO_2$toluene	OH	H	C	C	37 ± 5.3	12 ± 1.4	2.6 ± 1.4

Me = methyl, Ac = acetyl, Bn = benzyl, n.d = not determined.
Data represent the mean and standard deviation from two independent experiments.

still showed activity. This suggests that the position of the amine is important, but it can be slightly modified. To investigate an *ortho* substitution to the amine, a methoxy was inserted (**20**). This reduced activity compared to **13**, but was still reasonably active. Next, the hydroxyl group was investigated. Both replacing it with a nitrile (**15**) as well as introducing a carbon spacer (**21**), completely abolished activity. When the hydroxyl moiety was substituted with a methyl (**22**), it retained reasonable activity, but the acetyl and benzyl (**23**–**24**) substituted compounds lost activity, suggesting that the free hydroxyl is important for activity and there is little

space for substituents here. Subsequently, substitutions to the amine were investigated. The dimethylated amine (**25**) showed good potency, but the acetylated (**26**) and propionylated (**27**) amine lost activity. Strikingly, substituting with a long aliphatic tail (**28**) recovered activity again, which is probably due to lipophilic interactions. The preference of KAT8 for lipophilic substitutions has been previously observed with the anacardic acid derived inhibitors as well [18]. A phenyl instead of a aliphatic tail (**29**) also showed better activity than the propionyl, suggesting that a lipophilic or aromatic interaction is gained close to the aminonaphthol scaffold. This was observed with two sulphonamide derivatives as well (**30**, **31**). These derivatives showed good activity for KAT8 and the toluene sulphonyl derivative was approximately twice as active as the methyl sulphonyl, suggesting a gained lipophilic or aromatic interaction. However, none of the derivatives was significantly more active than compound **13**. Therefore, compound **13** was chosen for further investigation.

2.3. Inhibitor properties

An often encountered problem with high-throughput screening is the discovery of hits that turn out to be pan assay-interfering compounds (PAINS) [19]. Computational tools are available to easily test structural entities for PAIN properties [20], but recent criticism warns against blind use of these tools, since it was observed that the tools will not recognize all PAIN structures [21]. It is therefore necessary to experimentally test the properties of the high throughput hit to test for PAINS behavior. Therefore, compound **13** was investigated for thiolreactivity, stability, reversibility, anti-oxidant properties and selectivity.

Compounds similar to compound **13** were discovered to be assay interfering compounds (PAINS), seeming to inhibit the HAT Rtt109 in an assay based on detection of the product CoA, but instead reacting with the thiol group of the product, which prevented detection of CoA [22]. The proposed mechanism was a Michael addition-elimination reaction in which the thiol, glutathion, replaced a thiol or halogen substituent on position 2 from the hydroxyl moiety. Therefore, the thiolreactivity of compound **13** was investigated in an assay containing all elements of the IC_{50} assay, but where Ac-CoA was replaced with CoA (Fig. S3). No reduction in fluorescence was found in this assay, suggesting that compound **13** does not react with thiols under these conditions. Additionally, the stability of compound **13** was investigated using HPLC and no degradation of the compound was observed up till 4 h of incubation with the assay buffer (Fig. S4). Longer incubation times were not tested.

To investigate whether **13** shows irreversible inhibition, a pre-incubation assay was performed (Fig. 1A). Three different concentrations (0.75, 2 and 10 $\times$ the IC_{50}) of compound **13** were pre-incubated with KAT8 for 2, 5 or 10 min before adding the enzyme substrates and measuring enzyme activity. An irreversible inhibitor will show time-dependent inhibition and therefore give more inhibition when pre-incubated with the enzyme for a longer time. However, no difference was observed between 2, 5 or 10 min pre-incubation with any of the concentrations, indicating that **13** is a reversible inhibitor of KAT8. This was confirmed using a dilution experiment (Fig. 1A) in which KAT8 was pre-incubated with **13** at a concentration of >5 times the IC_{50} value and subsequently diluted 100 times in a solution containing the substrates. The enzyme activity was measured over time and was shown to recover linearly, indicating a fully reversible inhibitor [23]. These data are consistent with a model where **13** is a reversible inhibitor of KAT8.

Due to their phenolic structure, **13** and several other derivatives have anti-oxidant activity. Although KAT8 does not depend on redox cycling mechanisms in its enzymatic reaction, it may interfere with the assay, which could lead to observation of inhibition, where no true inhibition of KAT8 takes place. Therefore, the anti-oxidant activity of **13** and all derivatives was determined in a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, a widely used assay for determination of anti-oxidant activity of single molecules [24]. The concentration that gave 50% reduction in DPPH absorbance (EC_{50}) was determined for all compounds (Fig. S5A). As expected, many derivatives containing the phenolic structure were anti-oxidants, showing very similar EC_{50}'s between 7 and 26 μM. However, their anti-oxidant activity did not show a relationship with their inhibitory activity on KAT8. For example, many compounds showing anti-oxidant activity, did not show KAT8 inhibition (**9**, **23**, **26**, **27**). Compound **19** showed no anti-oxidant activity due to the strong deactivating properties of the nitro substituent, but did show inhibitory activity on KAT8. Additionally, the IC_{50} of compound **13** was determined in the presence of non-thiol containing redox active substances (tris(2-carboxyethyl)phosphine (TCEP, 1 μM), NAD^+ (nicotinamide adeninedinucleotide, 100 μM) and NADH (100 μM)) (Fig. S5B). The presence of these redox active substances in the assay did not significantly influence the IC_{50} of **13**. Taken together, these data indicate that the anti-oxidant activity of compound **13** does not influence the inhibition of KAT8.

The selectivity of **13** and the active derivatives was investigated using KAT3B and KAT2B, representing two main other HAT families; p300/CBP and GNAT. An assay similar to that of KAT8 based on fluorescence detection of CoA was set up for KAT2B and KAT3B and the IC_{50} values of **13** and all derivatives showing activity on KAT8, were determined for KAT2B and KAT3B (Table 2, Figs. S6 and S7). Compound **13** showed inhibitory activity for both KAT2B ($IC_{50} = 3.6 \pm 1.1$) and KAT3B ($IC_{50} = 1.4 \pm 0.1$). Although the derivatives showed in general no selectivity over KAT2B and KAT3B, based on IC_{50} values, some differences were observed. For example, compound **22** shows activity for KAT8 and KAT3B, but seems inactive on KAT2B, suggesting that substitutions on the hydroxyl group are even more constrained for this enzyme. Additionally, compound **19** shows moderate activity on KAT8 and is preferred above for example **20** and **28**, but on KAT2B and KAT3B, it is less active compared to these others. This suggests that the nitro group is not preferred on KAT2B and KAT3B as on KAT8. Therefore, although **13** and derivatives are generally non-selective between KAT8, KAT2B and KAT3B based on IC_{50} values, the different enzymes were inhibited to different extents by the derivatives.

However, due to differences in catalytic mechansims, substrates and substrate affinities of the different HATs, which all influence the IC_{50} values, for accurate comparison of the potency for different enzymes, the K_i values should be calculated. For the calculation of the K_i values it is essential to determine the K_m values of the substrates for KAT2B and KAT3B. Therefore, both KAT2B and KAT3B were kinetically investigated for their catalytic mechanisms as initially done for KAT8 (Fig. S8A/B). KAT2B showed sigmoidal kinetics, suggesting a catalytic process with multiple steps of different velocities, for example cooperative subunits or two preferred catalytic mechanisms which depend on the concentration of Ac-CoA present [10] (Fig. S8A). This deviation from Michaelis-Menten kinetics is clearly observed when transforming the data to a Lineweaver-Burke plot. The normally linear regression of the double reciprocal is not linear, but curves upward (Fig. S8A). In case of KAT3B, it was not possible to fully reach saturation of the velocity (Fig. S8B). The histone 3 peptide substrate (H3 substrate), for which p300 has affinity as well, showed the same behavior. Unfortunately, since the determination of K_m values is based on model enzymes that follow Michaelis-Menten kinetics, in both of these cases it is not currently described how to determine K_m values of the substrates. Therefore, calculation of the K_m values, and

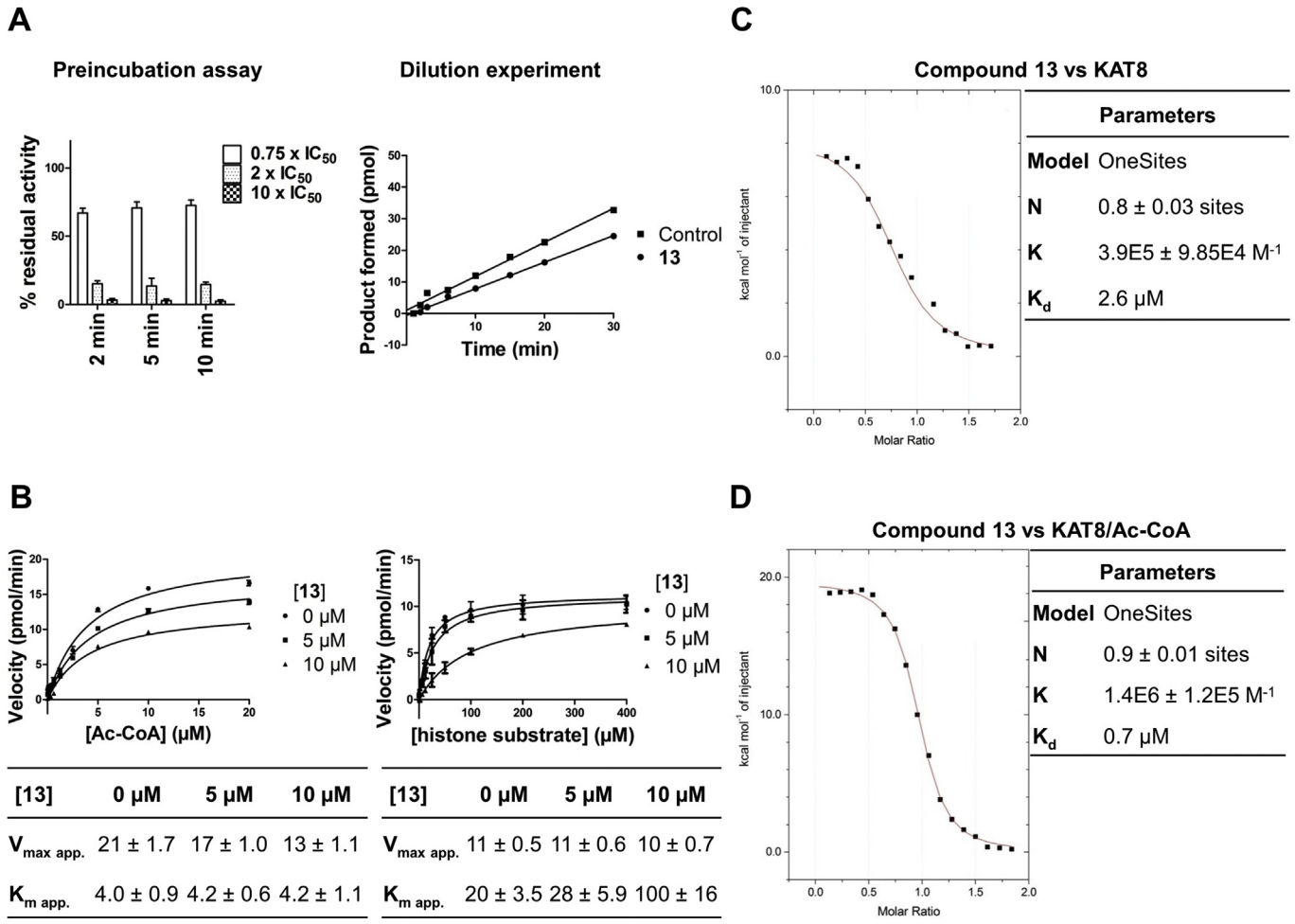


Fig. 1. A) A preincubation assay was performed to investigate whether **13** is an irreversible inhibitor. Compound **13** (0.75, 2 and 10 × IC_{50}) was preincubated for 2, 5 or 10 min with KAT8. Subsequently, the substrates were added and the enzyme reaction was performed. No difference in percentage inhibition was observed between 2, 5 or 10 min preincubation with any of the concentrations. This indicates that **13** is a reversible inhibitor. A dilution experiment was done to further confirm that **13** is a reversible inhibitor. KAT8 was preincubated with **13** at a concentration of 50 µM (>5 times the IC_{50}) and subsequently diluted 100 times in a solution containing the substrates. The enzyme activity was recovered linearly over time, indicating fully reversible inhibition. B) The velocity of the substrate conversion of KAT8 was measured at increasing concentrations of Ac-CoA in the presence of different concentrations of **13**. An increase in **13** concentration resulted in a decrease in V_{max}, but no change in the K_m of Ac-CoA. This indicates non-competitive behavior with Ac-CoA. The velocity of the substrate conversion of KAT8 was measured at increasing concentrations of histone substrate in the presence of different concentrations of **13**. An increase in **13** concentration did not influence the V_{max}, but resulted in an increase in K_m of the histone substrate. This indicates competitive behavior with the histone substrate. C) The equilibrium dissociation constant (K_d) of **13** to KAT8 was determined using isothermal titration calorimetry (ITC). Compound **13** showed a K_d of 2.6 µM for KAT8. N = stoichiometry, K = association constant ($1/K_d$), K_d = equilibrium dissociation constant (1/K). D) The equilibrium dissociation constant (K_d) of **13** to KAT8 in the presence of Ac-CoA was determined using isothermal titration calorimetry (ITC). Compound **13** showed a K_d of 0.7 µM. N = stoichiometry, K = association constant ($1/K_d$), K_d = equilibrium dissociation constant (1/K).

an accurate determination of the selectivity of the inhibitors, could not be done and remains to be investigated.

Additionally, the selectivity of compound **13** was investigated towards two unrelated enzymes, human arginase 1 and histone deacetylase 3 (HDAC3) (Fig. S9). Compound **13** was tested for activity on HDAC3 in a biochemical enzyme activity assay and on human arginase 1 in a binding assay based on differential scanning fluorimetry (DSF). Compound **13** showed inhibition of HDAC3 at higher concentrations (estimated $IC_{50} \approx 80–90$ µM). In the DSF assay, compound **13** showed no change in the melting temperature (T_m) of human arginase 1, suggesting that compound 13 did not bind this enzyme.

Taken together, considering the lack of selectivity for other HATs and HDAC3 and its anti-oxidant properties, which could cause effects unrelated to KAT8 inhibition in more advanced systems such as cells, we would not suggest using compound **13** for development of a selective drug targeting KAT8. However, control experiments suggest compound **13** does not interfere in the KAT8 assay due to thiolreactivity or its anti-oxidant properties. Since the aim of this study was to investigate the kinetic behavior of an inhibitor structurally unrelated to the current inhibitors on KAT8, compound **13** was considered suitable for further investigation of the mechanism of inhibition.

2.4. Mechanism of inhibition

To investigate the mechanism of inhibition by **13**, kinetic studies were done using Michaelis-Menten enzyme kinetics. The velocity of substrate conversion by KAT8 was measured at increasing concentrations of Ac-CoA or histone substrate in the presence of different concentrations of **13**. The apparent maximal velocity ($V_{max\ app.}$) and Michaelis constants ($K_{m\ app.}$) of Ac-CoA and the histone substrate were determined. In case of Ac-CoA, an increase in **13** concentration resulted in a decrease in $V_{max\ app.}$, but no change in the $K_{m\ app.}$ of Ac-CoA (Fig. 1B). This indicates non-competitive behavior with Ac-CoA, where binding of **13** does not influence binding of Ac-CoA. In case of the histone substrate, an increase in **13** concentration did not influence the $V_{max\ app.}$, but resulted in an

increase in $K_{m\ app.}$ of the histone substrate. This indicates competitive behavior with the histone substrate. KAT8 has been shown to operate via a ping-pong mechanism [18] in which Ac-CoA has to bind first to KAT8, followed by an acetylation of the enzyme, which creates a second intermediate form of the enzyme. Subsequently the histone substrate binds and is acetylated, generating the acetylated lysine and returning KAT8 back to its free form. Competitive behavior of **13** with the histone substrate therefore suggests that **13** binds not only to the free KAT8 enzyme, but also to the acetylated intermediate.

To further investigate this, binding studies of **13** to KAT8 were done using isothermal titration calorimetry (ITC). Compound **13** was titrated to KAT8 in the absence of Ac-CoA (the free enzyme) and the equilibrium dissociation constant (K_d) of **13** was determined (2.6 ± 0.7 μM, Fig. 1C, Fig. S10A). The stoichiometry of the interaction was close to one, suggesting that one molecule of **13** binds to one molecule of KAT8. Compound **13** thus directly interacts with KAT8. Additionally, **13** was titrated to KAT8 in the presence of Ac-CoA to generate a certain amount of the acetylated/Ac-CoA bound enzyme form. The binding data showed a K_d of 0.7 ± 0.06 μM (Fig. 1D, Fig. S10B). The K_d for Ac-CoA bound KAT8 is lower than for free KAT8, indicating that **13** binds with a higher affinity to the acetylated intermediate than to the free enzyme. These data suggest that compound **13** inhibits KAT8 by interacting both with the free enzyme form as well as with the Ac-CoA bound form, but has higher affinity for the acetylated intermediate.

2.5. Determination of the K_i values

The IC_{50} value determined in an enzyme inhibition assay is dependent on the conditions used in the assay, such as the concentration of the enzyme substrates and their respective K_m values. This value is therefore not reproducible unless exactly the same assay conditions are used. The K_i value is a potency value independent of the substrate concentration and K_m values and is therefore much more representative as potency of the inhibitor [6]. Calculation of the K_i value from the IC_{50} is crucial for the comparison of the potency with known inhibitors or between different assays and for the determination of selectivity. KAT8 is a bi-substrate enzyme that converts two substrates (Ac-CoA and the histone substrate) to two products (CoA and the acetylated histone substrate). Therefore, knowledge on the catalytic mechanism of the enzyme, as well as the mechanism of inhibition by the inhibitor is needed to calculate the K_i value [9]. It was previously reported by us that KAT8 follows a ping-pong mechanism in which Ac-CoA binds first and acetylates a residue on the enzyme. Subsequently the histone substrate binds and is acetylated [18]. Combining the knowledge that KAT8 follows a ping-pong mechanism and that **13** inhibits KAT8 by interacting both with the free enzyme form as with the Ac-CoA bound form as determined from the kinetic experiments, equation (1) can be used to calculate the K_i values [9]. It must be noticed that this equation yields two K_i values, one for the free KAT8 enzyme (K_{i1}) and one for the acetylated intermediate (K_{i2}). Fig. 2 schematically shows the ping-pong mechanism of KAT8 and the inhibition of **13** of the free enzyme (E) and the acetylated enzyme form (AcE) and its respective K_i values. The K_m values of Ac-CoA and the histone substrate were derived from a kinetic assay with both substrates as described by Segel [10] and were consistent with previously reported values (Fig. S10C/D) [18]. We assumed that the K_{i1} was equal to the K_d of **13** to KAT8 as determined by ITC, since for the process of inhibiting the free enzyme (E), only binding of **13** is of influence. The K_{i1} of **13** was therefore assumed to be 2.6 μM and the K_{i2} was 0.017 μM as derived from the equation (see SI for calculation). This suggests that the inhibitory potency was much better for the acetylated intermediate than for the free

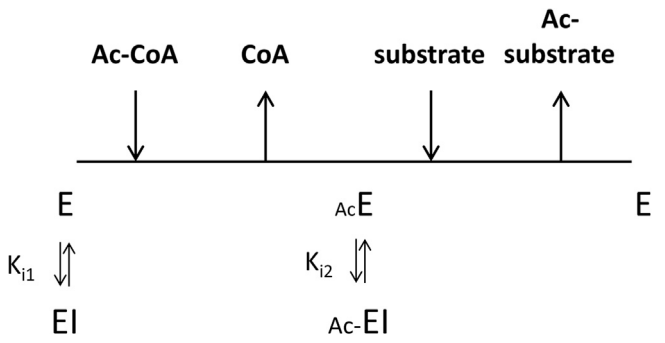


Fig. 2. KAT8 uses a ping-pong mechanism in which the cofactor, acetyl coenzyme A (Ac-CoA), binds first to the free enzyme form (E) and acetylates a residue on the enzyme (AcE). Coenzyme A (CoA) leaves the enzyme as product. Subsequently, the histone substrate can bind and is acetylated by the enzyme. The enzyme returns to its free form (E). Compound **13** (I) can interact with the free enzyme (E), with an inhibitory potency (K_{i1}). It can also interact with the acetylated enzyme form (AcE) with an inhibitory potency (K_{i2}).

enzyme. This stronger interaction was also observed in the K_d value of **13** for KAT8 in presence Ac-CoA as determined by ITC, which was lower than the K_d for KAT8 in absence of Ac-CoA. Additionally, the kinetic behavior of **13** is different from the behavior of the previously reported anacardic acid derivatives, which did not interact with the free enzyme [18]. This kinetic behavior has a large influence on the calculation of the resulting K_i values, suggesting that kinetic evaluations of inhibitors for this enzyme are crucial for determining inhibitory potency. Taken together, this suggests that the inhibition of compound **13** is mostly due to inhibition of the acetylated enzyme intermediate by competition with the histone substrate and that these kinetic evaluations are crucial for determining the inhibitory potency of KAT8 inhibitors.

$$k_bA + K_aB + AB = \left(\frac{K_b}{B}\frac{1}{K_{i2}}\frac{K_a}{A}\frac{1}{K_{i1}}\right)IC_{50} \quad (1)$$

IC_{50} = the IC_{50} determined in the enzyme inhibition assay
K_a = K_m of Ac-CoA
K_b = K_m of the histone substrate
A = the concentration of Ac-CoA used in the IC_{50} assay
B = the concentration of the histone substrate used in the IC_{50} assay
K_{i1} = the K_i of **13** for the free enzyme
K_{i2} = the K_i of **13** for the acetylated intermediate

3. Conclusion

In conclusion, this study describes the discovery of a potent fragment inhibitor of KAT8, compound **13**, via a fragment screening approach. A SAR study was done, which showed that **13** could be modified, although no derivatives were found that were significantly more potent. Investigation of the compound properties suggested that compound **13** was not selective for other HATs or HDAC3 and had anti-oxidant properties. However, experiments with CoA and redox active substances suggest compound **13** was a reversible inhibitor that did not interfere in the KAT8 assay due to thiolreactivity or its anti-oxidant properties. Therefore it was used for investigation of its mechanism of inhibition of KAT8. The results of kinetic studies and ITC are consistent with a model where the fragment interacts with both the free enzyme and the acetylated intermediate form. This enabled the calculation of the assay-independent K_i values of **13** for both the free enzyme form of

KAT8 (K_{i1} = 2.6 μM), and the acetylated form (K_{i2} = 0.017 μM), which suggested that its inhibition is mostly due to interaction with the acetylated form of the enzyme. Taken together, in this study a fragment screening is presented that provides a fragment inhibitor of KAT8 that is further characterized by enzyme kinetics and biophysics. This combination reveals a striking difference in binding affinity between the acetylated enzyme and the free enzyme that is not revealed by the IC_{50} determinations. This shows that kinetic characterization of inhibitors and calculation of K_i values is crucial for determining the binding constants of HAT inhibitors. We anticipate that more comprehensive characterization of enzyme inhibition, as described here, is needed to advance the field of HAT inhibitors.

Acknowledgements

We acknowledge the European Research Council for providing an ERC starting grant (309782) and the NWO for providing a VIDI grant (723.012.005) to F. J. Dekker. This work was supported by PRIN 2012 (prot. 2012CTAYSY) to D. Rotili and PRIN 2016 (prot. 20152TE5PK) to A. Mai.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2017.05.015.

References

[1] A. Ganesan, Multitarget drugs: an epigenetic epiphany, ChemMedChem 11 (12) (2016 Jun 20) 1227–1241.
[2] A. Bird, Perceptions of epigenetics, Nature 447 (7143) (2007) 396–398.
[3] B.D. Strahl, C.D. Allis, The language of covalent histone modifications, Nature 403 (6765) (2000) 41–45.
[4] S. Kaypee, D. Sudarshan, M.K. Shanmugam, D. Mukherjee, G. Sethi, T.K. Kundu, Aberrant lysine acetylation in tumorigenesis: implications in the development of therapeutics, Pharmacol. Ther. 162 (2016 Jun) 98–119.
[5] R.P. Simon, D. Robaa, Z. Alhalabi, W. Sippl, M. Jung, KATching-up on small molecule modulators of lysine acetyltransferases, J. Med. Chem. 59 (4) (2016 Feb 25) 1249–1270.
[6] H. Wapenaar, F.J. Dekker, Histone acetyltransferases: challenges in targeting bi-substrate enzymes, Clin. Epigenetics 8 (2016 May 26), 59–016-0225-2.
[7] H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, J. Am. Chem. Soc. 56 (3) (1934 03/01) 658–666.
[8] M. Dixon, The determination of enzyme inhibitor constants, Biochem. J. 55 (1) (1953 Aug) 170–171.
[9] Y. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction, Biochem. Pharmacol. 22 (23) (1973 Dec 1) 3099–3108.
[10] I.H. Segel, Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems, Wiley Classics Library, 1993.
[11] R. Marmorstein, Structure and function of histone acetyltransferases, Cell Mol. Life Sci. 58 (5–6) (2001 May) 693–703.
[12] E.R. Smith, C. Cayrou, R. Huang, W.S. Lane, J. Cote, J.C. Lucchesi, A human protein complex homologous to the Drosophila MSL complex is responsible for the majority of histone H4 acetylation at lysine 16, Mol. Cell Biol. 25 (21) (2005 Nov) 9175–9188.
[13] Y. Dou, T.A. Milne, A.J. Tackett, E.R. Smith, A. Fukuda, J. Wysocka, et al., Physical association and coordinate function of the H3 K4 methyltransferase MLL1 and the H4 K16 acetyltransferase MOF, Cell 121 (6) (2005 Jun 17) 873–885.
[14] X. Li, L. Wu, C.A. Corsa, S. Kunkel, Y. Dou, Two mammalian MOF complexes regulate transcription activation by distinct mechanisms, Mol. Cell 36 (2) (2009 Oct 23) 290–301.
[15] Y. Cai, J. Jin, S.K. Swanson, M.D. Cole, S.H. Choi, L. Florens, et al., Subunit composition and substrate specificity of a MOF-containing histone acetyltransferase distinct from the male-specific lethal (MSL) complex, J. Biol. Chem. 285 (7) (2010 Feb 12) 4268–4272.
[16] S. Rea, G. Xouri, A. Akhtar, Males absent on the first (MOF): from flies to humans, Oncogene 26 (37) (2007 Aug 13) 5385–5394.
[17] D.G. Valerio, H. Xu, C.W. Chen, T. Hoshii, M.E. Eisold, C. Delaney, et al., Histone acetyltransferase activity of MOF is required for MLL-AF9 leukemogenesis, Cancer Res. 77 (7) (2017 Apr 1) 1753–1762.
[18] H. Wapenaar, P.E. van der Wouden, M.R. Groves, D. Rotili, A. Mai, F.J. Dekker, Enzyme kinetics and inhibition of histone acetyltransferase KAT8, Eur. J. Med. Chem. 105 (2015 Nov 13) 289–296.
[19] J.B. Baell, Observations on screening-based research and some concerning trends in the literature, Future Med. Chem. 2 (10) (2010 Oct) 1529–1546.
[20] C. Aldrich, C. Bertozzi, G.I. Georg, L. Kiessling, C. Lindsley, D. Liotta, et al., The ecstasy and agony of assay interference compounds, ACS Chem. Biol. 12 (3) (2017 Mar 17) 575–578.
[21] S.J. Capuzzi, E.N. Muratov, A. Tropsha, Phantom PAINS: Problems with the utility of alerts for Pan-Assay INterference CompoundS, J. Chem. Inf. Model 57 (3) (2017 Mar 27) 417–427.
[22] J.L. Dahlin, J.W. Nissink, J.M. Strasser, S. Francis, L. Higgins, H. Zhou, et al., PAINS in the assay: chemical mechanisms of assay interference and promiscuous enzymatic inhibition observed during a sulfhydryl-scavenging HTS, J. Med. Chem. 58 (5) (2015 Mar 12) 2091–2113.
[23] R. Copeland, Evaluation of Enzyme Inhibitors in Drug Discovery: a Guide for Medicinal Chemists and Pharmacologists, second ed., Wiley, 2013.
[24] S.B. Kedare, R.P. Singh, Genesis and development of DPPH method of antioxidant assay, J. Food Sci. Technol. 48 (4) (2011 Aug) 412–422.