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Research paper

Enzyme kinetics and inhibition of histone acetyltransferase KAT8

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ABSTRACT

Lysine acetyltransferase 8 (KAT8) is a histone acetyltransferase (HAT) responsible for acetylating lysine 16 on histone H4 (H4K16) and plays a role in cell cycle progression as well as acetylation of the tumor suppressor protein p53. Further studies on its biological function and drug discovery initiatives will benefit from the development of small molecule inhibitors for this enzyme. As a first step towards this aim we investigated the enzyme kinetics of this bi-substrate enzyme. The kinetic experiments indicate a ping-pong mechanism in which the enzyme binds Ac-CoA first, followed by binding of the histone substrate. This mechanism is supported by affinity measurements of both substrates using isothermal titration calorimetry (ITC). Using this information, the KAT8 inhibition of a focused compound collection around the non-selective HAT inhibitor anacardic acid has been investigated. Kinetic studies with anacardic acid were performed, based on which a model for the catalytic activity of KAT8 and the inhibitory action of anacardic acid (AA) was proposed. This enabled the calculation of the inhibition constant K_i of anacardic acid derivatives using an adaptation of the Cheng–Prusoff equation. The results described in this study give insight into the catalytic mechanism of KAT8 and present the first well-characterized small-molecule inhibitors for this HAT.

1. Introduction

Epigenetic modifications of histones, such a lysine acetylation, play a key role in the regulation of gene transcription. Histone acetyltransferases (HATs) are a class of acetyltransferases that catalyze the acetylation of ε-amino groups on lysine residues in both histone and non-histone proteins. Through histone acetylation, they play a regulatory role in the chromatin structure, thereby influencing gene transcription. The acetylation of non-histone proteins, for example transcription factors, is involved in the regulation of many processes, such as cell growth and inflammatory signaling [1]. This enzyme class has been linked to the pathology of various diseases, including cancer [2–4], inflammatory diseases [5–7], viral infections [8] and neurological diseases [9,10]. However, knowledge on their role in specific diseases and drug discovery efforts towards this class of enzymes are still limited. In particular the HAT lysine acetyltransferase 8 (KAT8) is marginally explored in drug discovery projects.

The HATs are a disparate group of enzymes that can be divided into different families based on their structural homology. The three main families are GNAT (Gcn5-related N-acetyltransferases), MYST (MOZ, YBF2/SAS3, SAS2 and TIP60) and p300/CBP (CREB binding protein). The HAT of our interest, KAT8, is a member of the MYST family. This enzyme was originally discovered in *Drosophila*, where it is involved in dose-compensation of the X-chromosome gene transcription in male flies. KAT8 functions in two protein complexes, MSL and MSL1v1, that are conserved throughout the eukaryotic kingdom, including humans [11]. Both KAT8 complexes have been described to be responsible for acetylating lysine 16 on histone H4 (H4K16) and were shown to play a role in cell cycle progression [12]. However, only the MSL1v1 complex seems to be involved in acetylation of the tumor suppressor protein p53 [13]. KAT8 has also been shown to play a role in embryonic stem cell renewal. Embryonic stem cells lacking KAT8 lose differentiation potential and show changes in morphology and gene expression of essential transcription factors [14]. Thus, it is clear that KAT8 plays a

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very important role in normal physiology and disease.

KAT8 is a bi-substrate enzyme that binds two substrates; acetyl coenzyme A (Ac-CoA) and histone H4 containing free lysine ε-amino groups. Development of inhibitors for bi-substrate enzymes requires knowledge of the catalytic mechanism. It is important to understand if the substrates, acetyl coenzyme A and histone H4, bind simultaneously or consequently and if the individual binding events are inter-dependent. In addition, knowledge on the catalytic mechanism combined with inhibitor kinetics, enables the calculation of the assay-independent inhibition constant (K_i) from the assay-dependent inhibitory concentration (IC_{50}) as described by Cheng and Prusoff [15]. Therefore, we investigated the catalytic mechanism of KAT8 using enzyme kinetic studies based on models described by Copeland [16]. We demonstrated that the non-selective HAT inhibitor anacardic acid (AA) [17] also inhibits KAT8 and performed kinetic studies to further investigate this inhibitor. Based on the results, we proposed a model comprising the catalytic activity of KAT8 and the inhibitory action of AA. We employed this knowledge to study the inhibitory potency of a small collection of anacardic acid derived inhibitors and to calculate their respective binding constants (K_i). Inhibition studies on p300 did not reveal selectivity between both enzymes for the compound collection that was investigated.

2. Results and discussion

2.1. Catalytic mechanism

As described by Copeland[16], steady state kinetic experiments can be employed to determine by which catalytic mechanism the enzyme operates. In our studies we adapted an enzyme activity assay based on fluorescence detection of the HAT reaction product CoA, as described by Gao et al. [18] for use with KAT8. In this assay the CoA thiolate is detected by the thiol sensitive fluorescent dye 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM). As histone substrate, a synthetic peptide corresponding to the first 20 amino acids of the histone H4 *N*-terminal (histone H4 peptide) was used. First, the K_m of the histone substrate and k_{cat} of the enzyme were determined using increasing concentrations of histone substrate at constant concentration of Ac-CoA (Fig. 1A). Then the catalytic mechanism was determined according to procedures described by Copeland. The velocity of recombinantly expressed KAT8 was determined at different concentrations of Ac-CoA in the presence of varying concentrations of histone substrate (Fig. 1B). The K_m and V_{max} of Ac-CoA were determined. Both K_m and V_{max} of Ac-CoA increased at increasing concentrations of histone substrate (Table 1), which is characteristic for a ping-pong mechanism. In a ping-pong mechanism the donor substrate binds first to the enzyme. In case of KAT8, Ac-CoA acts as an acetyl donor. Subsequently the acetyl group is transferred temporarily to a residue on the enzyme and CoA leaves the binding pocket. Then the histone substrate binds and the acetyl group is transferred onto its lysine residue, upon which the second product is formed.

From the V_{max} and the enzyme concentration, the turnover number of the enzyme (k_{cat}) can be calculated. This is the maximum number of substrate molecules that the enzyme can convert per catalytic site per unit of time. The k_{cat} ranged between 0.2 and 1.1 molecules per minute, which is lower than the k_{cat} observed by Yang et al. [19]. This difference can be explained by the concentration dependency of V_{max} and k_{cat} on the concentration of both substrates (Table 1).

As described by Copeland, in case of a ping-pong mechanism the histone peptide should have little or no affinity for the enzyme in absence of Ac-CoA. To find further evidence for this mechanism isothermal titration calorimetry (ITC) experiments were performed (Fig. 2). Titration of Ac-CoA to KAT8 indicated binding with an equilibrium dissociation binding constant (K_d) of 8.7 μM (Table 2). This is close to the K_d determined for GNAT family HATs [20] and the yeast HAT ESA1 in the picNuA4 complex [21]. The stoichiometry (N) of the interaction was close to 1, showing that one molecule of Ac-CoA binds to one molecule of enzyme. Subsequent titration of histone substrate to the KAT8/Ac-CoA complex demonstrated a K_d of 6.7 μM for the histone substrate. In contrast, titration of the histone substrate solution to the KAT8 enzyme in absence of Ac-CoA showed no measurable binding. These observations are consistent with the ping-pong mechanism for the KAT8 acetyltransferase activity as postulated based on the enzyme kinetic experiments, providing further proof for this mechanism.

It is of interest to mention that binding of both substrates has an unfavorable enthalpy component that is compensated for by a strongly favorable entropy component (Table 2). Strongly favorable entropy components in equilibrium binding kinetics are often linked to the release of bound water molecules upon substrate

Table 1
K_m, V_{max} and k_{cat} of Ac-CoA at varying concentrations of histone H4 peptide (Fig. 1).

Histone H4 peptide	V_{max} (pmol/min)	K_m (μM)	k_{cat} (min^{-1})
15 μM	3.4 ± 0.2	1.1 ± 0.3	0.2 ± 0.02
30 μM	4.5 ± 0.3	1.2 ± 0.3	0.3 ± 0.02
60 μM	9.9 ± 0.3	2.1 ± 0.3	0.7 ± 0.04
90 μM	14.7 ± 0.6	2.8 ± 0.5	1.1 ± 0.06

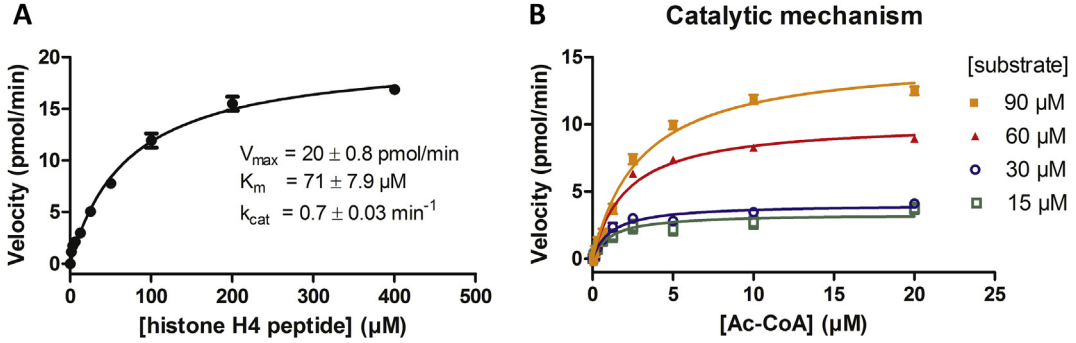


Fig. 1. Kinetics and catalytic mechanism of the bi-substrate enzyme KAT8. A) Determination of V_{max}, K_m and k_{cat} of the histone substrate (histone H4 peptide). The steady-state velocity was determined of recombinantly expressed KAT8 using 0–400 μM histone substrate and 4 μM Ac-CoA. V_{max}, K_m and k_{cat} of the histone substrate were derived from the non-linear Michaelis–Menten regression. B) KAT8 operates via a ping-pong mechanism. The steady-state velocity was determined of KAT8 at 0–20 μM of Ac-CoA in the presence of 15, 30, 60 and 90 μM of histone substrate. Both K_m and V_{max} of Ac-CoA increased at increasing concentrations of histone substrate (H4peptide), which suggests a ping-pong mechanism.

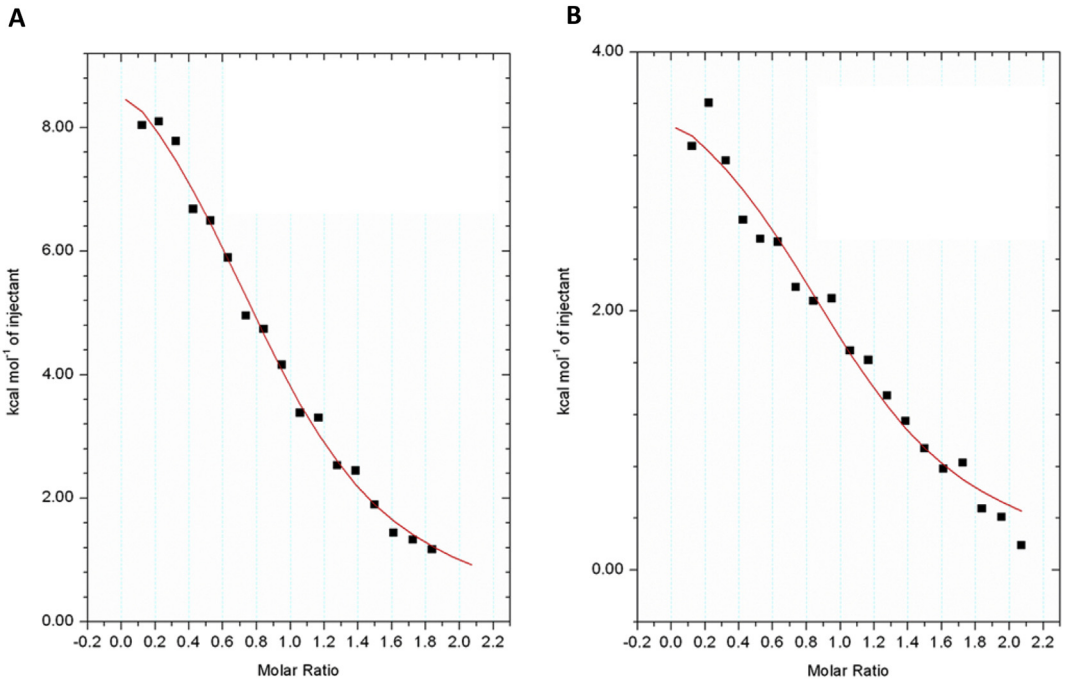


Fig. 2. ITC experiments support a ping-pong mechanism. ITC experiments were done to determine the affinity of the substrates for KAT8. A) Titration of Ac-CoA to KAT8 demonstrated a K_d of 8.7 μM. B) Titration of the histone substrate (histone H4peptide) to the KAT8/Ac-CoA complex demonstrated a K_d of 6.7 μM. However, this peptide showed no affinity for KAT8 in absence of Ac-CoA. Based on these data the binding energies were calculated as shown in Table 2.

Table 2
Parameters for Ac-CoA and the histone substrate as derived from ITC experiments (Fig. 2).

	Ac-CoA vs KAT8	Histone H4 peptide vs Ac-CoA/KAT8
Kd (μM)	8.7 ± 1.2	6.7 ± 2.1
N (stoichiometry)	0.95	1.03
ΔH (cal/mol)	10,470 ± 470	3732 ± 300
−TΔS (cal/mol/degK)	−17228	−10665
ΔG	−6758	−6933

binding, which may also be the case in KAT8.

A requirement for a ping-pong mechanism is that the binding pocket has a residue that can temporarily accept the acetyl group before transfer to the substrate. This role is usually taken by a cysteine residue because acetylation of a cysteine residue results in a thioester, which is prone to aminolysis by the lysine ε-amine functionalities of the histone substrates. Previously published KAT8 crystal structures (PDB: 2GIV, 3TOA [19]) indicate that cysteine-143 is close to the binding site of Ac-CoA (Fig. 3A). It seems reasonable to presume that this cysteine residue plays a role as an initial acceptor of the acetyl group in the postulated ping-pong mechanism, although none of these published crystals structures contain an acetylated cysteine residue in this position. Surprisingly, close to this cysteine, both crystal structures show an acetylated lysine residue (Ac-Lys-101) that is auto-acetylated by KAT8 itself. As shown by mutagenesis studies, both the cysteine and lysine residues were essential for HAT activity [19]. The observation that the cysteine residue is essential for catalysis also supports the postulated ping-pong mechanism.

Further support for the ping-pong mechanism in KAT8 catalysis comes from the crystal structure of the yeast HAT ESA1, which has a high sequence and structural similarity with KAT8. Importantly, the Ac-CoA binding pocket is almost identical and the aforementioned cysteine and lysine residues are conserved (Cys-304 and Lys-262). Interestingly, a crystal structure of ESA1 co-crystallized with Ac-CoA showed bound CoA rather than Ac-CoA and acetylation of cysteine-304 (PDB: 1MJA, Fig. 3B) [22]. Taken together, the postulated ping-pong mechanism as observed in the enzyme kinetic experiments can be rationalized by structural data for KAT8 and closely related HATs thus further supporting the evidence for this mechanism.

Despite this evidence we should note that enzyme kinetics for HATs frequently seem to depend on the assay conditions. For example, in contrast to the structural data described before, a kinetic study on the yeast analog ESA1 indicated the formation of a ternary complex between the enzyme, Ac-CoA and the substrate in catalysis [21]. Comparable complications were encountered in the analysis of the catalytic mechanism of p300. Based on enzyme kinetics a ping-pong mechanism was proposed [23]. However, it was demonstrated that an electrophilic acetyl-CoA affinity labelling-based probe did not target a residue that is critical for catalysis, arguing against a ping-pong mechanism [24], but a ternary complex mechanism could not be confirmed either. Despite the fact that p300 contains a cysteine in the binding pocket no mutagenesis studies have yet been performed to investigate whether this cysteine is important for HAT activity. Based on structural and biochemical data, it was proposed that p300/CBP uses a modified mechanism denoted as the Theorell–Chance ('hit-and-run') catalytic mechanism. In the Theorell–Chance mechanism, there is no stable ternary complex as formed in a standard ternary complex mechanism. After acetyl-CoA binds, the histone substrate associates weakly with the p300 surface, allowing the lysine to react with the acetyl group, but kinetically only the interaction with acetyl-CoA is important [25]. Therefore, we do not exclude the possibility that a more refined enzyme kinetic model will be assigned to KAT8 in the future. Nevertheless, the steady state kinetic study here clearly indicates a ping-pong mechanism in which Ac-CoA binds first followed by binding of the histone H4 peptide. Therefore, we applied this mechanism for calculation of the K_i values of inhibitors of this enzyme using an adaptation of the Cheng–Prusoff equation

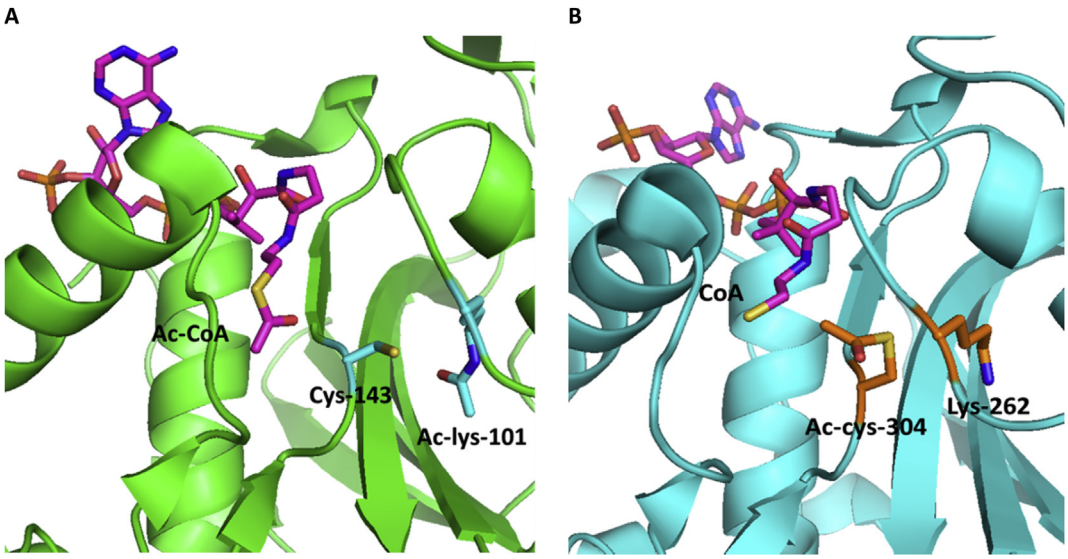


Fig. 3. Structural support for a ping-pong mechanism. A) A crystal structure of KAT8 (PDB:2GIV) shows Ac-CoA bound to the enzyme. Close to the binding pocket, a cysteine is situated, which could facilitate a ping-pong mechanism. Additionally, an acetylated lysine is shown, which is essential for activity of KAT8[19]. B) A crystal structure of yeast ESA1, a MYST family HAT and homologue to KAT5, shows an almost identical binding pocket with an acetylated cysteine. It is possible that either the cysteine or the lysine, or both, contribute to a ping-pong mechanism.

as described by Copeland [16].

2.2. Inhibitors – chemistry

Anacardic acid is a known natural product HAT inhibitor, which also shows activity on KAT8. A focused compound collection inspired by AA was assembled from newly synthesized compounds **8a-b**, **11c-e** and **13,** previously synthesized compounds **14** and **15** [26] and the known p300 inhibitor C646. The compound collection was designed to vary the position of alkylation of the salicylate core and the length and polarity of the aliphatic substituent. The compounds were synthesized, using a convenient and flexible synthetic route employing Sonogashira coupling as a key step as published previously [27]. Using this strategy, different salicylate triflates or halides were linked to various alkynes.

6-alkyl substitution of the salicylate core was achieved using Sonogashira coupling of terminal alkynes to triflate **5**. Triflate **5** was synthesized from 2,6-dihydroxybenzoic acid using a previously published two-step synthesis [28]. 5-alkyl substitution of the salicylate core was achieved with aryl halides **9** as starting materials, which were prepared using known procedures [29,30].

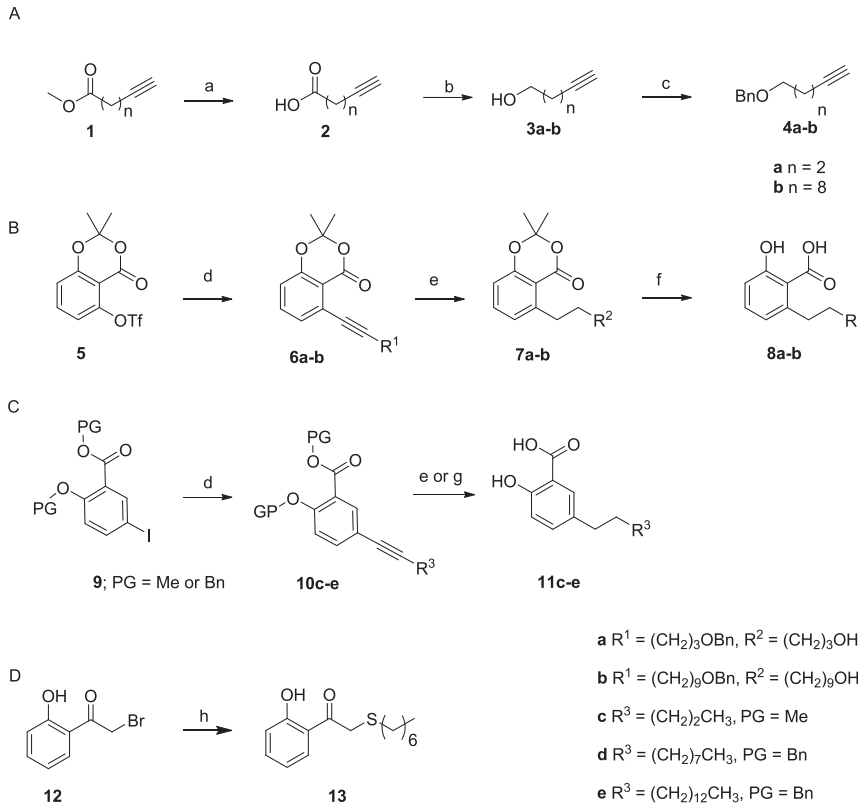
Commercially available terminal alkynes were used, except for terminal alkynes **4a** and **4b**. Alkyne **4a** was synthesized by benzylation of **3a** using previously published procedures [31] (Scheme 1A). Alkyne **4b** was synthesized from methyl undec-10-ynoate (**1**) by hydrolysis of the ester (**2**), reduction of the acid (**3b**) and subsequent benzylation in an overall yield of 44% over 3 steps.

Sonogashira couplings in the salicylate 6-position (Scheme 1B) and the 5-position (Scheme 1C) were performed with moderate to good yields (46–98%). The purification was in some cases demanding due to side products formed in the reaction. The resulting alkynes were reduced by hydrogenation, which simultaneously resulted in the removal of the benzyl moiety from the aliphatic alcohols. Intermediate **6b** could not be completely purified after the Sonogashira coupling due to presence of a side product with similar polarity. After an initial hydrogenation step, which proved to reduce the triple bond, the impurity could be removed. Removal of the benzyl required a separate hydrogenolysis step giving **7b** in 60% yield. Hydrolysis of the acetonide of **7a** using the published procedures of 5 N KOH in THF/water at 55 °C required 5 days giving **8a** in 24% yield. Therefore **7b** was deprotected by acid using 11 M HCl in dioxane/water. An improved yield of 46% was observed under these conditions. The methyl protecting group of **10c** was removed with boron tribromide in 53% yield. The removal of the benzyl protective group could be done simultaneously with the hydrogenation and was therefore used for the other compounds. Hydrogenation and hydrogenolysis of **10d-e** was done very efficiently, giving **11d-e** in 88–97% yield.

To investigate whether the salicylic acid could be replaced by a 2-hydroxy acetophenone group, product **13** was synthesized (Scheme 1D). A substitution reaction using 2-bromo-2′-hydroxyacetophenone **12** and 1-heptane thiol provided **13** in one step in 85% yield.

2.3. Inhibitor kinetics-KAT8

To investigate the binding kinetics of the inhibitors, we conducted enzyme kinetic studies on KAT8 with AA. The velocity of KAT8 was determined at different concentrations of Ac-CoA and constant concentration of the histone substrate in the presence of varying concentrations of AA (Fig. 4A). A clear decrease in V_{max} and K_m with increasing concentrations of AA was observed (Table 3). The decrease in V_{max} indicates that AA binds a site allosteric from the Ac-CoA binding pocket. The decrease in K_m suggests that the binding of AA stabilizes the binding of Ac-CoA. This is a characteristic of uncompetitive inhibition, where the substrate must be present for the inhibitor to bind. The same experiment was done using different concentrations of the histone substrate and a constant concentration of Ac-CoA (Fig. 4B). Strikingly, the curves do not follow Michaelis–Menten kinetics when AA is present, but have a sigmoidal appearance. This shows that there is cooperativity resulting in a Hill coefficient, a measure for the slope of the curve, that is not equal to 1 as described in the Monod–Wyman–Changeux (MWC) model [32]. It is not possible to derive a true K_m from a sigmoidal curve and therefore the concentration of peptide that gives half-maximal velocity (k_{half}) is determined, which resembles the K_m, but is dependent on the hill slope (for calculation of k_{half} see SI). At increasing concentrations of



Scheme 1. Synthesis of anacardic acid derivatives. a) lithium hydroxide, THF/water, RT; b) $LiAlH_4$, THF, 0 °C - RT; c) Benzyl bromide, NaH, THF, 0 °C - RT; d) $PdCl_2(PPh_3)_2$, CuI, Et_2NH, CH_3CN, respective aliphatic alkynes or alkyne 4a-b, 100 °C, MW; e) Hydrogenation triple bond and hydrogenolysis of benzyl protected salicylic acids or alkynes: H_2 (3 atm), Pd/C, MeOH/ethyl acetate, 40 °C, PARR apparatus; f) 5 N KOH, THF, 55 °C or HCl (37%), dioxane, RT; g) deprotection of methyl protected salicylic acids: 1 M BBr_3 in DCM, acetonitrile, −78 °C to RT; h) 1-heptanethiol, KOH, MeOH, 0 °C to RT.

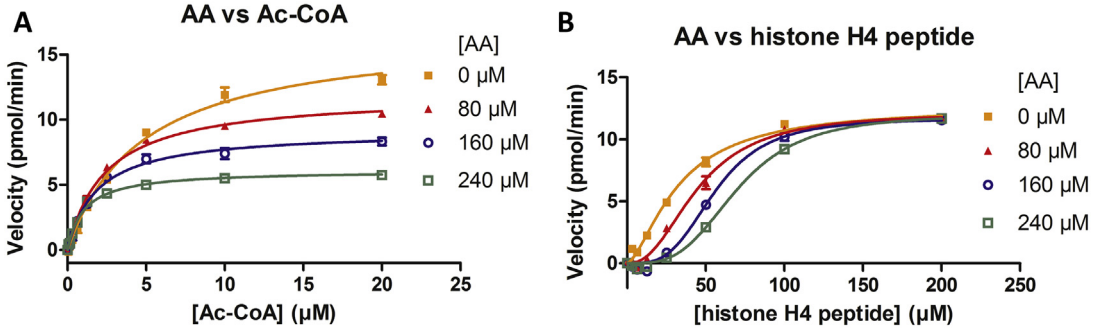


Fig. 4. Inhibitor kinetics of AA on KAT8. The velocity of KAT8 was determined at 0–20 μM Ac-CoA in the presence of 0, 80, 160 and 240 μM AA respectively. Both K_m and V_{max} decrease at increasing concentrations of AA. The velocity of KAT8 was determined at 0–200 μM of the histone substrate (histone H4 peptide) in the presence of 0, 80, 160 and 240 μM AA respectively.

Table 3
V_{max} and K_m for Ac-CoA at different concentrations of inhibitor AA. V_{max}, hill slope and k_{half} for histone H4 peptide at different concentrations of inhibitor AA (Fig. 4).

[AA] (μM)	Ac-CoA		Histone H4 peptide		
	V_{max} (pmol/min)	K_m (μM)	V_{max} (pmol/min)	Hill slope	k_{half} (μM)
0	17 ± 0.7	4.8 ± 0.5	13 ± 0.7	1.5 ± 0.2	36
80	12 ± 0.3	2.4 ± 0.2	12 ± 0.5	2.3 ± 0.3	47
160	9.2 ± 0.3	1.9 ± 0.2	12 ± 0.5	3.4 ± 0.6	54
240	6.1 ± 0.2	1.0 ± 0.1	12 ± 0.4	3.4 ± 0.3	514

AA, the V_{max} is constant, but an increase is observed in k_{half}. This suggests that the binding of AA opposes the binding of the histone substrate.

The cooperativity may be explained using a model based on the existence of two conformations of the enzyme, E and E* (Fig. 5). Ac-CoA binds the free enzyme (E), which has low catalytic activity. The

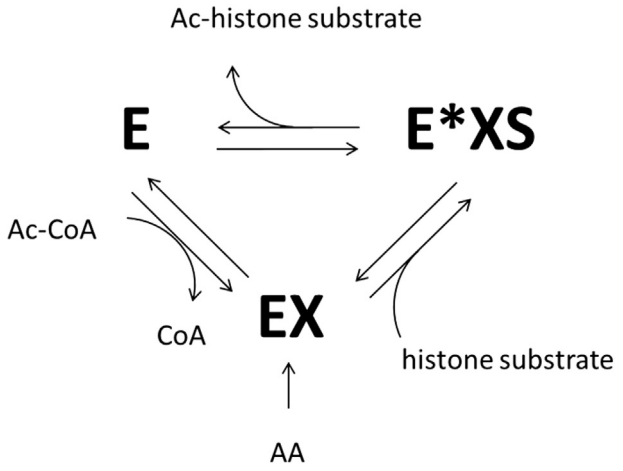


Fig. 5. Proposed model for the inhibitory and catalytic activity of AA and KAT8. Ac-CoA binds to the free enzyme (E), which has low catalytic activity. The histone substrate is not able to bind this conformation. Following the ping-pong mechanism, the acetyl group (X) is transferred to the enzyme (EX). Binding of the histone substrate (S) induces a conformational change of the enzyme (E*XS), which is catalytically active. Ac-CoA has no or lower affinity for this conformation. The histone substrate is acetylated and leaves the enzyme as product (Ac-histone substrate) upon which the free enzyme conformation (E) is regenerated. The inhibitor AA binds to EX and stabilizes the catalytically inactive conformation, therefore inhibiting the catalytic activity of the enzyme, but increasing the affinity of Ac-CoA.

histone substrate is not able to bind this conformation, which is shown by the ITC data (Fig. 2). Upon binding of Ac-CoA, the acetyl group (X) is transferred onto the enzyme (EX) and the histone substrate (S) can bind. This is shown by the ITC experiments as well as the mechanistic studies. Binding of the histone substrate induces a conformational change (E*XS), which is catalytically active. Ac-CoA has lower or no affinity for this conformation, which is shown by the increase in K_m of Ac-CoA induced by higher concentrations of histone H4 peptide (Fig. 1). The histone substrate is acetylated, leaves the enzyme as product (Ac-histone substrate) and free enzyme (E) is regenerated. The inhibitor AA binds to EX, thereby stabilizing the catalytically inactive conformation of the enzyme (E). This is shown by the increase in K_m of the histone substrate and especially by the decrease in K_m of Ac-CoA. Ac-CoA regains affinity for the enzyme, even though the enzyme activity is inhibited. Additionally, an increasing concentration of histone substrate, which induces conformation E* for which AA has little or no affinity, will eventually be able to restore activity of the enzyme. Cooperativity between an active and an inactive conformation can cause the sigmoidal behavior observed in the inhibitor kinetics with the histone substrate. This was also observed in case of the bi-substrate enzyme phosphofructokinase [33]. Michaelis–Menten kinetics can be observed when the enzyme has maximal activity. A shift towards sigmoidal kinetics is therefore only observed in case of the histone substrate where the k_{half} decreases due to the presence of an inhibitor. Based on mechanistic and inhibitor kinetic studies, we propose a model comprising the catalytic activity of KAT8 and the inhibitory action of AA, which can be used to further investigate the inhibitor properties.

2.4. Inhibitors – K_i calculation

The inhibitory potency for the HAT enzyme KAT8 was determined using the same fluorescence-based assay as used for the kinetic studies. The inhibitory concentrations 50% (IC_{50}) were determined if more than 50% inhibition was observed at 400 μM inhibitor concentration. As shown in the kinetic studies for the catalytic mechanism, the K_m of one substrate depends on the concentration of the other substrate. The observed IC_{50} values are therefore dependent on the concentrations of both substrates in the assay and their respective K_m values. This will give large variations in the IC_{50} values observed in different assays and makes direct comparison of inhibitors published in literature impossible. Correcting for the assay conditions by calculating the K_i enables comparison between assays and assay conditions. The K_i values were calculated from the IC_{50} values using a model that takes into account that the enzyme operates via a ping-pong mechanism as observed in the enzyme kinetic study. It also takes into account that the inhibitors like AA affect only one form of the enzyme, EX, as shown by the kinetic model (Fig. 5). According to Cheng and Prusoff [15] if these requirements are met, Equation (1) can be used, which includes the K_m values of both substrates and their respective concentrations used in the assay. The K_i values of the inhibitors were calculated (example calculation in SI) using Equation (1) in which K_a is the K_m of Ac-CoA at the concentration of histone substrate used in the assay (2.1 μM), K_b is the K_m of the histone substrate at the concentration of Ac-CoA used (71 μM) and A and B the concentrations of either substrate in the assay (4 and 60 μM respectively).

$$IC_{50} = K_i\left(1 + \frac{K_a B}{K_b A} + \frac{A}{K_a}\right) \quad (1)$$

The results show that the inhibitors **15** and **11d** with the 10 carbon atom aliphatic tail bind slightly better than the inhibitors with the 15 carbon atom tail **AA** and **11e**. The inhibitors with the 5 carbon atom tail **14** and **11c** completely lose their affinity within the investigated range of concentrations as was observed in a previous study on KAT5 [26]. This indicates that hydrophobic interactions with the aliphatic tail play a major role in the inhibitory potency of this type of compound for KAT8. The importance of hydrophobic interactions of the aliphatic tail is further confirmed by the loss of potency of compound **8b** that includes an aliphatic alcohol in the aliphatic tail. The substitution position of the salicylate makes a small difference in the observed potencies with the best potency observed in the salicylate 6-position. We also note that replacing of the salicylate moiety by a 2-hydroxy acetophenone moiety (**13**) completely removed activity, stressing the importance of the salicylate functionality.

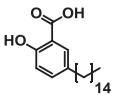
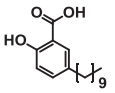
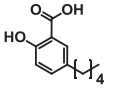
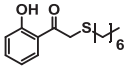
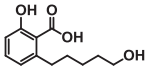
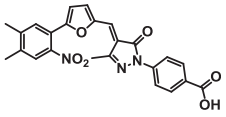
2.5. Inhibitors – P300

In order to assess the selectivity of the KAT8 inhibition by the compounds of this focused collection, we tested them for inhibitory potency on p300. Towards this aim we employed an assay based on radiolabeling of the histone substrate (Table 4). The reference compound C646 had an IC_{50} of 0.32 μM, which is consistent with literature [34]. AA showed 97% inhibition at a concentration of 50 μM. The salicylic acid derivatives inhibited p300 as well and showed comparable SAR as observed for KAT8. This suggests a similar binding mode and interactions, although KAT8 and p300 are structurally very different. It is not possible to align either the amino acid sequence or the 3D structures of KAT8 and p300 by conventional means. It is however possible that either the Ac-CoA or histone substrate pockets, due to the similarity of the ligands, show a certain resemblance thus resulting in a comparable SAR for these inhibitors.

3. Conclusions

In this study, the catalytic mechanism of KAT8 histone acetyltransferase has been investigated. Enzyme kinetic experiments

Table 4
Inhibitory potency of anacardic acid (AA) derivatives on KAT8 and p300. The inhibitory potency for KAT8 was measured using an assay based on fluorescent detection of CoA. The K_i values were calculated using an adaptation of the Cheng–Prusoff equation based on the postulated ping-pong mechanism and inhibitor kinetics (Equation (1)). The inhibition of p300 was measured using an assay based on radiolabeling of the histone substrate.

Compound	Structure	KAT8 K_i (μM)	P300 inhibition at 50 μM
AA		64 ± 8.9	97%
15		37 ± 7.0	88%
14		No inhibition[a]	No inhibition[b]
11e		79 ± 11	85%
11d		57 ± 6.6	95%
11c		No inhibition[a]	No inhibition[b]
13		No inhibition[a]	No inhibition[b]
8b		157 ± 7.2	No inhibition
8a		No inhibition[a]	No inhibition
C646		No inhibition[a]	IC_{50} = 0.32 μM

[a] No Inhibition at 400 μM.
[b] No Inhibition at 200 μM.

indicate that this bi-substrate enzyme operates by a ping-pong mechanism. This mechanism is supported by the observation that binding of the first substrate Ac-CoA is required for binding of the second substrate histone H4 as determined by ITC measurements. The presence of cysteine 143 in the KAT8 active site combined with the previous evidence that this residue is essential for catalysis further supports the evidence for a ping-pong mechanism for acetyltransferase activity of KAT8. We employed this model for calculation of the K_i values of inhibitors of this enzyme. In order to generate small molecule inhibitors for KAT8 we assembled a focused compound collection around the known non-selective HAT inhibitor AA. This compound collection was tested for inhibition of KAT8. Kinetic studies were performed with the reference compound AA and based on both inhibitor kinetics and mechanistic studies, a catalytic model was proposed involving two different conformations of the enzyme. The equilibrium binding constant K_i was calculated using an adaptation of the Cheng–Prusoff equation based on the catalytic mechanism and the proposed model. AA and its derivatives inhibited KAT8 and both the aliphatic tail and the salicylate functionality proved to be important for binding. The inhibitors were tested for activity on p300 and showed a similar SAR as on KAT8, suggesting a similar binding mode even though the two enzymes are structurally different.

This study gives insight in KAT8 through the catalytic mechanism and presents a series of small-molecule inhibitors for this HAT. Based on kinetic studies of AA and the catalytic mechanism, a model has been proposed comprising the catalytic activity of KAT8 and the inhibitory action of AA, which includes an active and an inactive conformation of the enzyme. This provides a basis for development of inhibitors and the interpretation of the enzyme inhibition studies, which will ultimately enable the exploitation of KAT8 as a novel drug target in disease.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.10.016.

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