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Improved inhibition of the histone acetyltransferase PCAF by an anacardic acid derivative

Massimo Ghizzoni [a], André Boltjes [a], Chris de Graaf [b], Hidde J. Haisma [a], Frank J. Dekker [a,*]

[a] Department of Pharmaceutical Gene Modulation, Groningen Research Institute of Pharmacy, Anthonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
[b] Leiden/Amsterdam Center for Drug Research (LACDR), Division of Medicinal Chemistry, Department of Pharmacochemistry, Faculty of Exact Sciences, VU University Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

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ABSTRACT

Several lines of evidence indicate that histone acetyltransferases (HATs) are novel drug targets for treatment of diseases like, for example, cancer and inflammation. The natural product anacardic acid is a starting point for development of small molecule inhibitors of the histone acetyltransferase (HAT) p300/CBP associated factor (PCAF). In order to optimize the inhibitory potency, a binding model for PCAF inhibition by anacardic acid was proposed and new anacardic acid derivatives were designed. Ten new derivatives were synthesized using a novel synthetic route. One compound showed a twofold improved inhibitory potency for the PCAF HAT activity and a twofold improved inhibition of histone acetylation in HEP G2 cells.

1. Introduction

For several diseases, treatment with the currently available drugs is not satisfactory, which demonstrates the need for conceptually new drugs for new therapeutic targets. The epigenetic regulation of gene transcription is an area of research in which new therapeutic targets are being discovered. Gene transcription is regulated by, among others, histone acetyltransferases (HATs) (Fig. 1).[1] Deregulation of histone acetylation has been linked to several diseases, including cancer and inflammation, which suggests that HATs are potential anti-inflammatory targets.[2–4]

Ultimately, we aim to explore the role of histone acetyltransferases in inflammation by development of small molecule inhibitors. Inflammation involves a variety of inflammatory mediators whose transcription is enhanced during the disease process by global histone acetylation as well as gene specific histone acetylation. Several studies showed a disturbed balance between HAT and HDAC activity in inflammatory diseases. Asthma patients show increased HAT activity in bronchial biopsies, whereas the activity and expression of HDACs are decreased.[5] The same was observed in lung tissue for chronic obstructive pulmonary disease (COPD) patients compared to healthy subjects. Tissue of patients with COPD showed reduced HDAC activity and increased histone H4 acetylation in the interleukin-8 promotor.[6]

Nuclear factor-κB (NF-κB) mediated signal transduction plays an important role in inflammation. When activated, NF-κB translocates to the nucleus, where it binds to the κB recognition site of target genes and to cofactors with intrinsic HAT activity such as p300/CBP and PCAF.[7] This leads to global acetylation of lysine residues in histone H4 and opening of the chromatin structure, enabling transcription of proinflammatory genes, including IL-8.[8] This mechanism is supported by the observation that *Legionella pneumophila*-induced IL-8 release from alveolar epithelial cells is decreased by HAT inhibition and enhanced by HDAC inhibition. These findings suggest that restoring the HAT/HDAC balance by small molecule inhibitors of histone acetylation provides opportunities for treatment of inflammation.[6,9–11]

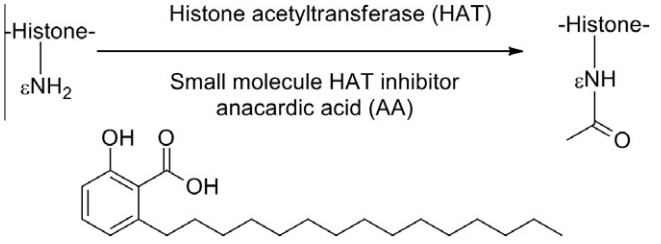

Figure 1. Acetylation of histone lysine residues by histone acetyltransferases provides potential anti-inflammatory drug targets. Anacardic acid is a cell-permeable small molecule inhibitor of the histone acetyltransferase PCAF.

* Corresponding author. Tel.: +31 50 3638030; fax: +31 50 3637953.
E-mail address: f.j.dekker@rug.nl (F.J. Dekker).

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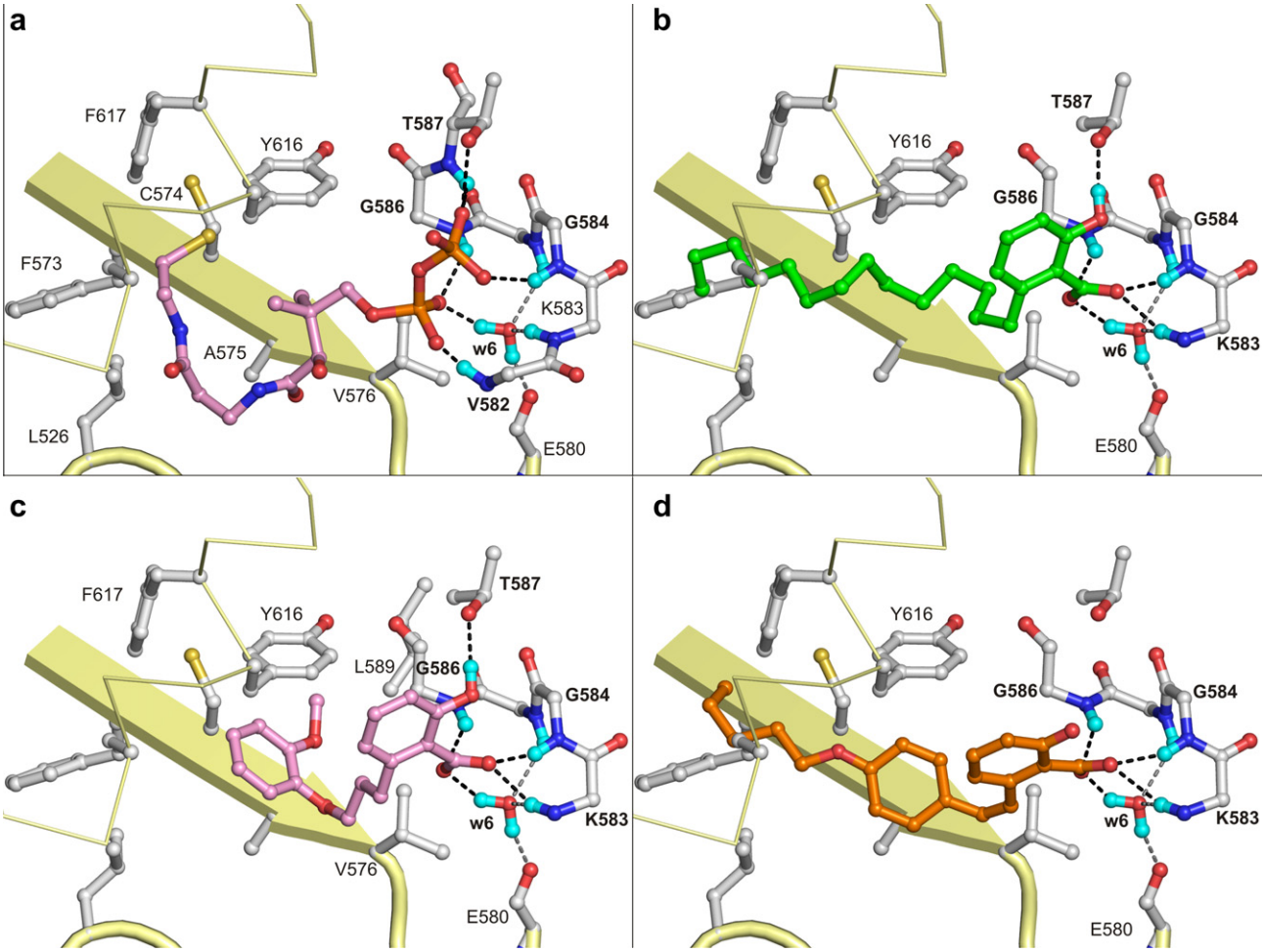


Figure 2. (a) Binding mode of the natural substrate CoA bound to the HAT PCAF active site (PDB code 1CM0). Only the pantothenic acid and the pyrophosphate of CoA are shown. The substrate carbon atoms are shown in purple. Docked binding configurations of anacardic acid (b) (carbons atoms in green), compound **4e** (c) (carbon atoms in pink), and compound **6d** (d) (carbon atoms in orange). Helix α4 and beta strand β4 are represented by yellow ribbons. Important binding residues are shown as ball-and-sticks with grey carbon atoms. Oxygen, nitrogen sulfur, phosphor, and hydrogen atoms are colored red, blue, yellow, orange, and cyan, respectively. Protein–ligand as well as w6-ligand and protein-w6 H-bonds described in the text are shown as black or grey dotted lines.

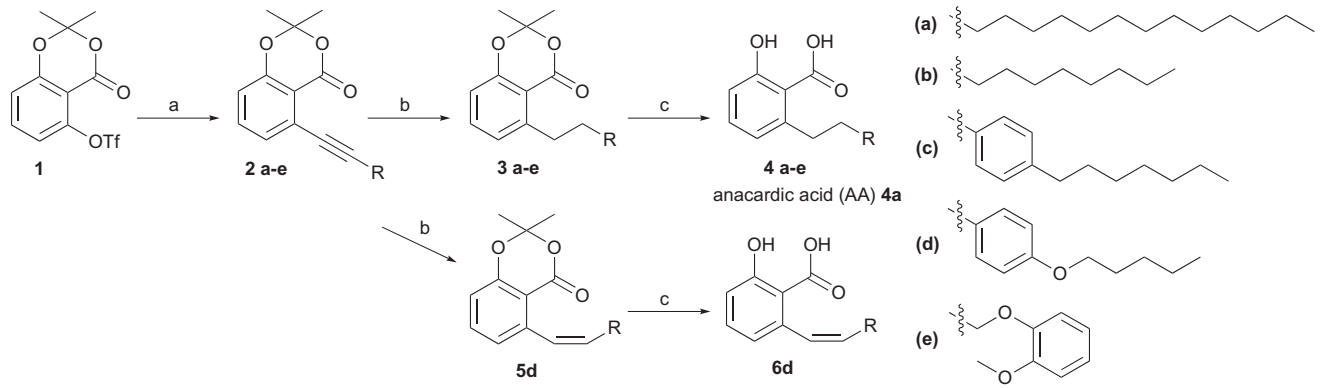
Only a few small molecule inhibitors of HATs are currently available. Potent and selective bisubstrate inhibitors for the HATs p300 and PCAF have been described, however the lack of cell permeability represents a disadvantage.[12] Another well known class of inhibitors is represented by isothiazolones, which are cell-permeable and potent; however their reactivity limits their specificity.[13–16] Furthermore, the natural products curcumin,[17] plumbagin,[18] garcinol[19] and anacardic acid[20] (AA, Fig. 1) have been reported as inhibitors of different classes of HATs. Interestingly, it has been demonstrated that anacardic acid suppresses the NF-κB pathway, which shows the potential of this compound to suppress inflammation.[21] Several benzamide derivatives of anacardic acid have been studied for inhibition of p300, however they were either equally potent p300 HAT inhibitors compared to anacardic acid or they activated the p300 HAT.[20,22]

In this study we describe hypothesis-driven optimization of the HAT inhibitory potency of anacardic acid. We proposed a binding model for anacardic acid in the PCAF active site using molecular modeling techniques. Based on this model, a series of new anacardic acid derivatives was designed. A convenient synthetic route was developed to synthesize these derivatives. We replaced the 6-alkyl chain of anacardic acid by different substituents in order to improve the potency and the drug likeness of these inhibitors. A series of 10 anacardic acid derivates was synthesized and their inhibitory potency for the recombinant HAT PCAF enzyme and for histone acetylation in HEP G2 cells was studied.

2. Results and discussion

2.1. Molecular docking studies

The crystal structure of PCAF in complex with Coenzyme A (CoA) has been published previously.[23] Using this structure, docking studies were performed in order to propose a possible binding mode for anacardic acid. We hypothesized that the salicylate group of anacardic acid mimics the pyrophosphate group of CoA, which forms an extensive H-bond network with the backbone amide nitrogens of V582, G584, G586, and T587, with the side chain hydroxyl of T587, and with the water molecule w6 (Fig. 2a). This active site water molecule is further stabilized by a H-bond network with the backbone of E580, K583, and Y585 (Fig. 2a). Docking simulations including this essential water molecule indeed yielded a (highest ranked) pose of anacardic acid similar to the CoA binding mode (Fig. 2b), in which the carboxylic group accepts H-bonds from the backbone of K583, G584, G586, and w6, the hydroxyl group donates a H-bond to the T587 side chain in the pyrophosphate binding pocket, and the long alkyl chain of anacardic acid protrudes deep into the hydrophobic pantothenic acid binding pocket between beta strand



Scheme 1. Synthesis of anacardic acid derivatives: Reagents and conditions: (a) CuI, $PdCl_2(PPh_3)_2$, Et_2NH, HC≡CR, CH_3CN, 70 °C; (b) H_2, Pd/C, MeOH, 40 °C; (c) KOH 5 N, THF, 55 °C.

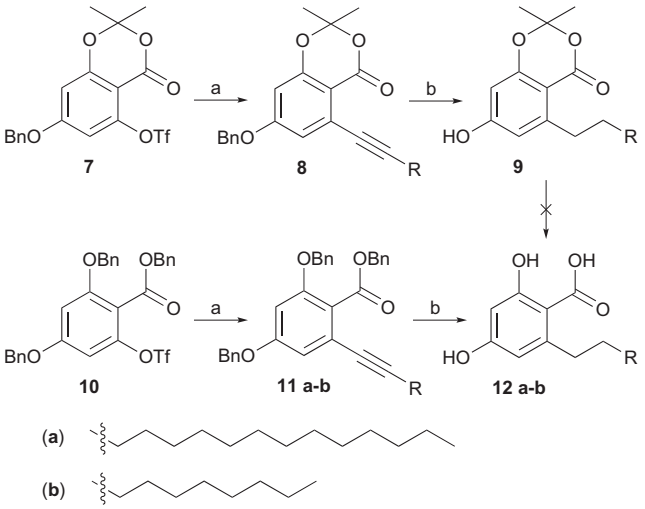
4 (β4) and alpha helix 4 (α4) (Fig. 2b). The anacardic acid binding mode is further stabilized by aromatic edge-to-face stacking between the salicylate ring and Y616. This molecular docking pose was used as starting point for the design of new molecules and to rationalize structure–activity relationships. First of all, the role of the hydrophobic chain in the binding to the pantothenic acid pocket between α4 and β4 was investigated. Secondly, it was hypothesized that incorporation of a second aromatic ring could further stabilize ligand binding via additional aromatic stacking interactions with Y616. Thirdly, the acidity of the carboxyl group was modulated by introduction of substituents on the salicylate ring. In silico predicted binding modes of novel potent anacardic acid-based PCAF ligands (Fig. 2c and d) will be discussed in Section 2.3.

2.2. Chemistry

A convenient synthetic method was developed in order to synthesize a diverse series of compounds with a salicylate functionality. Several syntheses of anacardic acid have been described so far; however these routes require harsh conditions that are not compatible with many functional groups.[24–26] Therefore, a new synthetic route was developed, in which a Sonogashira coupling is the key step (Scheme 1).

Triflate **1** was synthesized according to previously published procedures,[27] starting from commercially available 2,6-dihydroxybenzoic acid. Different terminal alkynes were coupled in presence of CuI, diethylamine, and $PdCl_2(PPh_3)_2$ to give the correspondent alkynyls **2**. The Sonogashira reaction, performed in acetonitrile at 70 °C on oil bath, required 2 h to give the product with high yields (80–90%). The same reaction was also performed in acetonitrile at 100 °C with microwave irradiation. In this case, the product was obtained in comparable yields after just 30 min. Hydrogenation, using palladium on activated carbon as catalyst, was used to reduce the akynyl triple bond to a single bond. The best yields (60–80%) were obtained if hydrogenation was performed for 16 h, with 3 atm H_2-pressure in methanol at 40 °C. In case of compound **2d**, hydrogenation under the described conditions gave the correspondent *cis*-alkene **5d** (J = 12.1 Hz). To obtain the alkane **3d**, it was necessary to perform hydrogenation for 40 h. The last step in this synthetic route was the cleavage of the acetonide protecting group. This was achieved by saponification with KOH 5 N at 55 °C with yields between 50% and 80%.

Structures of 4-hydroxy-6-alkyl-salicylates **12a** and **12b** (Scheme 2) are closely related to another natural compound known as olivetolic acid, an intermediate in the biosynthesis of cannabinoids.[28] A synthetic route towards these compounds has been described by Dushin and Danishefsky.[29] This route implicates a Sonogashira coupling on **7**, followed by hydrogenation to give the



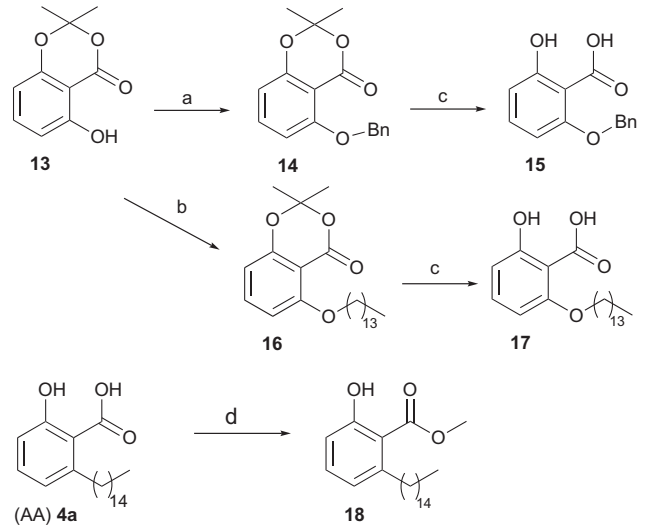
Scheme 2. Synthesis of olivetolic acid derivatives: Reagents and conditions: (a) CuI, $PdCl_2(PPh_3)_2$, Et_2NH, HC≡CR, CH_3CN, 70 °C; (b) H_2, Pd/C, MeOH, 40 °C.

alkane **9**. Cleavage of the acetonide **9** to yield **12** poses a challenge. Saponification by hydroxide (OH^-) can only be performed under forcing conditions due to deprotonation of the 4-hydroxy group and concomitant electron-donation to the carboxylate. Acidic cleavage of the acetonide will result in decarboxylation.[30] We designed another route to avoid these problems (Scheme 2). Building block **10**[31] was employed for Sonogashira coupling to terminal alkenes. The resulting alkynyls gave the desired product upon hydrogenation.

Anacardic acid derivatives with an alkoxy function in the 2-position were synthesized starting from **13** (Scheme 3). Compound **13** was converted to **14** using a Williamson ether synthesis with benzyl bromine (yield 83%). In contrast, reaction of **13** with tetradecylchloride did not give the desired product. Compound **16** was obtained by Mitsunobu coupling between **13** and tetradecanol (yield 29%). Saponification of **14** and **16** provided the corresponding free carboxylic acids **15** and **17** with yields of 49% and 66%, respectively. Compound **18** was synthesized from anacardic acid using a catalytic amount of H_2SO_4 in methanol.

2.3. Enzyme inhibition assay

The synthesized compounds were tested for inhibition of the HAT PCAF using an indirect ELISA assay in order to study their binding properties and structure–activity relationship. The ELISA was



Scheme 3. Reagents and conditions: (a) Benzylbromide, K_2CO_3, DMF; (b) tetradecanol, PPh_3, DIAD, THF; (c) KOH 5 N, THF, 55 °C; (d) H_2SO_4, MeOH, reflux.

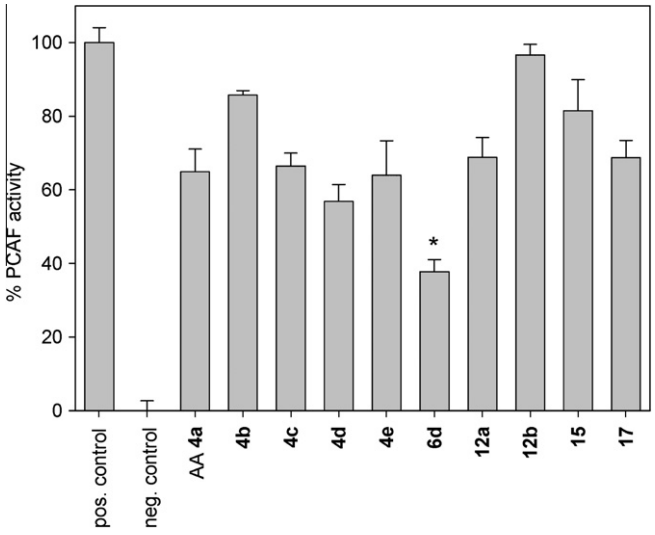


Figure 3. Inhibition of the HAT PCAF by different compounds at 1 mM. Data are expressed as percentage of PCAF activity ($n = 3$, $^{*}P < 0.05$ compared to AA **4a**).

based on detection of acetylated lysine residues on a histone H4 peptide using an anti-acetyl-lysine antibody. Human recombinant PCAF was used as source of HAT activity. Anacardic acid (AA) **4a** showed around 40% inhibition at 1 mM (Fig. 3), which would give an estimated IC_{50} value between 1 and 2 mM. The potency measured in our assay is 100-fold lower than the estimation by Balsubramanyam et al.[20] but in line with the IC_{50} reported by Wu et al.[32] The differences might be explained by the fact that Wu et al.[32] used comparable Ac-CoA concentrations, whereas Balsubramanyam et al.[20] used lower Ac-CoA concentrations in the enzyme inhibition assay. Furthermore, differences in assay buffer composition or differences in the enzyme source might cause differences in IC_{50} values.

Several modification of the salicylate moiety were investigated to slightly modulate the acidity of the carboxyl group. Compounds **12a** and **12b** showed comparable potency as observed for compounds AA **4a** and **4b**, respectively. These data show that the introduction of a hydroxyl group in the 4-position of the salicylate does not influence the inhibitory properties. Introduction of an alkoxy group in the 6-position of the salicylate ring **17** did not lead to significant changes in potency. The methyl ester of anacardic acid was synthesized to study the importance of a free carboxylic acid for the interaction with PCAF, however the water solubility of **18** is to low for testing at 1 mM concentration in our assay.

The role of the hydrophobic interactions for the ligand potency was investigated by modifications of the hydrophobic alkyl chain. Compounds **4c** and **4d** showed inhibition comparable to anacardic acid, indicating that introduction of an aromatic ring in the chain did not affect the inhibitory properties. On the other hand, compounds **4b** and **12b** were less potent than the correspondent AA **4a** and **12a**, indicating that one-third reduction of the chain length causes loss of potency. Also compound **15**, with a benzyl group instead of the long alkylic chain, showed less than 20% inhibition at 1 mM. These data suggest that hydrophobic interactions at the end of the CoA binding pocket are important for binding. This observation is in line with the proposed binding mode of anacardic acid (Fig. 2b), which is stabilized by hydrophobic interactions deep in the pantothenic acid pocket between α4 and β4 (Fig. 1a).

Interestingly, incorporation of a 3-(2-methoxyphenoxy)propyl moiety **4e** provided equal inhibition to AA **4a**. In the highest ranked docking pose of **4e** (Fig. 2c), the 2-methoxyphenoxy ring stacks between the aromatic rings of Y616 and F617, whereas the 2-methoxy group can be accommodated in a small additional pocket between L589, V576 and Y616. Compound **4e** provides a good platform for further structural variation in order to optimize the inhibitory potency of this compound class.

Compound **6d** is the only compound that proved to be more potent than anacardic acid in our assay. We measured for this compound an IC_{50} of 662 ± 64 μM (see Supplementary data). Remarkably, compounds **4c** and **4d**, also including a second benzyl group, but lacking the *cis*-double bond, did not show increased potency compared to anacardic acid **4a**. In the highest ranked docking pose of compound **6d**, the salicylate moiety is hydrogen bonded to the pyrophosphate binding pocket as described for anacardic acid **4a** (Fig. 2d). In addition, the geometry of the *cis*-double bond forces the phenoxy ring in an optimal conformation for face-to-edge aromatic stacking with Y616 and orientates the hydrophobic tail of the molecule deep into the hydrophobic binding cleft between α4 and β4 (Fig. 2d). This conformational restriction could explain the increased potency of **6d** compared to **4d**. Compound **6d** has improved druglike properties compared to anacardic acid, because it has a more favorable logP and the long aliphatic chain has been replaced by a more compact substituent. The salicylates are fully deprotonated at pH 7.4, which gives a *c* log *P* value of 5.1 for anacardic acid and 2.0 for **6d** (Chemical properties, Chemdraw). In conclusion, compounds **6d** and **4e** provide a starting point for the design of new PCAF inhibitors.

2.4. Cell-based study

Since compound **6d** was more potent than anacardic acid **4a**, we decided to test the effects of **6d** on histone acetylation in a cell-based study. Histone H4 acetylation levels in HEP G2 cells were measured using an anti-acetyl histone H4 antibody. The HDACs inhibitor suberoylanilide hydroxamic acid (SAHA) was used to increase the basal histone acetylation. Cells were treated for 24 h with 5 μM SAHA and co-treated with different concentration of **6d** and anacardic acid **4a**. The cells were treated with sub-toxic concentrations of the inhibitors (see Supplementary data). Figure 4a shows that SAHA increased histone H4 acetylation levels significantly. Compound **6d** decreased the acetylation induced by SAHA in HEP G2 cells at 30 and 60 μM. The decrease is stronger than observed for a comparable concentration of anacardic acid (AA) **4a**. Quantification of the western blot results (Fig. 4b) showed a significant increased inhibition for compound **6d** compared to AA **4a** ($P < 0.01$). These data demonstrate that compound **6d** is a useful tool to inhibit histone acetylation in cell-based studies. Differences between the

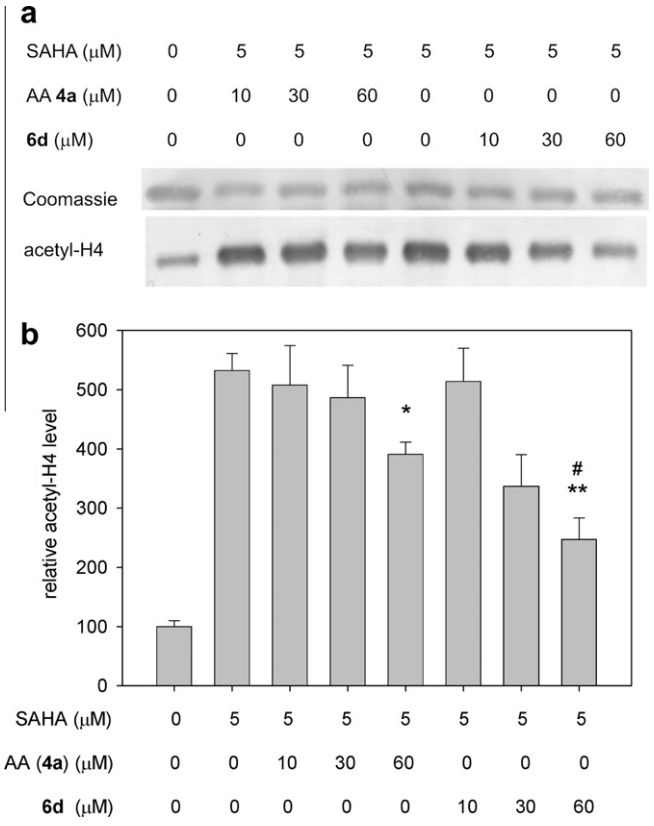


Figure 4. Cell-based studies on histone H4 acetylation levels in HEP G2 cells. (a) Western blot of histone extracts of HEP G2 cells after 24 h treatment with different concentrations of the indicated compounds. Coomassie brilliant blue staining was used to determine equal amount of loading. (b) Quantification of Histone H4 acetylation levels: values are shown in comparison to histone acetylation levels of non-treated cells (100%) and expressed as mean ± SEM of three independent experiments. $^{*}P$ <0.05, $^{**}P$ <0.01 compared to SAHA stimulated samples. #P <0.01 compared to SAHA—AA **4a** (60 μM) treated samples.

effective concentrations between the inhibition of the recombinant enzyme and the cell-based studies might originate from factors such as differences in the assay buffer composition, differences in the Ac-CoA concentration or differences in the enzyme source. Furthermore, the fact that higher concentrations of AA **4a** and **6d** were required to inhibit the recombinant enzyme PCAF compared to the cell-based studies could indicate that these compounds target different histone acetyltransferases in the cell-based studies.

3. Conclusion

In this study we describe the development of a salicylate derivative **6d** with improved inhibition of histone acetylation compared to the natural product anacardic acid. A binding configuration of anacardic acid in the HAT PCAF active site was proposed using molecular modeling. In this binding model, the salicylate is bounded in the pyrophosphate binding pocket and the alkyl chain is located in the CoA substrate binding cleft. Based on this model, new anacardic acid derivatives were designed in order to explore the role of the alkyl chain and the importance of the salicylate moiety. A convenient synthetic route towards anacardic acid was developed and 10 derivatives were synthesized. Testing of the inhibitory potency for the PCAF HAT activity showed that one derivative had a twofold improved inhibitory potency for the HAT PCAF recombinant enzyme. Moreover, this derivative showed also a twofold improved potency for inhibition of histone H4 acetylation in cell-based studies on HEP G2 cells. This is the first report on hypothesis-driven optimization of the HAT PCAF inhibition.

4. Experimental sections

4.1. Chemistry

Chemicals were purchased from Sigma-Aldrich and Acros Organics, and used without further purification. Solvents were dried before use according to known procedures and all the reactions were carried-out under a nitrogen atmosphere. Analytical thin-layer chromatography (TLC) was performed on aluminium sheets of Silica Gel 60 F254. Ultraviolet light, $KMnO_4$ solution or ninhydrin solution were used to visualize bands. All microwave irradiation reactions were carried out in a Biotage Initiator™ Microwave Synthesizer. Column chromatography was performed with MP Ecochrom Silica Gel 32–63, 60 Å. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini-200. ^{13}C spectra were recorded using the attached proton test (APT) pulse sequence. Chemical shift values are reported as part per million (δ) relative to residual solvent peaks ($CDCl_3$, ^1H δ = 7.26, ^{13}C δ = 77.16 or CD_3OD, ^1H δ = 3.31, ^{13}C δ = 49.00). The coupling constants (J) are reported in Hertz (Hz). Electrospray ionization mass spectra (ESI-MS) were recorded on an Applied Biosystems/SCIEX API3000-triple quadrupole mass spectrometer. High-resolution mass spectra (HR-MS) were recorded using a flow injection method on a LTQ-Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany) with a resolution of 60,000 at m/z 400. Protonated testosterone (lock mass m/z = 289.2162) was used for internal recalibration in real time. Melting points were determined on an Electrothermal digital melting point apparatus and are uncorrected.

4.1.1. Synthetic procedure 1

Freshly distilled diethylamine (3.0 mmol, 0.30 mL) and the alkyne (2.2 mmol) were subsequently added to a solution of the triflate (2.0 mmol), CuI (0.20 mmol, 38 mg), and $PdCl_2(PPh_3)_2$ (0.01 mmol, 70 mg) in degassed anhydrous acetonitrile (6.0 mL). The mixture was either stirred for 2 h at 70 °C on oil bath or subjected to microwave irradiation for 30 min at 100 °C (70 W). Water (10 mL) was added and the mixture was extracted with EtOAc (3 × 30 mL). The combined organic phases were washed with brine (1 × 60 mL), dried over Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography.

4.1.2. Synthetic procedure 2

A suspension of the starting material (1.5 mmol) and Pd/C (10%) (0.15 mmol) in methanol (30 mL) was shaken overnight at 40 °C, with 3 atm H_2-pressure in a Parr apparatus. The mixture was filtered through Celite, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography.

4.1.3. Synthetic procedure 3

The starting material (1.0 mmol) was dissolved in THF (2.0 mL). KOH 5 N (10 mmol, 2.0 mL) was added and the solution was stirred overnight at 55 °C. The reaction mixture was diluted with EtOAc (10 mL) and acidified with 1 N HCl (20 mL). The mixture was extracted with EtOAc (3 × 30 mL). The organic phases were collected, washed with brine (1 × 50 mL), dried over Na_2SO_4 and filtered. The solvent was evaporated and the product was purified by column chromatography or crystallization.

4.1.4. 2,2-Dimethyl-5-(pentadec-1-ynyl)-4*H*-benzo[*d*][1,3]-dioxin-4-one (2a)

The product was obtained using Section 4.1.1 starting from **1** (0.65 g, 2.0 mmol) and 1-pentadecyne (0.46 g, 2.2 mmol), using oil bath heating. The crude product was purified using column chromatography with EtOAc/Hex 1:6 (v/v) as eluent to yield **2a**

(0.65 g, 85%) as a brown solid; R_f = 0.60 (EtOAc/Hex 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.87 (3H, t, J = 6.5 Hz), 1.20–1.65 (22H, m), 1.70 (6H, s), 2.50 (2H, t, J = 7.1 Hz), 6.85 (1H, d, J = 8.5 Hz), 7.18 (1H, d, J = 7.6 Hz), 7.38 ppm (1H, t, J = 7.9 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.3, 20.2, 22.9, 25.9, 28.7, 29.2, 29.4, 29.6, 29.7, 29.9, 32.1, 78.9, 98.8, 105.7, 114.2, 116.6, 126.5, 129.1, 134.9, 156.6, 161.6 ppm; MS (ESI): m/z 385.5 $[M+H]^+$.

4.1.5. 2,2-Dimethyl-5-(dec-1-ynyl)-4*H*-benzo[*d*][1,3]dioxin-4-one (2b)

The product was obtained using Section 4.1.1 starting from **1** (0.65 g, 2.0 mmol) and 1-decyne (0.30 mg, 2.2 mmol) using oil bath heating. It was purified by column chromatography using EtOAc/Hex 1:5 (v/v) as eluent. The product was obtained as a brown solid (0.52 g, 86%); R_f = 0.58 (EtOAc/Hex 1:1); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.86 (3H, t, J = 6.5 Hz), 1.27–1.65 (12H, m), 1.68 (6H, s), 2.48 (2H, t, J = 6.9 Hz), 6.84 (1H, d, J = 8.2 Hz), 7.17 (1H, d, J = 7.6 Hz), 7.38 ppm (1H, t, J = 8.1 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.2, 20.1, 22.8, 25.8, 28.6, 29.1, 29.2, 29.3, 32.0, 78.8, 98.7, 105.5, 114.2, 116.5, 126.5, 129.0, 134.8, 156.6, 159.1 ppm; MS (ESI): m/z 315.3 $[M+H]^+$.

4.1.6. 5-((4-Heptylphenyl)ethynyl)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (2c)

The product was obtained using Section 4.1.1 starting from **1** (0.65 g, 2.0 mmol) and 1-ethynyl-4-heptylbenzene (0.44 g, 2.2 mmol) using oil bath heating. The residue was purified by column chromatography using EtOAc/Hex 1:14 (v/v) as eluent. The product was obtained as a brown solid (0.66 g, 88%); R_f = 0.62 (EtOAc/Hex 1:5); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.88 (3H, t, J = 6.6 Hz), 1.26–1.73 (8H, m), 1.59 (2H, m), 1.73 (6H, m), 2.62 (2H, t, J = 7.65 Hz), 6.89–6.93 (1H, m), 7.17 (2H, d, J = 8.2 Hz), 7.28–7.33 (1H, m), 7.42–7.46 (1H, m), 7.50–7.57 ppm (2H, d, J = 8.2 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.2, 22.8, 25.9, 29.2, 29.3, 31,3, 31.9, 36.1, 87.2, 96.8, 105.7, 114.0, 117.0, 120.2, 125.8, 128.5, 128.6, 132.1, 134.9, 144.2, 156.8, 159.0 ppm; MS (ESI): m/z 377.3 $[M+H]^+$.

4.1.7. 2,2-Dimethyl-5-((4-(pentyloxy)phenyl)ethynyl)-4*H*-benzo[*d*][1,3] dioxin-4-one (2d)

The product was obtained using Section 4.1.1 starting from **1** (0.65 g, 2.0 mmol) and 1-eth-1-ynyl-4-(pentyloxy)benzene (0.41 g, 2.2 mmol) using either oil bath heating or microwave irradiation. The residue was purified by column chromatography using EtOAc/Hex 1:6 (v/v) as eluent. The product was obtained as brown oil. The yield was 80% (0.58 g) after oil bath heating and 84% (0.61 g) after microwave irradiation; R_f = 0.57 (EtOAc/Hex 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.72 (3H, t, J = 6.9 Hz), 1.21–1.17 (4H, m), 1.50–1.52 (8H, m), 3.74 (2H, t, J = 6.6 Hz), 6.62–6.68 (3H, m), 7.03–7.08 (1H, m), 7.20 (1H, d, J = 7.9 Hz), 7.31–7.36 ppm (2H, m); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.1, 22.5, 25.8, 28.2, 29.0, 68.1, 86.7, 96.9, 105.7, 113.8, 114.6, 114.9, 116.7, 126.0, 128.2, 133.7, 134.9, 135.3, 156.7, 159.1, 159.8 ppm; MS (ESI): m/z 365.2 $[M+H]^+$.

4.1.8. 5-(3-(2-Methoxyphenoxy)prop-1-yn-1-yl)-2,2-dimethyl-4*H*-benzo [*d*][1,3]dioxin-4-one (2e)

The product was obtained using Section 4.1.1 starting from **1** (0.65 g, 2.0 mmol) and 1-methoxy-2-(prop-2-yn-1-yloxy)benzene (0.36 g, 2.2 mmol) using either oil bath heating or microwave irradiation. The residue was purified by column chromatography using EtOAc/Hex 1:5 (v/v) as eluent. The product was obtained as yellow oil in 88% yield (0.60 g) after bath heating and 86% yield (0.58 g) after microwave irradiation; R_f = 0.43 (EtOAc/Hex 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.79 (6H, s), 3.97 (3H, s), 5.16 (2H, s), 6.98–7.07 (4H, m), 7.29–7.35 (2H, m), 7.46–7.54 ppm (1H, m); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 25.8, 56.0, 57.9, 85.1, 91.2, 105.8, 111.8, 115.0, 117.8, 121.0, 122.2, 124.6, 129.3, 135.0, 147.2, 149.8, 156.6, 158.8 ppm; MS (ESI) m/z: 356.2 $[M+NH_4]^+$.

4.1.9. 2,2-Dimethyl-5-pentadecyl-4*H*-benzo[*d*][1,3]dioxin-4-one (3a)

The product was obtained using Section 4.1.2 starting from **2a** and it was purified by column chromatography using EtOAc/Hex 1:6 (v/v) as eluent. The product was obtained as colorless solid (0.31 g, 60%); R_f = 0.61 (EtOAc/Hex 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.87 (3H, t, J = 6.5 Hz), 1.20–1.60 (26H, m), 1.69 (6H, s), 3.08 (2H, t, J = 7,6 Hz), 6.79 (1H, d, J = 8.2 Hz), 6.92 (1H, d, J = 7.6 Hz), 7.38 ppm (1H, t, J = 7.9 Hz). ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.3, 22.8, 25.8, 29.5, 29.6, 29.8, 31.3, 32.1, 34.5, 105.1, 112.2, 115.2, 125.2, 135.2, 148.7, 157.2, 160.3 ppm; MS (ESI): m/z 389.5 $[M+H]^+$.

4.1.10. 2,2-Dimethyl-5-decyl-4*H*-benzo[*d*][1,3]dioxin-4-one (3b)

The product was obtained using Section 4.1.2 starting from **2b** and it was purified by column chromatography using EtOAc/Hex 1:5 as eluent. The product was obtained as a white solid (0.29 g, 65%); R_f = 0.60 (EtOAc/Hex 1:1); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.87 (3H, t, J = 6.8 Hz), 1.25–1.66 (16H, m), 1.69 (6H, s), 3.08 (2H, t, J = 7.6 Hz), 6.78 (1H, d, J = 8.2 Hz), 6.92 (1H, d, J = 7.6 Hz), 7.38 ppm (1H, t, J = 7.9 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.3, 22.8, 25.8, 28.7, 29.5, 29.6, 29.8, 31.3, 32.1, 33.4, 34.5, 105.1, 112.2, 115.2, 125.2, 135.2, 148.7, 157.2, 160.4 ppm; MS (ESI): m/z 319.3 $[M+H]^+$.

4.1.11. 5-(4-Heptylphenethyl)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (3c)

The product was obtained using Section 4.1.2 starting from **2c** and it was purified by column chromatography using EtOAc/Hex 1:12 as eluent. The product was obtained as dark yellow solid (0.55 g, 97%); R_f = 0.41 (EtOAc/Hex 1:5); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.88 (3H, t, J = 6.5 Hz), 1.30 (8H, m), 1.60 (2H, m), 1.69 (6H, m), 2.56 (2H, t, J = 7.6), 2.86 (2H, t, J = 8.0), 3.38 (2H, t, J = 8.0), 6.79–6.90 (2H, m), 7.06–7.19 (4H, m), 7.34–7.42 ppm (1H, m); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.2, 22.8, 25.8, 29.3, 29.4, 31.7, 31.9, 35.7, 36.8, 37.2, 105.1, 105.2, 112.3, 115.5, 125.5, 125.9, 127.2, 128.4, 128.6, 128.9, 135.2, 139.0, 140.6, 147.4, 157.2, 160.4 ppm; MS (ESI): m/z 381.3 $[M+H]^+$.

4.1.12. 2,2-Dimethyl-5-(4-(pentyloxy)phenethyl)-4*H*-benzo[*d*][1,3]dioxin-4-one (3d)

A suspension of the starting material **2d** (0.55 g, 1.5 mmol) and Pd/C 10% (16 mg, 0.15 mmol) in methanol (30 mL) was stirred under 3 atm H_2-pressure at 40 °C for 36 h in a PARR apparatus. The mixture was filtered through Celite and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography using EtOAc/Hex 1:14 (v/v) as eluent. The product was obtained as yellow oil (0.51 g, 93%); R_f = 0.38 (EtOAc/Hex 1:10); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.88 (3H, t, J = 3.5 Hz), 1.38–1.44 (4H, m), 1.70 (6H, s), 1.73–1.79 (2H, m), 2.84 (2H, t, J = 8.1 Hz), 3.36 (2H, t, J = 7.9 Hz), 3.93 (2H, t, J = 6.8 Hz), 6.80–6.84 (m, 2H), 6.86 (1H, d, J = 7.7 Hz), 7.06 (1H, d, J = 8.5 Hz), 7.14–7.16 (m, 2H), 7.35–7.39 ppm (m, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.1, 22.5, 25.7, 28.3, 29.1, 34.9, 36.7, 36.9, 68.0, 105.1, 112.2, 114.4, 115.4, 125.5, 129.3, 129.6, 133.7, 135.2, 147.2, 157.2 157.4, 160.0 ppm; MS (ESI): m/z 369.4 $[M+H]^+$.

4.1.13. 5-(3-(2-Methoxyphenoxy)propyl)-2,2-dimethyl-4*H*-benzo[*d*][1,3] dioxin-4-one (3e)

The product was obtained using Section 4.1.2 starting from **2e** and it was purified by column chromatography using EtOAc/Hex 1:8 (v/v) as eluent. The product was obtained as yellow oil (0.46 g, 90%); R_f = 0.49 (EtOAc/Hex 1:2); ^{1}H NMR (200 MHz, $CDCl_3$):

δ = 1.70 (3H, m), 2.13–2.24 (2H, m), 3.28 (2H, t, J = 7.6 Hz), 3.87 (3H, s), 4.09 (2H, t, J = 6.6 Hz), 6.82 (1H, d, J = 7.0 Hz), 6.90 (4H, m), 6.98 (1H, d, J = 7.6 Hz), 7.36–7.44 ppm (1H, t, J = 7.9 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 26.4, 31.1, 31.5, 56.7, 69.1, 105.8, 112.7, 114.1, 116.2, 121.7, 126.1, 126.1, 135.9, 147.7, 149.3, 150.3, 158.0, 161.0 ppm; MS (ESI) m/z 360.3 $[M+H]^+$.

4.1.14. 2-Pentadecyl-6-hydroxybenzoic acid (anacardic acid, 4a)

The product was obtained using Section 4.1.3 starting from **3a** and it was purified by crystallization from EtOAc/Hex 1:6 (v/v) at 4 °C. The product was obtained as a white solid (0.30 g, 85%); R_f = 0.31 (100% EtOAc); mp: 90–91 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.88 (3H, t, J = 6.5 Hz), 1.20–1.50 (24H, m), 1.55–1.60 (2H, m), 2.97 (2H, t, J = 7.6 Hz), 6.77 (1H, d, J = 7.3 Hz), 6.87 (1H, d, J = 8.2 Hz), 7.36 ppm (1H, t, J = 7.9 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.4, 22.9, 29.6, 29.7, 29.9, 30.1, 32.2, 32.3, 36.7, 110.6, 116.1, 123.0, 135.7, 148.1, 163.9, 176.4 ppm; HRMS: m/z $[M-H]^-$, calcd for $C_{22}H_{35}O_3$ 347.2586, found 347.2576.

4.1.15. 2-Decyl-6-hydroxybenzoic acid (4b)

The product was obtained using Section 4.1.3 starting from **3b** and it was purified by column chromatography using EtOAc/Hex 1:6 (v/v) as eluent. The product was obtained as pale yellow solid (0.14 g, 51%); R_f = 0.53 (100% EtOAc); mp: 81–82 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.87 (3H, t, J = 6.5 Hz), 1.20–1.45 (14H, m), 1.50–1.60 (2H, m), 2.98 (2H, t, J = 7,6 Hz), 6.78 (1H, d, J = 7.6 Hz), 6.87 (1H, d, J = 7.6 Hz), 7.36 ppm (1H, t, J = 7.9 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.3, 22.8, 29.4, 29.5, 29.6, 29.8, 30.0, 32.1, 32.2, 36.6, 110.5, 116.0, 122.9, 135.6, 148.0, 163.8, 176.3 ppm; HRMS: m/z $[M-H]^-$ calcd for $C_{17}H_{25}O_3$ 277.1804, found 277.1807.

4.1.16. 2-(4-Heptylphenethyl)-6-hydroxybenzoic acid (4c)

The product was obtained using Section 4.1.3 starting from **3c** and it was purified by column chromatography using EtOAc/Hex 1:2 (v/v) as eluent. The product was obtained as white powder (0.23 g, 67%); R_f = 0.4 (100% EtOAc); mp: 110–111 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.03 (3H, t, J = 5.5 Hz), 1.43–1.46 (8H, m), 1.71–1.78 (2H, m), 2.72 (2H, t, J = 7.6 Hz), 3.00–3.08 (2H, m), 3.40–3.48 (2H, m), 6.86 (1H, d, J = 7.5 Hz), 7.06 (1H, d, J = 7.5 Hz), 7.20 (4H, m), 7.39–7.56 ppm (1H, m); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.2, 22.8, 29.3, 29.5, 31.7, 32.0, 35.7, 38.0, 38.7, 110.6, 116.4, 123.2, 127.0, 128.4, 128.5, 129.0, 135.8, 139.0, 140.8, 146.7, 163.8, 176.1 ppm; HRMS: m/z $[M-H]^-$ calcd for $C_{22}H_{27}O_3$ 339.1965, found 339.1969.

4.1.17. 2-Hydroxy-6-(4-(pentyloxy)phenethyl)benzoic acid (4d)

The product was obtained using Section 4.1.3 starting from **3d** and it was purified by column chromatography using EtOAc/Hex 1:5 (v/v) as eluent. The product was obtained as yellow solid (0.17 g, 51%); R_f = 0.43 (100% EtOAc); mp: 98–99 °C; ^{1}H NMR (200 MHz, CD_3OD): δ = 0.94 (3H, t, J = 7.0 Hz), 1.38–1.45 (m, 4H), 1.72–1.75 (m, 2H), 2.70–2.85 (2H, m), 3.10–3.20 (2H, m), 3.92 (t, J = 6.5 Hz), 6.66–6.80 (m, 4H), 7.08 (2H, d, J = 8.5 Hz), 7.23–7.27 ppm (m, 1H); ^{13}C NMR (50 MHz, CD_3OD): δ = 13.4, 22.5, 28.4, 29.2, 37.7, 38.7, 68.0, 106.4, 114.3, 115.1, 122.2, 129.2, 133.2, 134.4, 144.9, 157.8, 161.7, 171.7 ppm; HRMS m/z $[M-H]^+$ calcd for $C_{20}H_{23}O_4$ 327.1601, found 327.1614.

4.1.18. 2-Hydroxy-6-(3-(2-methoxyphenoxy)propyl)benzoic acid (4e)

The product was obtained using Section 4.1.3 starting from **3e** and it was purified by column chromatography using EtOAc/Hex 1:4 (v/v) as eluent. The product was obtained as white powder (0.22 g, 73%); R_f = 0.42 (EtOAc); mp: 147–148 °C; ^{1}H NMR (200 MHz, CD_3OD): δ = 2.03–2.11 (2H, m), 3.09 (2H, t, J = 7.6 Hz), 3.83 (3H, s), 3.98 (2H, t, J = 6.5 Hz), 6.75–6.79 (2H, m), 6.89–6.94 (4H, m), 7.12–7.29 ppm (1H, m); ^{13}C NMR (50 MHz, CD_3OD): δ = 32.4, 21.7, 56.6, 69.7, 106.4, 113.6, 115.1, 116.1, 122.2, 122.4, 123.2, 134.3, 145.6, 149.9, 151.0, 162.5, 174.1; HRMS m/z $[M-H]^+$ calcd for $C_{17}H_{17}O_5$ 301.1081, found 301.1074.

4.1.19. (*Z*)-2,2-Dimethyl-5-(4-(pentyloxy)styryl)-4*H*-benzo[*d*][1,3]dioxin-4-one (5d)

The product was obtained using the Section 4.1.2 starting from **2d** and purified by column chromatography using EtOAc/Hex 1:14 (v/v) as eluent. The product was obtained as yellow oil (0.44 g, 80%); R_f = 0.51 (EtOAc/Hex 1:2); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.10–1.19 (3H, m), 1.54–1.67 (4H, m), 1.92–2.07 (8H, m), 4.09–4.18 (2H, m), 6.90–6.93 (2H, m), 7.01–7.10 (2H, m), 7.16–7.23 (3H, m), 7.23–7.25 (1H, m), 7.35–7.39 ppm (1H, m); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.8, 23.2, 26.5, 28.9, 29.7, 68.6, 106.2, 112.8, 114.8, 115.1, 116.1, 116.6, 126.1, 128.3, 129.7, 131.1, 131.2, 134.4, 135.7, 143.0, 159.0, 161.0 ppm; MS (ESI): m/z 367.3 $[M+H]^+$.

4.1.20. (*Z*)-2-Hydroxy-6-(4-(pentyloxy)styryl)benzoic acid (6d)

The product was obtained using Section 4.1.3 starting from **5d** and it was purified by column chromatography using EtOAc/Hex 1:5 (v/v) as eluent. The product was obtained as yellow solid (0.18 mg, 55%); R_f = 0.67 (100% EtOAc); mp: 94–95 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.91 (3H, t, J = 7.0 Hz), 1.33–1.42 (4H, m), 1.71–1.78(2H, m), 3.89 (2H, t, J = 6.4), 6.58 (1H, d, J = 12.1), 6.68 (2H, d, J = 8.4), 6.77 (1H, d, J = 8.3), 6.83 (1H, d, J = 12.1), 6.93 (1H, d, J =8.2), 7.00 (2H, d, J = 8.4), 7.27–7.32 ppm (1H, m); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.1, 22.6, 28.3, 29.0, 68.0, 110.3, 114.2, 114.6, 116.9, 122.9, 128.7, 129.0, 129.3, 130.6, 135.8, 142.6, 158.4, 163.6, 175.5 ppm; HRMS: m/z $[M-H]^+$ calcd for $C_{20}H_{21}O_4$ 325.1445, found 325.1443.

4.1.21. Benzyl 2,4-bis(benzyloxy)-6-(((trifluoromethyl)sulfonyl)-oxy) benzoate (10)

Triflic anhydride (35 mmol, 5.9 mL) was added dropwise to a solution of benzyl 2,4-bis(benzyloxy)-6-hydroxybenzoate (30 mmol, 13 g) and pyridine (0.11 mol, 8.1 mL) in dry dichloromethane (30 mL) at 0 °C. The resulting mixture was stirred for 1.5 h at 0 °C. The excess of triflic anhydride was quenched by dropwise addition of water (5 mL). The mixture was slowly poured into a saturated solution of $NaHCO_3$ (50 mL), the two layers were separated in a separation funnel, and the organic layer was collected. The water layer was washed with dichloromethane (2 × 50 mL). The combined organic layers were washed with water (1 × 100 mL), brine (1 × 100 mL), and dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography using EtOAc/Hex 1:10 (v/v) as eluent to yield the product as a white solid (9.6 g, 56%); R_f = 0.46 (EtOAc/Hex 1:4); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.04 (s, 2H), 5.05 (s, 2H) 5.33 (s, 2H), 6.54 (s, 1H), 6.59 (s, 1H), 7.34 ppm (m, 15H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 67.9, 71.1, 71.3, 100.7, 100.8, 110.9, 127.5, 127.8, 128.5, 128.7, 128.8, 128.9, 129.1, 135.5, 135.7, 148.3, 158.8, 161.6, 163.3 ppm; MS (ESI): m/z 590.2 $[M+NH_4]^+$.

4.1.22. Benzyl 2,4-bis(benzyloxy)-6-(pentadec-1-yn-1-yl)-benzoate (11a)

The product was obtained using Section 4.1.1 starting from **10** (1.2 g, 2.0 mmol) and 1-pentadecyne (0.46 mg, 2.2 mmol) using either oil bath heating or microwave irradiation. It was purified by column chromatography using EtOAc/Hex 1:25 (v/v) as eluent. The product was obtained as colorless oil in 83% yield (1.1 g) for oil bath heating and 88% (1.1 g) for microwave irradiation; R_f = 0.53 (EtOAc/Hex 1:7 v/v); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.88 (3H, t, J = 6.3 Hz,), 1.20–1.60 (22H, m), 2.28 (2H, t, J = 6.6 Hz,), 5.00 (1H, s), 5.02 (1H, s), 5.33 (1H, s), 6.50 (1H, d, J = 2.4 Hz), 6.63 (1H, d,

J = 2.4 Hz), 7.24–7.39 ppm (15H, m); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.2, 19.5, 22.7, 28.6, 29.0, 29.2, 29.4, 29.6, 29.7, 31.9, 66.9, 70.2, 70.5, 77.8, 94.6, 101.1, 109.6, 119.8, 124.1, 127.1, 127.6, 127.9, 128.0, 128.2, 128.4, 128.5, 128.7, 136.0, 136.2, 136.3, 156.8, 160.2, 166.9 ppm; MS (ESI): m/z 631.4 $[M+H]^+$.

4.1.23. Benzyl 2,4-bis(benzyloxy)-6-(dec-1-yn-1-yl)benzoate (11b)

The product was obtained using Section 4.1.1 starting from **10** (1.2 g, 2.0 mmol) and 1-decyne (0.30 g, 2.2 mmol) using microwave irradiation. It was purified by column chromatography using EtOAc/Hex 1:20 (v/v) as eluent. The product was obtained as brown oil (0.89 g, 79%); R_f = 0.66 (EtOAc/Hex 1:4); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.6, 3H), 1.27–1.55 (m, 12H), 2.28 (t, J = 6.9, 2H), 4.99 (s, 2H), 5.02 (s, 2H), 5.33 (s, 2H), 6.50 (1H, d, J = 4.3 Hz), 6.62 (1H, d, J = 4.3 Hz), 7.27–7.40 ppm (m, 15H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.1, 19.4, 22.6, 28.5, 28.9, 29.1, 29.2, 31.8, 66.9, 70.2, 70.5, 94.6, 101.1, 109.6, 119.8, 124.1, 127.0, 127.5, 127.8, 127.9, 128.2, 128.3, 128.5, 128.6, 135.9, 136.2, 136.3, 156.8, 160.2, 166.9 ppm; MS (ESI): m/z 561.4 $[M+H]^+$.

4.1.24. 2,4-Dihydroxy-6-pentadecylbenzoic acid (12a)

The product was obtained using Section 4.1.2 starting from **10a** and purified by column chromatography using EtOAc/Hex 1:3 (v/v) as eluent. The product was obtained as a white solid (0.49 g, 89.5%); R_f = 0.19 (100% EtOAc); mp: 124–125 °C; ^{1}H NMR (400 MHz, CD_3OD): δ = 0.88 (3H, t, J = 6.8 Hz), 1.22–1.31 (24H, m), 1.51–1.56 (2H, m), 2.86 (2H, t, J = 7.7 Hz), 6.14 (1H, d, J = 2.2 Hz), 6.18 ppm (1H, d, J = 2.2 Hz); ^{13}C NMR (50 MHz, CD_3OD): δ = 13.1, 22.4, 29.1, 29.2, 29.4, 29.6, 31.7, 31.8, 36.2, 100.3, 103.5, 110.4, 148.7, 162.2, 165.5, 173.4 ppm; MS (ESI): m/z 363.4; $[M-H]^-$; HRMS: m/z $[M-H]^-$ calcd for $C_{22}H_{35}O_4$ 363.2540, found 363.2542.

4.1.25. 2-Decyl-4,6-dihydroxybenzoic acid (12b)

The product was obtained using Section 4.1.2 starting from **10b** and it was purified by column chromatography using EtOAc/Hex 1:3 (v/v) as eluent. The product was obtained as a pale orange solid (0.34 g, 76.5%); R_f = 0.48 (100% EtOAc); mp: 107–108 °C; ^{1}H NMR (400 MHz, CD_3OD): δ = 0.90 (3H, t, J = 6.8 Hz), 1.29–1.33 (m, 14H), 1.54–1.57 (2H, m), 2.86–2.90 (2H, t, J = 7.9 Hz), 6.14 (1H, d, J = 2.6 Hz), 6.19 ppm (1H, d, J = 2.6 Hz); ^{13}C NMR (50 MHz, CD_3OD): δ = 14.4, 23.7, 30.5, 30.7, 30.8, 30.9, 33.1, 33.2, 37.6, 101.7, 111.4, 111.7, 150.2, 163.5, 166.8, 173.5 ppm; HRMS: m/z $[M-H]^-$ calcd for $C_{17}H_{25}O_4$ 293.1758, found 293.1761.

4.1.26. 5-(Benzyloxy)-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (14)

The starting material **13** (2.2 g, 11 mmol) was dissolved in DMF (20 mL), and cooled to 0 °C. K_2CO_3 (3.1 g, 22 mmol) was added and benzylbromide (1.5 mL, 12 mmol) was added dropwise, and the mixture was stirred for 1 h at 0 °C and then overnight at room temperature. The reaction mixture was extracted with EtOAc (3 × 20 mL), and the combined organic layers were washed with brine (1 × 40 mL), dried with $MgSO_4$, and filtered. The solvent was evaporated to give the pure compound as a yellow solid (2.6 g, 84%); R_f = 0.23 (EtOAc/Hex 1:5); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.71 (6H, s), 5.25 (2H, s), 6.53 (1H, d, J = 8.2 Hz), 6.63 (1H, d, J = 8.5 Hz), 7.26–7.43 (5H, m), 7.53–7.57 ppm (1H, m); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 25.8, 70.8, 104.2, 105.1, 105.4, 107.3, 109.6, 126.8, 127.9, 128.7, 136.4, 158.0, 158.2, 160.5 ppm; MS (ESI): m/z 285.2 $[M+H]^+$.

4.1.27. 2-(Benzyloxy)-6-hydroxybenzoic acid (15)

The product was obtained using Section 4.1.3 starting from **14** and it was purified by column chromatography using EtOAc/Hex 1:2 (v/v) as eluent. The product was obtained as white solid (0.12 g, 49%); R_f = 0.39 (100% EtOAc); mp: 122–123 °C; ^{1}H NMR (200 MHz, CD_3OD): δ = 5.27 (2H, s), 6.59 (1H, d, J = 8.2), 6.67 (1H, d, J = 8.2), 7.35 (5H, m), 7.48–7.53 ppm (1H, m). ^{13}C NMR (50 MHz, CD_3OD): δ = 73.0, 105.2, 112.0, 112.2, 121.1, 129.8, 130.1, 136.4, 137.8, 160.5, 164.5, 171.5 ppm; HRMS: m/z $[M-H]^-$ calcd for $C_{14}H_{11}O_4$ 243.0662, found 243.0666.

4.1.28. 2,2-Dimethyl-5-(tetradecyloxy)-4*H*-benzo[*d*][1,3]dioxin-4-one (16)

5-Hydroxy-2,2-dimethyl-4*H*-benzo[*d*][1,3]dioxin-4-one (**13**) (0.99 g, 5.1 mmol) and 1-tetradecanol (1.6 g, 7.5 mmol) were dissolved in dry THF (15 mL), and the reaction mixture was cooled down to 0 °C. PPh_3 (2.0 g, 7.5 mmol) and DIAD (1.5 mL, 7.5 mmol) were added, and the mixture was first stirred for 1 h at 0 °C and then overnight at room temperature. The reaction mixture was concentrated under reduced pressure, and the compound was purified by column chromatography using EtOAc/Hex 1:12 as eluent. The product was obtained as white solid (0.54 g, 27%); R_f = 0.33 (EtOAc/Hex 1:10); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.84 (3H, m), 1.09–1.33 (22H, m), 1.51 (2H, m), 1.70 (6H, s), 4.03 (2H, t, J = 6.8 Hz), 6.50 (1H, d, J = 7.4 Hz), 6.57 (1H, d, J = 8.5 Hz), 7.32–7.39 ppm (1H, m); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.09, 22.8, 25.7, 25.9, 26.8, 27.5, 29.1, 29.5, 29.6, 29.7, 32.2, 69.5, 103.6, 105.2, 106.5, 109., 136.3, 157.9, 158.0, 161.2 ppm; MS (ESI): m/z 391.3 $[M+H]^+$.

4.1.29. 2-Hydroxy-6-(tetradecyloxy)benzoic acid (17)

The product was obtained using Section 4.1.3 starting from **16** and it was purified by column chromatography using EtOAc/ Hex 1:5 (v/v) as eluent. The product was obtained as pale pink solid (0.23 g, 66%); R_f = 0.63 (100% EtOAc); mp: 93–94 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 0.87 (3H, t, J = 6.8 Hz), 1.21–1.26 (22H, m), 1.86–1.90 (2H, m), 4.21 (2H, t, J = 6.2), 6.46 (1H, d, J = 8.2), 6.69 (1H, d, J = 8.5), 7.33–7.41 ppm (1H, m). ^{13}C NMR (50 MHz, $CDCl_3$): δ = 14.2, 22.8, 25.9, 26.0, 26.7, 27.5, 28.9, 29.2 29.5, 29.6, 29.7, 32.1, 70.9, 101.8, 102.3, 112.2, 134.2, 135.6, 158.2, 171.0 ppm; HRMS: m/z $[M-H]^-$ calcd for $C_{21}H_{33}O_4$ 349.2384, found 349.2383.

4.1.30. Methyl 2-hydroxy-6-pentadecylbenzoate (18)

The starting material **4a** (0.17 g, 0.5 mmol) was dissolved in 5 mL methanol. Concentrated H_2SO_4 (0.5 mL) was slowly added and the solution was stirred for 4 h at reflux. The mixture was then cooled down to room temperature, diluted with 20 mL dichloromethane and extracted with NaOH 1 M (3 × 20 mL). The organic layer was washed with brine (1 × 15 mL), dried over Na_2SO_4 and filtered. The solvent was evaporated to give the pure product as a pail yellow solid (0.12 g, 65%); R_f = 0.73 (EtOAc/Hex 1:6); mp: 77–78 °C; ^{1}H NMR (200 MHz, CD_3OD): δ = 0.85–0.88 (3H, m), 1.26–1.53 (26H, m), 2.88 (2H, t, J = 7.6), 3.96 (3H, s), 6.72 (1H, d, J = 6.8), 6.82 (1H, d, J = 7.8), 7.25–7.29 ppm (1H, m); ^{13}C NMR (50 MHz, CD_3OD): δ = 14.3, 22.8, 25.1, 25.7, 29.5, 29.7, 29.8, 30.0, 32.1, 32.3, 34.1, 36.8, 52.2, 112.0, 115.7, 122.6, 134.3, 146.3, 162.7, 172.1 ppm; HRMS: m/z $[M-H]^-$ calcd for $C_{23}H_{37}O_3$ 361.2746, found 361.2748.

4.2. Cell culture and histone extraction

All cell culture reagents were purchased from Invitrogen. The human cancer cell line HEP G2 (liver) was cultured in Dulbecco's modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal serum bovine (FBS), 50 IU/mL penicillin, and 50 mg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. For the experiments, cells were seeded in T-175 flasks, allowed to grow until about 70% confluency and FBS-starved for 16 h. Cells were then treated at sub-toxic (see Supplementary data) concen-

tration of inhibitor for 24 h. The histone extraction was performed as previously described in literature with minor modifications.[33] Protein concentrations of samples were determined using the Bradford Assay using BSA as a standard for calibration.

4.3. Immunoblot protocol

Approximately 5 μg of extracted histones were loaded on a 12.5% polyacrylamide gel, resolved by SDS–PAGE electrophoresis and electroblotted to PVDF membranes. The membranes were incubated with rabbit anti-acetyl-Histone H4 antibody (Millipore, 06-598) followed by a swine anti-rabbit HRP conjugated antibody (DakoCytomation, P0217). Bands were visualized using AEC detection kit (Sigma). Bands were scanned using a BioRad GS-710 densitometer and quantified using ImageJ quantification software. Equal amount of loading was determined by Coomassie blue staining. The experiments were performed in triplicate.

4.4. Histone acetyltransferase assay

An indirect ELISA was used to test the inhibition of the HAT PCAF activity. A solution 0.1% of bovine serum albumin (BSA) in 100 mM HEPES 7.4 was used as buffer unless differently specified. C96 Maxisorp 96-well plate (Nunc) was coated overnight at 4 °C with 50 μL per well of a solution 19 μg/mL streptavidin in 0.2 M Na_2CO_3 pH 9.4. After washing with buffer, the wells were blocked with 200 μL of a solution 3% BSA in buffer for 1 h at room temperature (rt). The plate was washed with buffer, and then coated with 50 μL per well of a solution 300 nM biotinylated histone H4 peptide (Millipore, 12-372) for 1 h at rt. After washing, 40 μL reaction buffer (0.1% BSA + 0.16% Triton X-100 in 100 mM HEPES pH 7.4) was added to the wells, followed by 20 μL recombinant PCAF 240 nM in reaction buffer, and 10 μL of the selected inhibitor solution at different concentration in reaction buffer. After 15 min incubation, 10 μL solution 40 μM Ac-CoA in reaction buffer was added to the well, except for the negative control. The enzymatic reaction was allowed to proceed for 15 min before washing. Each well was then incubated for 1 h with 50 μL rabbit anti-acetyl-Lys antibody (Millipore, AB3879) dilution 1:1800. After washing, wells were incubated with 50 μL swine anti-rabbit HRP-conjugated dilution 1:1000 (DakoCytomation, P0217). The plate was washed with buffer and incubated with 100 μL per well of chromogen solution (0.1 mg/ml 3,3′,5,5′-tetramethyl benzidine + 0.003% H_2O_2 in 0.1 M acetate buffer pH 5.5). The reaction was terminated by addition of 100 μL H_2SO_4 1.0 M and the optical extinction read at 450 nm. The positive controls were the values of wells with no inhibitor, which were set as 100%. The negative controls were the values of wells with no Ac-CoA and were set as 0%. Stock solutions of inhibitors were prepared in dimethyl sulfoxide (DMSO) and diluted in reaction buffer. The final concentration of DMSO was tested not to have any effect on the assay. Each compound was tested in triplicate on one plate.

4.5. Molecular docking

The Crystal structure of PCAF chain B was downloaded form Protein Data Bank (code 1CM0). All molecules were drawn using Chemaxon MarvinSketch (www.chemaxon.com) and prepared (structure recognition and protonation) using SPORES (www.tcd.uni-konstanz.de/research/spores.php). Molecular docking simulations were performed using PLANTS v1.6.[34,35] The docking site center was determined by considering all residues within 5 Å from the co-crystallized CoA. One active site water molecule (pdb ID6) was kept for the docking. Fifteen poses were generated for each compound. The docking results were analyzed using MOE 2008.10 (www.chemcomp.com) and Molegro Virtual Docker (www.molegro.com). Pictures were generated using PyMOL (www.pymol.org).

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.089. These data include MOL files and InChiKeys of the most important compounds described in this article.

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