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Quinoline-Based p300 Histone Acetyltransferase Inhibitors with Pro-apoptotic Activity in Human Leukemia U937 Cells

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Chemical manipulations performed on 2-methyl-3-carbethoxyquinoline (**1**), a histone acetyltransferase inhibitor previously identified by our research group and active at the sub-millimolar/millimolar level, led to compounds bearing higher alkyl groups at the C2-quinoline or additional side chains at the C6-quinoline positions. Such compounds displayed at least three-fold improved inhibitory potency toward p300 protein lysine acetyltransferase activity; some of them decreased histone H3 and H4 acetylation levels in U937 cells and induced high degrees of apoptosis (three compounds >10-fold higher than compound **1**) after treatment of U937 cells.

Reversible protein acetylation is controlled by the opposite action of protein lysine acetyltransferases (KATs) and deacetylases (KDACs).[1] Respectively, such enzymes add ("writers") or remove ("erasers") an acetyl group to or from the ε-amino group of lysine residues in histone tails as well as in non-histone proteins.[2] In histones, these modifications affect the accessibility of transcription factors to DNA and regulate gene expression. In particular, hyperacetylated histones are associated with transcriptionally active euchromatin, whereas histone hypoacetylation involves gene silencing and arrest of transcription (heterochromatin).[3] Nuclear KATs can be grouped into at least four different families, based on sequence similarity with the acetyltransferase domain and biochemical mechanism of acetyl transfer: the GNAT (Gcn5-related *N*-acetyltransferase) family (KAT2), including GCN5 and PCAF; the p300/CBP (CREB binding protein) family (KAT3); the MYST family (KAT6), named for the founding members MOZ, Ybf2/Sas3, Sas2, and Tip60; and the Rtt109 family, named for identification as regulator of Ty1 transposition gene product 109 (KAT11).[4]

The KAT p300 and its paralogue CBP (KAT3B and -3A) have more than 75 identified histone and non-histone substrates, including p53, MyoD, STAT3, and NF-κB, and are able to interact with and bind more than 400 cellular partners.[5] The broad substrate specificity of p300 can be explained by its unique catalytic mechanism proposed after elucidation of the X-ray crystal structure of p300 in complex with the bi-substrate analogue Lys-CoA.[6] It consists in an initial stable binding of acetyl-CoA to p300, followed by a weak and transient interaction with the histone substrate for acetyl transfer (Theorell–Chance mechanism).[6] The intrinsic acetyltransferase activity of p300 is weak, but it is amplified after autoacetylation.[5d] SIRT2, a NAD^+-dependent deacetylase, counterbalances this acetylating activity;[7] however, p300 is also able to inactivate SIRT2 through acetylation, thereby boosting robust p300 autoacetylation and catalytic activity.[8]

Aberrant expression of p300 has been reported in advanced human malignancies such as prostate, liver, and breast cancer, seeming to be correlated with poor prognosis. CBP and p300 have also been found to be involved in multiple rare chromosomal translocations associated with acute myeloid leukemia (AML).[9] At the molecular level, activation of STAT3, NF-κB, and HIF-1α by p300 can lead to cytokine- or hypoxia-induced cancer cell survival and proliferation,[10] and acetylation by p300 of proteins involved in metabolism, autophagy, and motility may have a significant impact on cancer metastasis.[11] Therefore, p300 is a reliable target to inhibit for obtaining an anticancer effect.

In the last decade, there was a boom in the design, synthesis, and biological evaluation of KDAC inhibitors in cancer as well as non-cancer diseases, and two of them [SAHA (vorinostat) and FK-228 (romidepsin)] have been approved by the US Food and Drug Administration (FDA) for the treatment of refractory cutaneous T-cell lymphoma.[12]

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Only a few diverse KAT inhibitors have been described thus far, belonging to the series of bi-substrate inhibitors,[13] natural products,[14] or small molecules.[15] Focusing on p300, Lys-CoA is an example of a bi-substrate inhibitor,[13a] curcumin and plumbagin showed p300 specificity among the natural substances,[14c,d] isothiazolones have been reported as covalent p300 inhibitors,[15d] and C646 was recently identified by virtual screening.[15f]

In our experience, we have described some quinoline/hydroxyquinoline compounds as KAT inhibitors,[16] a number of bis-3,5-dibromo-4-hydroxyphenyl derivatives defined as epigenetic multiple ligands endowed with histone methyltransferase/sirtuin/p300 mixed inhibitory activities,[17] and a series of long-chain alkylidenemalonates (LoCAMs) carrying the unique profile of p300 inhibitors/PCAF activators.[18] Herein we report the results of our studies performed on novel quinoline compounds starting from the prototype MC1626 (**1**), identified through a phenotypic screen in yeast and then evaluated in human U937 leukemia cells.[16a,b] Once tested, **1** displayed both inhibition of yeast cell growth and of human KAT activity in U937 cell nuclear extracts at sub-millimolar concentrations, inducing 27% apoptosis at 1 mM after 24 h incubation.[16b]

As the quinoline ring is a privileged scaffold in medicinal chemistry, pursuing our interest in improving both KAT inhibitory activity and pro-apoptotic effects on U937 cells, we started to explore the chemical space around the quinoline nucleus by introducing some chemical modifications at fairly accessible positions of the 2-methyl-3-carbethoxyquinoline scaffold.

First, we prepared the methyl ester analogue of **1**, compound **2**, and we replaced the methyl group at the quinoline C2 position with higher alkyl chains such as ethyl (**3**), isopropyl (**4**), *n*-propyl (**5**), and benzyl (**6**) groups. Then, considering **1** as the active fragment against KAT, we thought to explore the chemical space around **1** by functionalizing its structure with some additional groups, and hence introducing a hydroxy group at the C6 position of the quinoline ring (compound **7**), which was in turn alkylated with a benzyl, 2-phenylethyl, 3-phenypropyl, 4-phenylbutyl, 5-phenylpentyl, or 2-oxo-2-phenylethyl chain (compounds **8**–**13**). Furthermore, some lysine-mimicking substituents were inserted at the C6-hydroxy group of the quinoline scaffold, likely to improve the competition of the compounds with the lysine substrate. Thus, C6-*tert*-butoxycarbonylaminopentyloxy-(**14**), -aminopentyloxy-(**15**), -(methylamino)pentyloxy-(**16**), -(dimethylamino)pentyloxy-(**17**), -(*n*-propylamino)pentyloxy-(**18**), -(cyclopropylamino)pentyloxy-(**19**), -(allylamino)pentyloxy-(**20**), -(prop-2-yn-1-ylamino)pentyloxy-(**21**), and -5-oxo-5-(prop-2-yn-1-ylamino)pentyloxy-(**22**) quinolines were prepared for testing as KAT inhibitors. Finally, we also synthesized the C6-hexyloxy analogue **23** to dissect the role of the lysine-mimicking amino terminal function in KAT inhibition (Figure 1).

Compounds **1**–**7** were prepared by Friedlander condensation between the properly substituted *ortho*-nitrobenzaldehyde and the appropriate commercially available β-keto ester at 70 °C in anhydrous ethanol in the presence of stannous chloride, zinc chloride, and 3 Å molecular sieves (Scheme 1).

Compounds **8**–**23** were synthesized by starting from 6-hydroxyquinoline **7** as depicted in Scheme 2. Alkylation of **7** in dry acetonitrile at 95 °C with the appropriate (aryl)alkyl bromide or iodide in the presence of anhydrous potassium carbonate and sodium iodide afforded compounds **8**–**13** and **23**. Similarly, compound **14** was obtained under the same conditions and in the presence of *tert*-butyl (5-bromopentyl)carbamate **24**, which was in turn synthesized from the corresponding alcohol by reaction of tetrabromomethane and triphenylphosphine in dry dichloromethane. The Boc-protected quino-

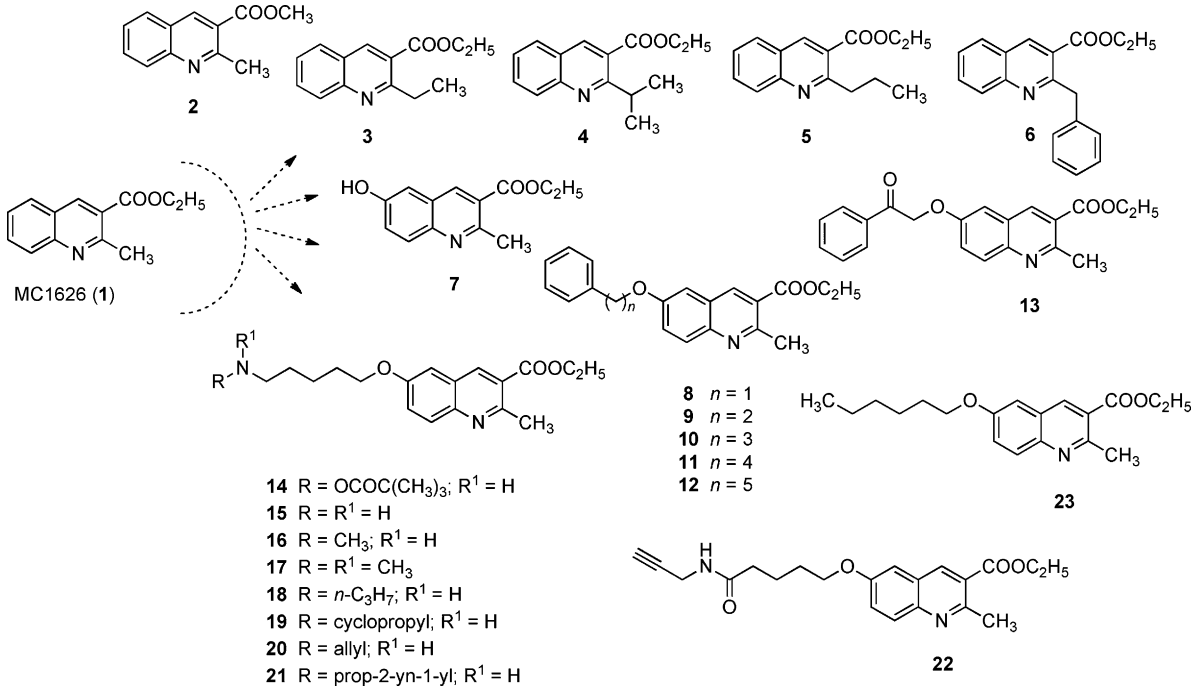
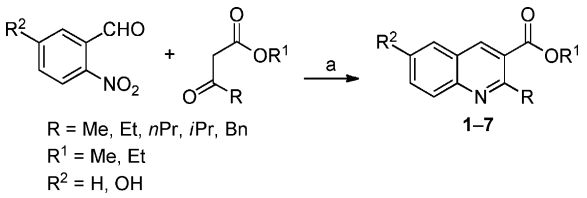


Figure 1. Novel quinoline-based KAT inhibitors.



Scheme 1. *Reagents and conditions*: a) $ZnCl_2$, $SnCl_2$, 3 Å molecular sieves, dry EtOH, 70 °C, 2–4 h.

line **14** was subsequently deprotected in acidic medium to provide the desired amine hydrochloride **15** (Scheme 2). Reaction between our common intermediate **7** and 1,5-dibromopentane afforded bromide **25**, which was then substituted with the appropriate primary and secondary amines in dry *N*,*N*-dimethylformamide (DMF) to obtain compounds **16**–**21** (Scheme 2). Finally, compound **22** was furnished by alkylation of **7** with 5-bromo-*N*-(prop-2-yn-1-yl)pentanamide (**26**), which was prepared by acylation of propargylamine with 5-bromopentanoyl chloride in dry dichloromethane (Scheme 2).

Compounds **1**–**23** were tested against p300 (KAT3B) (Table 1) and PCAF (KAT2B) (Table S1 in the Supporting Information) at 100 μM. Compounds showing >30% inhibition were also tested at 200 and 400 μM (Table 1).

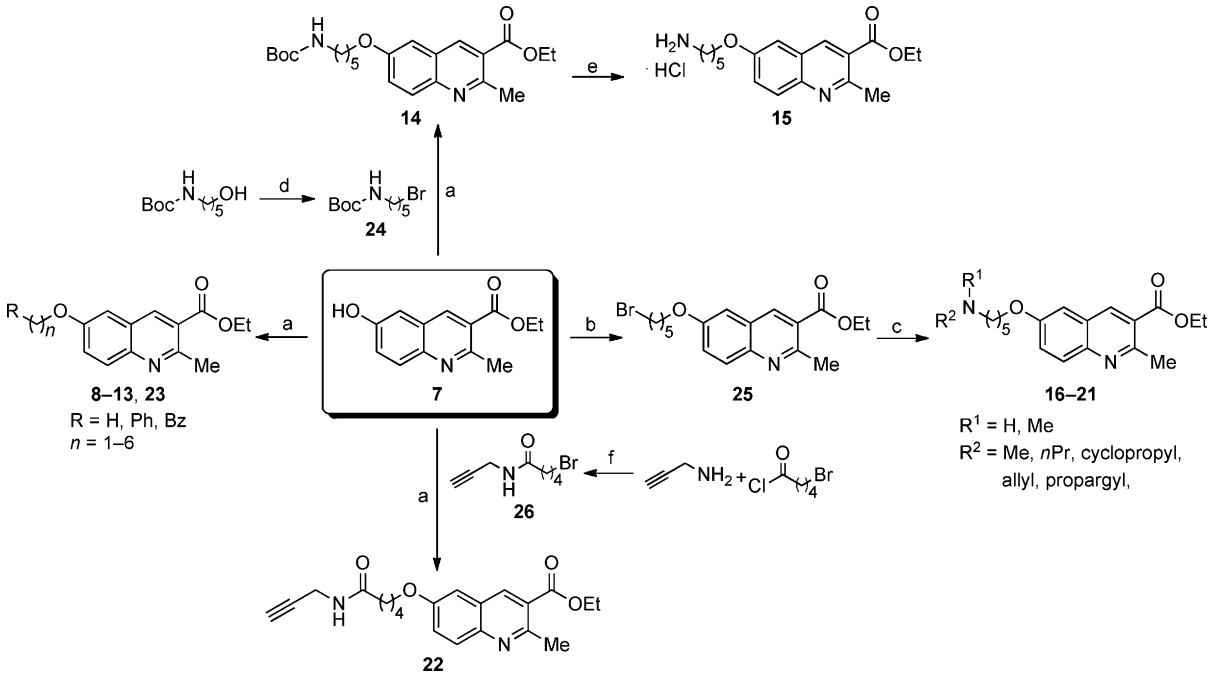
When tested on PCAF, the presented quinolines did not exceed 25% inhibition at 100 μM (Table S3, Supporting Information), and therefore this activity was not further investigated.

Against p300, the prototype **1** displayed 22.6% inhibition at 100 μM, according to its sub-millimolar/millimolar range of activity.[16b] The methyl ester analogue **2** showed the same potency as **1**, whereas derivatives in which the C2-methyl was replaced with a higher alkyl chain (**3**–**6**) were more efficient, with 2-benzyl-3-carbethoxyquinoline **6** being the most potent. The

Table 1. p300 (KAT3B) inhibitory activities of **1**–**23** at fixed doses.[a]

Compd	Inhibition [%] (100 μM)	(200 μM)	(400 μM)
1	22.6 ± 2.1	ND	ND
2	20.5 ± 0.6	ND	ND
3	33.4 ± 0.3	35.6 ± 1.9	44.0 ± 1.3
4	27.0 ± 0.8	ND	ND
5	42.8 ± 0.1	54.0 ± 0.4	67.6 ± 3.4
6	60.3 ± 1.5	64.1 ± 0.3	66.3 ± 0.4
7	16.5 ± 1.0	ND	ND
8	62.0 ± 1.3	65.3 ± 0.5	69.2 ± 0.2
9	5.6 ± 2.8	ND	ND
10	10.6 ± 0.2	ND	ND
11	6.7 ± 0.2	ND	ND
12	0	ND	ND
13	58.5 ± 2.3	67.3 ± 5.8	85.2 ± 1.5
14	0	ND	ND
15	16.2 ± 0.6	ND	ND
16	9.0 ± 1.5	ND	ND
17	7.2 ± 1.2	ND	ND
18	12.1 ± 1.5	ND	ND
19	3.4 ± 1.7	ND	ND
20	0	ND	ND
21	69.3 ± 0.3	83.3 ± 1.1	96.4 ± 0.8
22	27.9 ± 1.3	ND	ND
23	0	ND	ND

[a] Data are the mean ± SD of $n = 2$ independent experiments performed in duplicate. ND: not determined.



Scheme 2. *Reagents and conditions*: a) appropriate (aryl)alkyl bromide/iodide, anhyd K_2CO_3, NaI, dry CH_3CN, 95 °C, 4–8 h; b) 1,5-dibromopentane, anhydrous K_2CO_3, dry CH_3CN, 95 °C, 3 h; c) appropriate amine, anhydrous K_2CO_3, NaI, dry DMF, 60 °C, 2–5 h; d) CBr_4, PPh_3, dry CH_2Cl_2, RT, 2 h; e) 4 N HCl in dioxane, dry THF, RT, 25 h; f) Et_3N, dry CH_2Cl_2, RT, 20 h.

insertion of a hydroxy group at the C6 position of the quinoline scaffold (compound **7**) did not improve the potency of the prototype, but it furnished the key intermediate for the synthesis of C6-alkyloxy and -(amino)alkyloxy derivatives carrying a further substituent in addition to the 2-methyl-3-carbethoxyquinoline fragment. The insertion of a benzyloxy group at C6 (compound **8**) clearly increased the potency of **1**, while the use of longer chains such as 2-phenylethyloxy (**9**), 3-phenylpropyloxy (**10**), 4-phenylbutyloxy (**11**), and 5-phenylpentyloxy (**12**) did not. Interestingly, such inhibitory activity was fully restored if a 2-oxo-2-phenethyloxy chain was introduced at C6, yielding one of the most potent derivatives (**13**, IC_{50}: 74.0 ± 10.8 μM) among those described herein.

When an (amino)pentyloxy chain, designed as a lysine-mimicking portion, was inserted at C6 of the quinoline ring, in general, scarcely potent p300 inhibitors (if any) were obtained (compounds **14**–**20**), regardless of the length and complexity of the alkyl substituent(s) introduced at the amino group, or of the change of such amino group into the corresponding *tert*-butyl carbamate. The sole important exception to this behavior was compound **21**, which carries a C6-(prop-2-yn-1-ylamino)-pentyloxy chain linked to the 2-methyl-3-carbethoxyquinoline fragment, and is the most potent p300 inhibitor described herein (IC_{50}: 57.5 ± 8.2 μM). Replacing the propargylamino function with the corresponding amide (**22**) or deleting the amino function altogether (**23**) furnished less potent or totally inactive compounds, respectively.

Selected compounds **5**, **6**, **13**, and **21** were tested in human U937 leukemia cells at 100 μM to study their effects on acetylation levels of histones H3, H4, and α-tubulin, as functional assays for KAT inhibition in cells. The assays were performed either by treating the cells directly with the compounds, or after pre-treatment (1 h) with 5 μM SAHA and subsequent washout, to increase the starting signals of acetylation for H3, H4, and α-tubulin (Figure 2).

Western blot analyses showed that **5**, **6**, and **21** are highly efficient in decreasing the acetyl-H3 and acetyl-H4 signals with respect to control, with or without SAHA pre-treatment, whereas **13** was observed to be ineffective under both conditions. In addition, among the tested compounds, only **21** decreased α-tubulin acetylation levels (Figure 2).

U937 cells were also treated with **5**, **6**, **13**, and **21** at 100 μM for 30 h to determine effects on cell-cycle progression, apoptosis, and granulocytic differentiation. SAHA (5 μM) was used as a positive control. Under the tested conditions, the compounds did not show clear alteration of cell cycle, with the exception of **21**, which showed a slight increase in the G2/M phase percentage, and **5**, the effect on cell cycle of which could not be clearly evaluated due to the intense extent of apoptosis (see below; Figure 3 A). The percentage of cells in the pre-G_1 peak was taken as an index of apoptosis: in this assay, **5** displayed extensive apoptosis, and **6** and **21** induced apoptosis to 39 and 15%, respectively, relative to control (Figure 3 B). Granulocytic differentiation was evaluated as the percentage of cells positive to the superficial antigen CD11c and negative to propidium iodide ($CD11c^+/PI^-$ cells): in general, low differentiation activity was detected with the tested com-

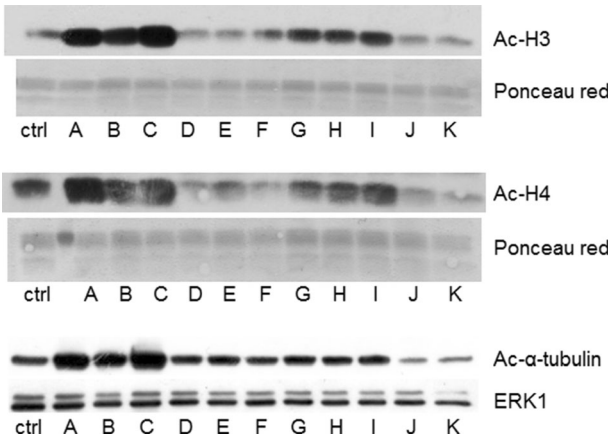


Figure 2. Effects of compounds **5**, **6**, **13**, and **21** (at 100 μM) on acetylation levels of histone H3, histone H4, and α-tubulin in human leukemia U937 cells; ctrl = control; A, SAHA 24 h; B, SAHA 1 h; C, SAHA 1 h washout + SAHA 24 h; D, compound **5**; E, SAHA 1 h washout + **5**; F, compound **6**; G, SAHA 1 h washout + **6**; H, compound **13**; I, SAHA 1 h washout + **13**; J, compound **21**; K, SAHA 1 h washout + **21**.

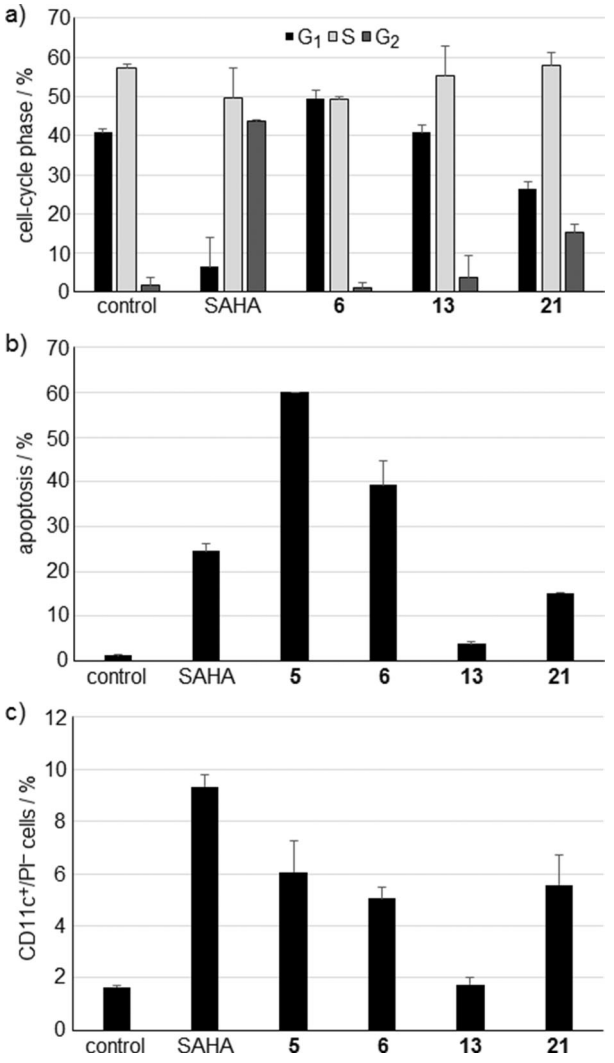


Figure 3. Effects on a) cell cycle, b) apoptosis induction, and c) granulocytic differentiation in U937 cells treated with **5**, **6**, **13**, and **21** for 30 h at 100 μM. SAHA (5 μM) was used as a positive control.

pounds, with **5** and **21** reaching ~6% of $CD11c^+/PI^-$ cells (Figure 3C).

In conclusion, we performed chemical manipulations on the scaffold of **1**, a KAT inhibitor prototype previously disclosed by us and active at sub-millimolar/millimolar levels, to improve its potency both in enzyme and cellular assays. Through replacement of the C2-methyl substituent of **1** with *n*-propyl (**5**) or benzyl (**6**) groups, as well as by inserting additional 2-oxo-2-phenethyloxy (**13**) or (prop-2-yn-1-ylamino)pentyloxy (**21**) side chains at the C6 position of the quinoline ring of compound **1**, we obtained two- to threefold increased potency of such derivatives against p300 (Table 1), with **21** being the most potent (IC_{50}: 57.5 μM). Compounds **5**, **6**, **13**, and **21** (100 μM) were tested in human leukemia U937 cells to determine their effects on histones H3, H4, and α-tubulin acetylation levels, as functional assays for KAT inhibition in cells. Western blot results showed a pronounced decrease in H3 and H4 acetylation in cells treated with **5** and **21**, whereas **6** was less efficient and **13** totally inactive, likely due to cell permeability problems. Accordingly, when the selected compounds were tested in U937 cells to study cell cycle, apoptosis induction, and cell differentiation (at 100 μM for 30 h), **5** effected extensive apoptosis, followed by **6** (39%) and **21** (15%). In a similar assay, the prototype **1** induced 27% apoptosis when tested at 1 mM (10×).[16] The intense degree of apoptosis displayed by **5** correlates well with its ability to highly decrease H3 and H4 acetylation levels (Figure 2), but is not in full agreement with enzyme inhibition data (**21**, for instance, was the most potent KAT inhibitor in enzyme assays, strongly hypoacetylated H3, H4, and α-tubulin, and induced 15% apoptosis in U937 cells); therefore, for compound **5**, off-target effects in producing apoptosis cannot be ruled out. Compound **13** did not show any effect on cell cycle or in apoptosis induction. Despite low efficiency, **5**, **6**, and **21** also displayed slight granulocytic differentiation under the conditions tested. Taking into account both enzyme (p300 KAT) and cellular data, compounds **5** and **21** can be retained as valuable starting points for further exploration of the chemical space around the quinoline nucleus, in order to acquire additional data and to improve the potency of the compounds.

Experimental Section

Chemistry

General: Melting points (m.p.) were determined on a Büchi 530 melting point apparatus. ^{1}H NMR spectra were recorded at 400 MHz using a Bruker AC 400 spectrometer; chemical shifts (δ) are reported in parts per million (ppm) relative to the internal reference (Me_4Si). Mass spectra (MS) were recorded on an API-TOF Mariner instrument (Perspective Biosystems, Stratford, TX, USA); samples were injected by a Harvard pump at a flow rate of 5–10 μL min^{-1}, infused using an electrospray (ESI) system.

All reactions were routinely checked by thin-layer chromatography (TLC) and ^{1}H NMR. TLC was performed on aluminum-backed silica gel plates (Alufolien Kieselgel 60 F_{254}, Merck) with spots visualized by UV light or an alkaline $KMnO_4$ solution. All solvents were reagent grade and, when necessary, were purified and dried by standard methods before use. The removal of solvent from reaction mixtures or after extraction involved the use of a rotary evaporator operating at a reduced pressure of ~20 Torr. Organic solutions were dried over anhydrous Na_2SO_4. Elemental analysis was used to determine purity of the described compounds, which were determined in all cases to be >95%, with analytical results within 0.40% of theoretical values. All chemicals were purchased from Sigma–Aldrich s.r.l. (Milan, Italy) or from TCI Europe N.V. (Zwijndrecht, Belgium) and were of the highest purity available. As a rule, samples prepared for physical and biological studies were dried under high vacuum over P_2O_5 for 20 h at temperatures ranging from 25 to 40 °C, depending on the sample melting point.

Example procedures for the syntheses of final compounds **1**–**23** are reported below. Chemical, physical, and analytical data for the final compounds (**1**–**23**) are reported in the Supporting Information, along with the syntheses and chemical and physical data of intermediate compounds **24**–**26**.

Example procedure for the preparation of 1–7: Ethyl 6-hydroxy-2-methylquinoline-3-carboxylate (7): A 250 mL round-bottom flask, equipped with a stir bar and reflux condenser, was flame dried under an atmosphere of N_2. 5-Hydroxy-2-nitrobenzaldehyde (1 equiv, 2.2 g, 13.2 mmol) and ethyl acetoacetate (1.2 equiv, 2.0 mL, 15.8 mmol) were added, followed by 80 mL of anhyd EtOH. Anhyd $SnCl_2$ (5 equiv, 12.5 g, 65.8 mmol), $ZnCl_2$ (5 equiv, 9.0 g, 65.8 mmol) and 3 Å molecular sieves (~1 g) were added to the solution. This mixture was then heated at 70 °C under N_2 atmosphere; the reaction was finished after 2 h. The mixture was cooled to RT and filtered. The EtOH was removed in vacuo. The residue was then made basic with 10% aq $NaHCO_3$ (100 mL), transferred to a 500 mL separator funnel, and extracted with EtOAc (4×100 mL). The organic extracts were combined, washed thoroughly with saturated aq NaCl, dried over anhyd Na_2SO_4, filtered and concentrated in vacuo. Purification by recrystallization from EtOAc gave **7** as a white solid (2.1 g, 68%):[19] mp: 211–213 °C (EtOAc); ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.37 (t, 3H, $COOCH_2CH_3$), 2.78 (s, 3H, CH_3), 4.37 (q, 2H, $COOCH_2CH_3$), 7.26 (d, 1H, quinoline proton), 7.40 (dd, 1H, quinoline proton), 7.83 (d, 1H, quinoline proton), 8.62 (s, 1H, quinoline proton), 10.11 ppm (s, 1H, OH); MS (ESI): m/z = 232 $[M+H]^+$.

Example procedure for the preparation of 8–14,22–23: **Ethyl 2-methyl-6-phenethoxyquinoline-3-carboxylate (9)**: A round-bottom flask containing a solution of **7** (1 equiv, 0.43 mmol, 100 mg) in dry CH_3CN (2 mL) was treated with, in sequence, NaI (1.3 equiv, 0.56 mmol, 84 mg), anhyd K_2CO_3 (1.75 equiv, 0.76 mmol, 104.6 mg) and 2-bromoethylbenzene (1.5 equiv, 0.56 mmol, 76.7 μL). The mixture was then stirred at 95 °C for 5 h. The reaction mixture was stopped by the addition of saturated aq NaCl (40 mL) and extracted with EtOAc (4×50 mL). The organic layers were combined, washed with saturated aq NaCl, dried over anhyd Na_2SO_4, filtered and concentrated in vacuo. Purification by chromatography on a silica gel column (EtOAc/*n*-hexane, 1:9) gave compound **9** as a white powder (85.1 mg, 59%): mp: 68–71 °C (cyclohexane); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.47 (t, 3H, $COOCH_2CH_3$), 2.97 (s, 3H, CH_3), 3.20 (t, 2H, CH_2CH_2O), 4.32 (t, 2H, CH_2CH_2O), 4.45 (q, 2H, $COOCH_2CH_3$), 7.13 (d, 1H, quinoline proton), 7.35–7.39 (m, 5H, phenyl proton), 7.45 (dd, 1H, quinoline proton), 7.94 (d, 1H, quinoline proton), 8.61 ppm (s, 1H, quinoline proton); MS (ESI): m/z = 336 $[M+H]^+$.

Ethyl 6-((5-aminopentyl)oxy)-2-methylquinoline-3-carboxylate hydrochloride (15): A solution of **14** (1 equiv, 0.4 mmol, 170 mg) in dry THF (10 mL) was treated with 4 N HCl in dioxane (60 equiv, 24.5 mmol, 6.1 mL) at 0 °C, and the resulting reaction mixture was

left under stirring at RT for 25 h. After the end of the reaction, the resulting precipitate was isolated by filtration, and washed first with dry THF and then dry Et_2O to give **15** as a white solid (103.3 mg, 87%): mp: 213–215 °C (EtOAc); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 1.39$ (t, 3H, $COOCH_2CH_3$), 1.52 (t, 2H, $H_2NCH_2CH_2CH_2$), 1.65 (t, 2H, $H_2NCH_2CH_2$), 1.83 (t, 2H, $H_2N(CH_2)_3CH_2$), 2.82 (m, 2H, H_2NCH_2), 2.98 (s, 3H, CH_3), 4.14 (t, 2H, $H_2N(CH_2)_4CH_2O$), 4.41 (q, 2H, $COOCH_2CH_3$), 7.67 (dd, 1H, quinoline proton), 7.75 (d, 1H, quinoline proton), 7.92 (br, 3H, NH_2·HCl), 8.18 (d, 1H, quinoline proton), 9.10 ppm (s, 1H, quinoline proton); MS (ESI): $m/z = 317$ $[M+H]^+$ (relative to free amine).

Example procedure for the preparation of 16–21: **Ethyl 6-((5-(allylamino)pentyl)oxy)-2-methylquinoline-3-carboxylate (20)**: A round-bottom flask was charged, in sequence, with **25** (1 equiv, 0.26 mmol, 100 mg), dry DMF (3.6 mL), NaI (1.1 equiv, 0.29 mmol, 43.4 mg), anhyd K_2CO_3 (1.5 equiv, 0.39 mmol, 54.5 mg) and allylamine (15 equiv, 3.9 mmol, 0.3 mL). The reaction mixture was left under stirring at 60 °C for 4 h. Upon completion, the reaction was cooled and then quenched with saturated aq NaCl (20 mL). The solution was then extracted with EtOAc (4×40 mL). The combined organic phases were washed with saturated aq NaCl, dried over anhyd Na_2SO_4, filtered and concentrated in vacuo. Purification by chromatography on a silica gel column ($CHCl_3$/MeOH, 25:1) gave **20** as a white solid (78.8 mg, 85%): mp: 104–106 (petroleum ether); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.47$ (t, 3H, $COOCH_2CH_3$), 1.57–1.62 (m, 2H, $NHCH_2CH_2CH_2$), 1.66–1.73 (m, 2H, $NHCH_2CH_2$), 1.86–1.93 (m, 2H, $NH(CH_2)_3CH_2$), 2.31 (br, 1H, NH), 2.73 (t, 2H, $NHCH_2CH_2$), 2.96 (s, 3H, CH_3), 3.35 (d, 2H, $CH_2=CHCH_2NH$), 4.08 (t, 2H, $NH(CH_2)_4CH_2O$), 4.45 (q, 2H, $COOCH_2CH_3$), 5.16–5.19 (d, 1H, $CH=CHCH_2NH$), 5.23–5.27 (d, 1H, $CH=CHCH_2NH$), 5.91–6.01 (m, 1H, $CHH=CHCH_2NH$), 7.11 (d, 1H, quinoline proton), 7.43 (dd, 1H, quinoline proton), 7.94 (d, 1H, quinoline proton), 8.63 ppm (s, 1H, quinoline proton); MS (ESI): $m/z = 357$ $[M+H]^+$.

Enzyme-based assays

KAT assays: The effects of test compounds on the catalytic activity of p300 and PCAF were determined in a HotSpot HAT activity assay (Reaction Biology Corp., Malvern, PA, USA), performed according to the company's instructions. In brief, the recombinant catalytic domains of PCAF (aa 492–658) or p300 (aa 1284–1673) were incubated with histone H3 as a substrate (5 μM) and [acetyl-^{3}H]-acetyl coenzyme A (3.08 μM) as an acetyl donor in reaction buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% DMSO) for 1 h at 30 °C in the presence or absence of a dose titration of the test compound. Histone H3 acetylation was assessed by liquid scintillation. Anacardic acid and curcumin served as positive controls against PCAF and p300 activity, respectively. Data at fixed doses were performed twice, each in duplicate. IC_{50} values were analyzed using Excel and GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA).

Cell-based assays

Cell culture: Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), penicillin G (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), L-glutamine (2 mM), amphotericin B (250 mg mL^{-1}). At the time indicated, inductions were performed with ligands.

Western blot analysis: After removal of the culture medium, the cells were washed with cold 1X phosphate-buffered saline (PBS) and lysed using a lysis buffer supplemented with protease and phosphatase inhibitors (50 mM Tris-HCl, pH 8.0, 150 mm NaCl, 1% NP-40, 10 mM NaF, 0.1 mm Na_3VO_4, 40 mg mL^{-1} PMSF, 20 g mL^{-1} aprotinin, 20 mg mL^{-1} leupeptin, 2 mg mL^{-1} antipain, 10 mM *para*-nitrophenyl phosphate (pNPP), 10 mg mL^{-1} pepstatin A and 20 nM okadaic acid). Then the cells were centrifuged for 15 min at 4 °C (13 000 rpm), and the protein concentration of the supernatant was determined by colorimetric assay (Bio-Rad, Italy). The cell extracts were diluted 1:1 in 2X Laemli sample buffer (0.217 M Tris-HCl, pH 8.0, 52.17% sodium dodecyl sulfate (SDS), 17.4% glycerol, 0.026% bromophenol blue, 8.7% β-mercaptoethanol), and then were boiled for 3 min. Equal amounts of protein (50 micrograms) were run and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Primary antibodies used were: anti-alpha tubulin acetylated (Sigma) and anti-ERK1 (Santa Cruz Biotechnology).

Histone extraction: Cells were harvested, washed twice with cold PBS, and lysed in triton extraction buffer (TEB): PBS containing 0.5% Triton X 100 (*v*/*v*), 2 mM PMSF, 0.02% (*w*/*v*) NaN_3, at a cellular density of 10^7 cells/mL for 10 min on ice, with gentle stirring. After brief centrifugation (2000 rpm at 4 °C for 10 min), the supernatant was removed, and the pellet was washed in half the volume of TEB and centrifuged as before. The pellet was suspended in 0.2 M HCl at a cell density of 4×10^7 cells/mL, and acid extraction was left to proceed overnight at 4 °C on a rolling table. Next, the samples were centrifuged at 2000 rpm for 10 min at 4 °C, the supernatant was removed, and the protein content was determined using a Bradford assay. Antibodies against acetylated histones H3 and H4 (Upstate Biotechnologies) at concentrations of 2 mg mL^{-1} were used. Ponceau red was used for normalization.

Flow cytometry: Cells were plated (2×10^5 cells mL^{-1}) and collected after stimulation. They were then centrifuged (1200 rpm for 5 min at 4 °C) and suspended in a solution of 1X PBS containing 0.1% sodium citrate, 0.1% NP-40 and 50 mg mL^{-1} propidium iodide. After 30 min incubation at room temperature in the dark, cell cycle was evaluated by fluorescence-activated cell sorting (FACS) flow cytometry (FACSCalibur, Becton Dickinson) and analyzed with the program ModFit V3 (Verity Software House, Inc.).

FACS analysis of apoptosis: Apoptosis was measured as the pre-G1 cell peak, analyzed by FACS with Cell Quest software (Becton Dickinson) as previously reported.[20]

FACS analysis of differentiation: After centrifugation, cells were resuspended in 10 μL of phycoerythrine-conjugated CD11c (CD11c-PE, PharMingen). Control samples were incubated with 10 μL of phycoerythrin-conjugated mouse IgG1; after incubation for 1 h at 4 °C in the dark, cells were washed with PBS and resuspended in 500 μL PBS containing propidium iodide (0.25 μg mL^{-1}). The samples were then analyzed by flow cytometry.

Supporting Information

Synthetic procedures and characterization data for intermediate and all remaining final compounds are given in the Supporting Information, along with ^{1}H NMR and mass spectra for compounds **1**–**23**. The results of the PCAF inhibition assay for compounds **1**–**23** are also given.

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