Brief Articles

Cinnamoyl Compounds as Simple Molecules that Inhibit p300 Histone Acetyltransferase

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Cinnamoly compounds **1a**−**c** and **2a**−**d** were designed, synthesized, and in vitro tested as p300 inhibitors. At different degrees, all tested compounds were proven to inactivate p300, particularly, derivative **2c** was the most active inhibitor, also showing high specificity for p300 as compared to other histone acetyltransferases. Most notably, **2c** showed anti-acetylase activity in mammalian cells. These compounds represent a new class of synthetic inhibitors of p300, characterized by simple chemical structures.

Introduction

DNA is a charged polymer that is highly packaged in the nucleus of eukaryotic cells. This extreme compaction is achieved through association of DNA with a set of basic histone proteins to form a structure known as chromatin. The fundamental repeat unit of chromatin is the nucleosome, in which 146 base pairs of DNA are wound around a histone octamer comprising two copies of each histones H2A, H2B, H3, and H4.[1,2] Nucleosomes are in turn folded into progressively higher-order structures. Though apparently repressive, the precise organization of chromatin is essential for replication, repair, recombination, and chromosomal segregation. Modification in the chromatin organization modulates the expression of underlying genes. The dynamic changes in the chromatin structure are brought about by post-translational modifications of the amino terminal tails of the histones and the ATP-dependent chromatin remodeling. Specific amino acids within the histone tails are the sites of a variety of modifications including phosphorylation, acetylation, methylation, ADP-ribosylation, and ubiquination.[3] Among these modifications, acetylation has been most widely studied in the context of gene expression.

The dynamic equilibrium between acetylation and deacetylation is maintained by the activity of hystone acetyltransferases (HATs[a]) and deacetylases (HDACs) that regulate the expression of the genome. Specifically, HATs function enzymatically by transferring an acetyl group from acetyl-coenzyme A to the ϵ-amino group of certain lysine side chains within a histone's basic N-terminal tail region.[4] HATs are divided into five families including the GNAT family, the MYST group, p300/CBP HATs, the general transcription factor, and the nuclear hormone-related HATs.[5] p300 is a ubiquitously expressed global transcriptional coactivator that has critical roles in a wide variety of cellular phenomena including cell cycle control, differentiation, and apoptosis.[6] Mutations in p300 enzyme have been proven to be associated with certain cancers and other human disease processes.[7] Therefore, these enzymes could be considered useful targets for a novel approach in chemotherapy. During the past decade, significant progress has been made in the field of HDAC inhibitors as antineoplastic agents, and some of these compounds are already in clinical trial as anticancer drugs.[8] Conversely, specific inhibitors for p300 were not identified until recently.

The aim of the present work was the identification of novel anti-p300 agents that could be useful as potential lead molecules for anticancer as well as antiviral drug discovery. The anti-p300 agents so far identified are (i) the natural products garcinol,[9] anacardic acid,[10] and curcumin[11] and (ii) the synthetic derivative Lys-CoA,[12] a lysine analog of HAT substrate acetyl-CoA (Figure 1).

In particular, a screening of plant extracts from *Curcuma longa* rhizome led to the discovery of curcumin as a potent and specific inhibitor of p300.[11] Interestingly, in the course of our studies aimed at the discovery of antiviral agents targeted to HIV-1 integrase, we reported a group of curcumin-like derivatives characterized by a 3,4-dihydroxycinnamoyl pharmacophore.[13] Based on this preliminary evidence and because the studies on structure−activity relationships (SARs) in the field of anti-p300 agents are still limited and only a few Lys-CoA analogs have been described,[14] we set out to identify new synthetic polyhydroxylated aromatic derivatives related to curcumin, garcinol, and anacardic acid as p300 inhibitors. The results of this study may represent a groundwork for the development of novel anti-p300 agents as potential leading molecules for anticancer as well as antiviral drug discovery.

An examination of the chemical structures of these natural products led us to identify some structural features that characterize these compounds: (i) a α,γ-diketo group; (ii) a cinnamoyl moiety; (iii) a catechol ring; and (iv) a salicylic acid portion. Therefore, we decided to test the activity against p300 of cinnamoyl compounds, such as **1a (**related to curcumin), **2a**, and **2d** (cyclohexanone derivatives), previously reported by us in the course of our studies aimed at the discovery of antiviral agents targeted to HIV-1 integrase.[13] In fact, **1a**, **2a**, and **2d**

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[a] Abbreviations: HAT, histone acetyl transferase; HDAC, histone deacetylase; GNAT, Gcn5-related *N*-acetyltransferase; P300/CBP, p300/CREB binding protein; SAR, structure−activity relationship; H2B-EYFP, H2B-enhanced yellow fluorescent protein; FCS-DMEM, fetal calf serum-Dulbecco's modified eagle's medium.

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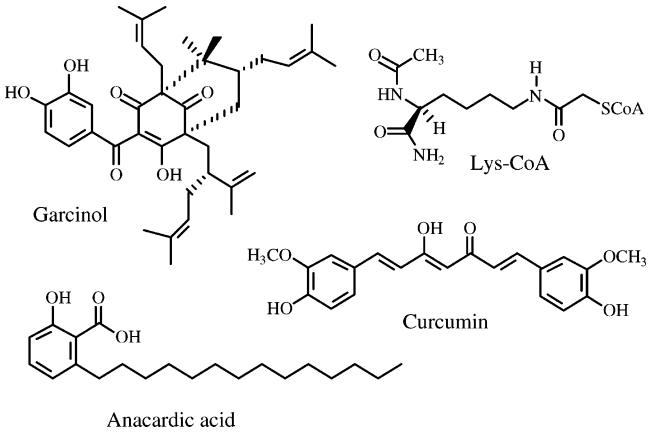
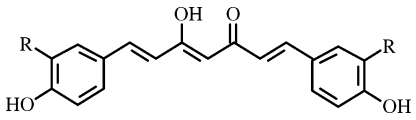
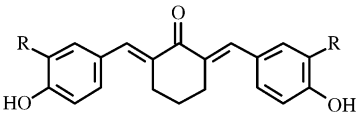


Figure 1. Structures of p300 inhibitors reported in literature.



1a-c

R = OH (**a**), COOH (**b**), Br (**c**)

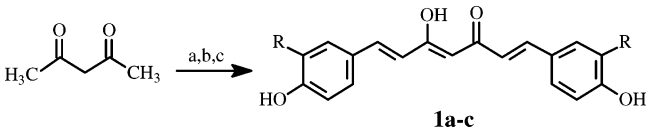


2a-d

R = OH (**a**), COOH (**b**), Br (**c**), OCH_3 (**d**)

Figure 2. Structures of the cinnamoyl derivatives **1a**−**c** and **2a**−**d** reported in the present study and tested as p300 inhibitors.

Scheme 1[a]



1a-c

[a] Reagents and conditions: (a) $B(OH)_3$ DMF, 100 °C, 5 min; (b) arylaldehydes, 1,2,3,4-tetrahydroquinoline, AcOH, DMF, 100 °C, 4 h; (c) AcOH, room temp, 1 h. Yields for the three-steps, one-pot synthesis: **1a**,[14] 10%; **1b**, 50%; and **1c**, 65%.

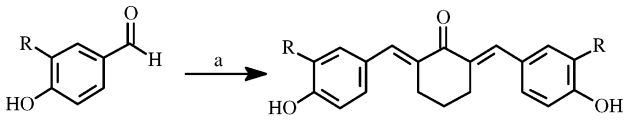
share some of the above chemical features such as (i) the α,γ-diketo group (**1a**), (ii) the cinnamoyl moiety (**1a**, **2a**, **2d**), and (iii) the catechol ring (**1a**, **2a**). Moreover, as a preliminary SAR study, we designed and synthesized salicylic derivatives **1b** and **2b** and compounds **1c** and **2c** that are characterized by the presence in the ortho position to OH groups by lipophilic and withdrawing bromine atoms. Compounds **1a**−**c** and **2a**−**d** (Figure 2) were tested in in vitro assays for their inhibitory activities against p300. To different extent, all synthesized molecules inhibited p300 enzymatic activity. The most active compound, **2c**, was selective for p300 as compared to other HATs and, most notably, was cell permeable, as demonstrated by decreased histones acetylation.

Results and Discussion

Chemistry. Synthesis of derivatives **1a**−**c** and **2a**−**d** is outlined in Schemes 1 and 2. The curcumin analogs **1a**−**c** were synthesized according to the Babu and Rajasekharan method (Scheme 1).[15]

The fundamental step in this reaction is the protection of the active methylene group by reacting with acetylacetone in the presence of boric acid to get acetylacetone−boric acid complex

Scheme 2[a]



2a-d

[a] Reagents and conditions: (a) cyclohexanone, montmorillonite K-10, 5 min, 100 W, 100 °C. Yields: **2a**, 75%; **2b**, 50%; **2c**, 89%; **2d**, 75%.

and reacting less reactive methyls with the appropriate aldehyde using 1,2,3,4-tetrahydroquinoline as a catalyst. Notably, the synthesis of derivative **1b** has already been reported in very low yields (<9%) by Subaraju[16] in three steps involving protection/deprotection procedures of carboxylic groups. However, we obtained **1b** in higher yields (50%) by the application of the Babu method to 5-formylsalicylic acid in the three steps, one-pot synthesis (Scheme 1).

Bis-arylidene derivatives **2a**−**d** were synthesized by condensation of cyclohexanone with the appropriate benzaldehyde (Scheme 2). A new procedure that did not require the preliminary protection of the OH groups was developed. In particular, a dispersion of cyclohexanone and the substituted benzaldehyde in montmorillonite K-10, was submitted to microwave-assisted heating (100 °C, 100 W) for 5 min. Interestingly, montmorillonite K-10 was used in this reaction as both an environmentally benign solid support and a heterogeneous acid catalyst. This procedure allowed (i) increased yields of these condensations if compared to those previously reported,[13] (ii) a reduction in the synthetic pathway from three steps to one step, and (iii) a minimization of the reaction time.

Evaluation of Biological Activities. The p300 inhibitory activities of the newly synthesized cinnamolyl compounds **1a**−**c** and **2a**−**d** were tested in an in vitro acetylation assay[17] using recombinant histones (H1, H2A, H2B, H3, and H4) and the HAT domain of p300 (Figure 3A).

The inhibitory activity of each compound was tested, with concentration ranging from 25 μM to 400 μM (data not shown), or starting with 0.19 μM for the derivative **2c**, to determine the IC_{50} value (Figure 3C and Table 1). Figure 3B shows the histone acetylation levels following incubation with p300 in the presence of scalar amounts of the derivative **2c**.

Compounds **1a**−**c** and **2a**−**d** could be divided into the following: (i) curcumin derivatives with different substituents on the aromatic moieties (**1a**−**c**) and (ii) 2,6-bis-arylidene cyclohexanone derivatives (**2a**−**d**). In general, compounds **1a**−**c** and **2a**−**d** showed good activities against p300, with IC_{50} values ranging from 5 to 233 μM (Table 1). Derivative **2c** was the most potent compound of these series (IC_{50} = 5 μM), being six times more potent than Lys-CoA used as a reference drug. Surprisingly, in our assays, curcumin was inactive at concentrations up to 400 μM. Due to this result, we tested a new stock of commercial curcumin (Fluka) after a further chromatography purification and ^1H NMR identification and tested with both HAT domain as well as the full length p300 enzyme.[18] In spite of this, the inactivity of curcumin was confirmed.

Interestingly, the curcumin derivatives **1a**−**c** were potent p300 inhibitors showing IC_{50} values from 21 to 46 μM, comparable to that found for Lys-CoA used as a reference drug in the same experiment (IC_{50} = 30 μM). The most active derivative among this group of molecules was **1b**, which was characterized by salicylic groups (1.4 times more potent than Lys-CoA). Replacement of the carboxylic function with a bromine or hydroxyl groups led to **1c** and **1a**, which were 1.5 and 2 times less potent than parent derivative **1b**, respectively. In general, the activities

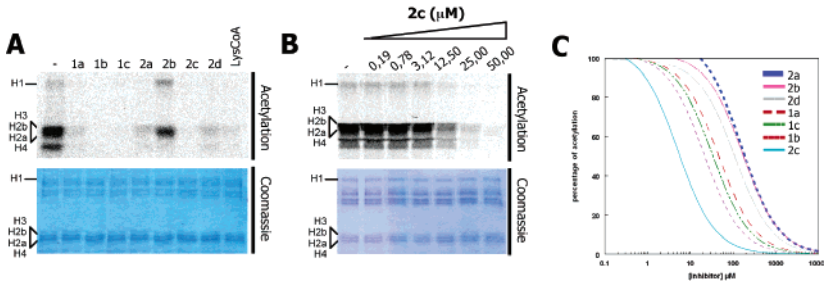
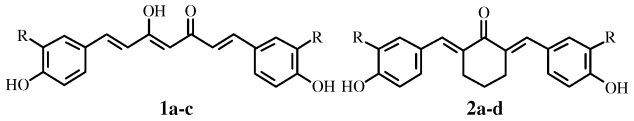


Figure 3. Inhibitory effects of compounds **1a**−**c** and **2a**−**d** on p300 activity. (A) Upper panel: autoradiography of a gel showing acetylated histones following incubation with p300 and ^{14}C acetyl-CoA in the presence of 400 μM of each indicated derivative, Lys-CoA (last lane) or DMSO (first lane). Lower panel: Coomassie blue staining of the same gel showing the total amounts of histones. (B) Upper panel: autoradiography of a gel showing acetylated histones following incubation with p300 in the presence of the indicated concentration of **2c** or DMSO alone (first lane). (C) Dose response curves obtained by densitometric analysis of the levels of histone acetylation mediated by p300 in the presence of **1a**−**c** and **2a**−**d**. The graph summarizes the results obtained from three independent experiments.

Table 1. Inhibitory Activity of Compounds **1a**−**c** and **2a**−**d** against p300 Enzyme



1a-c **2a-d**

cmpd	R	IC_{50}[a]
1a	OH	46 ± 3.9
1b	COOH	21 ± 8.7
1c	Br	33 ± 5.2
curcumin	OCH_3	>400
2a	OH	233 ± 120
2b	COOH	168 ± 12
2c	Br	5 ± 1.3
2d	OCH_3	111 ± 45
Lys-CoA		30 ± 1.6

[a] Inhibitory concentration of 50% (μM) determined from dose−response curves. Data represent the mean values of at least three separate experiments.

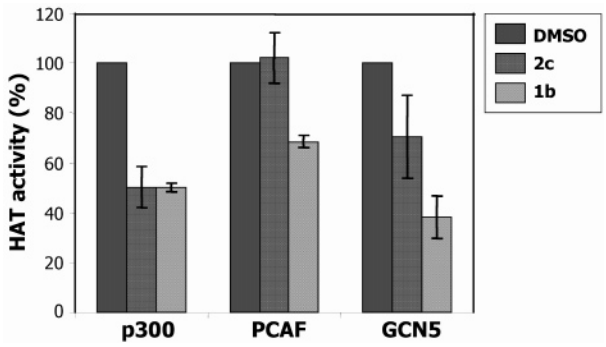


Figure 4. Inhibitory activities of derivatives **2c** (5 μM) and **1b** (21 μM) on different HATs were tested in in vitro assays using equal molar amounts of p300, PCAF, and GCN5. HAT activity for each enzyme is expressed as percent variation as compared to that of the DMSO-treated sample. The graph summarizes the mean densitometric values from three independent experiments (mean ± standard error).

in this series decreased if the electron-withdrawing groups (COOH, Br) were replaced by electron-donor groups (OH, OCH_3). The following order, depending on substituents in the 3-positions of the aromatic rings, was observed: COOH > Br > OH > OCH_3.

The cyclohexanone derivatives **2a**−**d** were active against p300 as well. The IC_{50} values obtained in the enzyme assays ranged from 5 to 233 μM. The activities of compounds **2a**−**d** decreased based on the substituents in the 3-positions of the aromatic rings in the following order: Br > OCH_3 > COOH > OH. In conclusion, the replacement of hydrophilic groups (COOH, OH) with the lipophilic ones (Br, OCH_3) in the **2a**−**d** series led to increased anti-p300 activities. In particular, the highest potency was obtained with the introduction of the lipophilic and electron-withdrawing bromine atom on the cinnamoyl portion.

The preliminary SARs in the series of cyclohexanone derivatives (compounds **2a**−**d**) were different if compared with those found in the curcumin series (compounds **1a**−**c** and curcumin). A direct comparison among the two series led to the following conclusions: (i) compounds **2a**−**d** were generally less potent than **1a**−**c** derivatives, showing IC_{50} values from 111 to 233 μM, with the exception of **2c**, which was the most potent derivative described in this work (IC_{50} = 5 μM); (ii) introduction of bromine atoms in the 3 position of the benzene rings gave derivatives **1c** and **2c**, which were both endowed with good activities; and (iii) introduction of OH or COOH groups in the same positions within the curcumin series gave compounds **1a** and **1b**, which showed good anti-p300 potency; opposite results were found when the same groups were introduced in the 3 position of benzene rings in the 2,6-bis-arylidene cyclohexanone series (**2a**, IC_{50} = 233 μM; **2b**, IC_{50} = 168 μM).

Derivatives **2c** and **1b**, which showed the highest inhibitory activity, were additionally tested on PCAF and GCN5, both belonging to a different class of HAT factors. The assays were performed using concentrations of **2c** and **1b** corresponding to the IC_{50} values formerly determined against p300 (5 μM and 21 μM for **2c** and **1b**, respectively; Table 1). As expected, the activity of p300 was reduced to 50% with both compounds, while the same concentration of derivative **2c** showed no effect on PCAF (100%) and only partial inactivation of GCN5 (70%), indicating a selective inhibition of p300 activity. Conversely, **1b** is partially active on PCAF (68%) and shows on GCN5 (38%) the same efficacy as for p300, indicating that this compound is active on HATs other than p300 (Figure 4).

Several previously described HAT inhibitors, such as Lys-CoA, are not cell permeable and cannot thus be used for in vivo studies. Therefore, we have tested for its anti-acetylase activity in cell culture system derivatives **1b** and **2c**, which showed the most potent inhibitory effect against p300. HeLa cells stably expressing fluorescent H2B histones (HeLa−H2B-EYFP)[18] were treated with various concentrations of **2c** and **1b** and subsequently immunostained with antibodies against acetylated H3 histones. The fluorescent H2B histones were used as internal control to monitor protein expression levels. Derivative **1b** at concentrations up to 200 μM did not alter either the H3 acetylation levels or the H2B protein expression. This experiment led us to hypothesize that **1b** is not cellular permeable (data not shown). Conversely, we found that at 20 μM and 40

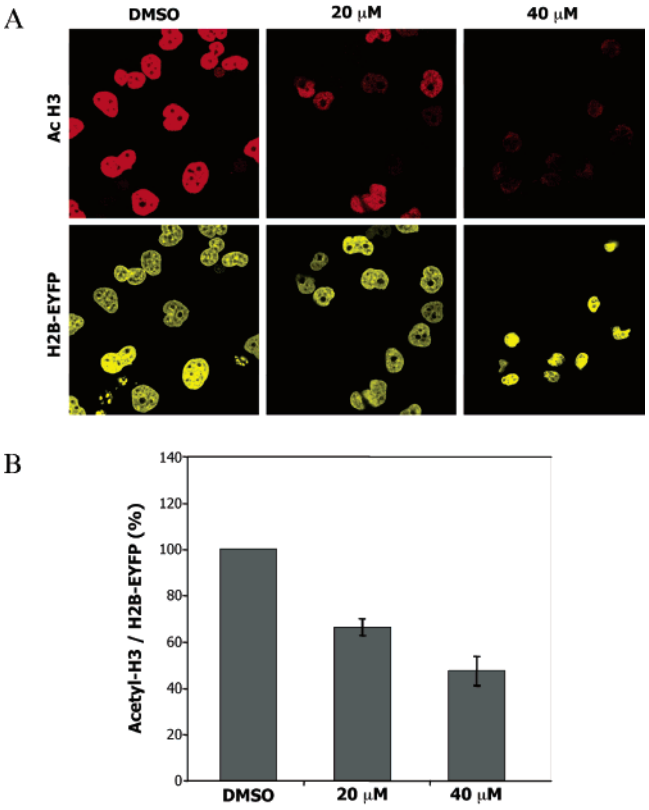


Figure 5. (A) HeLa—H2B-EYFP cells treated with derivative **2c** (20 μM or 40 μM) or DMSO were immunostained with antibodies anti-acetyl H3 and analyzed with appropriate wavelengths to visualize acetylated H3 or H2B-EYFP total protein levels. (B) The percent inhibition of histone H3 acetylation was obtained by measuring the mean fluorescence intensity with anti-acetyl H3 antibodies relative to the mean fluorescence intensity values of H2B-EYFP from the same cells. The graph summarizes data obtained from three independent experiments. Mean and standard error were derived analyzing 150 cells in each experiment.

μM of **2c** the levels of H3 acetylation decreased, while the H2B expression remained unaltered, indicating specificity of anti-acetylase treatment (Figure 5A).

Concentrations lower than 20 μM did not have any effect on the acetylation levels, while over 40 μM cell toxicity was observed as indicated by decreased H2B-EYFP fluorescence (data not shown). Interestingly, the effect of **2c** is not homogeneous in cell culture. Indeed, a high percentage of cells (estimated around 24%) showed no detectable H3 acetylation even though the H2B expression remained unaltered (Figure 5A, cell in the upper-center in the middle panels as a representative image). Finally, we observed that **2c** determined an overall increase of H2B-EYFP fluorescence intensity. This observation is indicative of decreased histone acetylation that results in chromatin condensation. This effect was visualized by increased fluorescence of the exogenously expressed histones as previously reported in similar cellular systems.[19,20] To quantify the different levels of histone H3 acetylation, the average fluorescence intensity obtained from the immunostaining with antibodies against acetylated H3 was measured and normalized with values obtained in the same cells with fluorescent histones H2B-EYFP. Results summarized in Figure 5B indicate that the acetylation levels were 30% reduced in cells treated with 20 μM of **2c**, and a reduction higher than 50% was observed using 40 μM as compared to that of DMSO control cells.

Similar results were obtained in parallel where the H3 acetylation level was normalized with the level of expression of the nuclear lamina in HeLa cells not expressing H2B-EYFP (data not shown).

Conclusions

In conclusion, herein we reported a new class of small synthetic molecules that inhibited the p300 activities in in vitro assays. In particular, we described the discovery of cinnamoyl compounds **1a**−**c** and **2a**−**d** as inhibitors of the p300 enzyme. Among them, derivative **2c** was proven the most potent anti-p300 agent, which was six times more active than Lys-CoA used as a reference drug and with a high selectivity for p300, as demonstrated by comparative assays performed with different HATs belonging to another family of enzymes. Most notably, derivative **2c** was active in mammalian cells, as demonstrated by the downregulation of histone H3 acetylation. For all the above-mentioned reasons, derivative **2c** might be considered a lead compound for further studies in this field.

Extensive SARs, as well as molecular modeling studies, are ongoing to increase the knowledge within these series of p300 inhibitors. Due to the vital role of p300 in the reversible processes of acetylation of histones and other cellular proteins, the development of these inhibitors might result in novel approaches to antitumor and antiviral chemotherapies.

Experimental Section

Chemistry. General. Melting points were determined with a Büchi 530 capillary apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Spectrum-one spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker AC 400 spectrometer, using tetramethylsilane (Me_4Si) as an internal standard. All compounds were routinely checked by TLC and ^{1}H NMR. TLC was performed by using aluminum-baked silica gel plates (Fluka F_{254}) and aluminum-baked aluminum oxide plates (Fluka F_{254}). Concentration of solutions after reactions and extractions involved the use of a rotatory evaporator operating at a reduced pressure of approximately 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. The microwave reactions were performed in a Discover CEM, which produced controlled irradiation with a power of 0−300 W.

Syntheses. Specific examples presented below illustrate general synthetic procedures.

1,7-Bis(3-bromo-4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (1c). A solution of 3-bromo-4-hydroxybenzaldehyde (1.0 g, 5.0 mmol) and acetylacetone (250 mg, 2.5 mmol) in *N*,*N*′-dimethylformamide (0.5 mL) was treated with boric acid (490 mg, 8.0 mmol), and the mixture was heated at 100 °C for 5 min. After this time, a solution of 1,2,3,4-tetrahydroquinoline (0.5 mL, 530 mg, 4.0 mmol) and acetic acid (0.15 mL) in *N*,*N*′-dimethylformamide (0.5 mL) was added. The resulting mixture was heated at 100 °C for 1.5 h, then cooled, diluted with 20% acetic acid (25 mL), and stirred at room temperature for 1 h. The precipitate that formed was extracted with ethyl acetate (3 × 50 mL), and the organic extracts were collected, washed with brine (3 × 100 mL), and dried. Evaporation of the solvent gave crude product, which was chromatographed on a silica gel column (chloroform/methanol, 20:1, as eluent) to obtain pure **1c** (760 mg, 65% yield); mp 175−176 °C (isopropanol/isopropyl ether). Anal. ($C_{19}H_{14}BrO_4$) C, H, Br. This procedure was used for the synthesis of compounds **1b** starting from 5-formylsalicylic acid. **1b**: 50%; mp >270 °C (dioxane). Anal. ($C_{21}H_{16}O_8$) C, H.

2,6-Bis(3-bromo-4-hydroxybenzylidene)cyclohexanone (2c). 3-Bromo-4-hydroxybenzaldehyde (300 mg, 1.5 mmol) was dissolved in MeOH and treated with montmorillonite K-10 (600 mg). Evaporation of the solvent gave a dispersion that was placed in a 5 mL glass tube and treated with cyclohexanone (75 mg, 0.75 mmol). The vessel was sealed with a septum and placed into the microwave cavity. Microwave irradiation of 100 W was used, the temperature being ramped from room temperature to 100 °C. Once

100 °C was reached, the reaction mixture was held at this temperature for 5 min. The reaction vessel was opened, and the mixture was diluted with methanol and filtered. Evaporation of the solvent gave crude product, which was chromatographed on a silica gel column (chloroform/methanol, 20:1, as eluent) to obtain pure **2c** (310 mg, 89% yield); mp 201−202 °C (isopropanol/water). Anal. ($C_{20}H_{16}Br_2O_3$) C, H, Br. This procedure was used for the synthesis of compounds **2a**, **2b**, and **2d** starting from 3,4-dihydroxybenzaldehyde, 5-formylsalicylic acid, and 4-hydroxy-3-methoxybenzaldehyde, respectively. Yield, mp, and recrystallization solvent are reported for each compound. **2a**: 75%, 244−246 °C, and methanol/water. **2b**: 50%, >270 °C, and DMF/water. Anal. ($C_{22}H_{18}O_7$) C, H. **2d**: 75%, 179−181 °C, and acetic acid.

Biological Assays. Acetylation Assay To Test the Efficacy of Curcumin Derivatives. To test the efficacy of derivatives **1a**−**c** and **2a**−**d**, the catalytic activity of p300 has been measured by an in vitro assay as previously reported.[17]

Acetylation Assays To Test the Efficacy of the Curcumin Derivatives in Mammalian Cells. HeLa cells, stably transfected with histones H2B fused to EYFP,[20] were cultured in 10% FCS DMEM. Histone H3 acetylation was analyzed as described in Supporting Information.

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Supporting Information Available: Spectroscopic data for derivatives **1b**,**c** and **2a**−**d**, elemental analyses for derivatives **1b**,**c** and **2b**,**c**, and biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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