Curcumin is an Inhibitor of p300 Histone Acetylatransferase

Monica G. Marcu[1], Yun-Jin Jung[1], Sunmin Lee[2], Eun-Joo Chung[2], Min-Jung Lee[2], Jane Trepel[2], and Len Neckers[1,*]

[1]*Urologic Oncology Branch and* [2]*Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA*

Abstract: Histone acetyltransferases (HATs), and p300/CBP in particular, have been implicated in cancer cell growth and survival, and as such, HATs represent novel, therapeutically relevant molecular targets for drug development. In this study, we demonstrate that the small molecule natural product curcumin, whose medicinal properties have long been recognized in India and Southeast Asia, is a selective HAT inhibitor. Furthermore the data indicate that α, β unsaturated carbonyl groups in the curcumin side chain function as Michael reaction sites and that the Michael reaction acceptor functionality of curcumin is required for its HAT-inhibitory activity. In cells, curcumin promoted proteasome-dependent degradation of p300 and the closely related CBP protein without affecting the HATs PCAF or GCN5. In addition to inducing p300 degradation curcumin inhibited the acetyltransferase activity of purified p300 as assessed using either histone H3 or p53 as substrate. Radiolabeled curcumin formed a covalent association with p300, and tetrahydrocurcumin displayed no p300 inhibitory activity, consistent with a Michael reaction-dependent mechanism. Finally, curcumin was able to effectively block histone hyperacetylation in both PC3-M prostate cancer cells and peripheral blood lymphocytes induced by the histone deacetylase inhibitor MS-275. These data thus identify the medicinal natural product curcumin as a novel lead compound for development of possibly therapeutic, p300/CBP-specific HAT inhibitors.

Key Words: Curcumin, p300/CBP, acetylation, Michael reaction acceptor.

INTRODUCTION

The posttranslational modification of core histones by acetylation and deacetylation, regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), has a profound impact on gene expression. Further, acetylation-dependent regulation of an increasing number of non-histone proteins has been demonstrated, including p53, tubulin, and heat shock protein 90 [1,2]. Dysfunction of HATS and HDACs is frequently associated with cancer. In particular, the p300/CBP family of HATs has been implicated in interleukin 6-mediated, androgen-independent transcriptional activity of the androgen receptor in hormone-refractory prostate cancer cells [3], and in cyclin E-dependent growth of melanoma cells [4]. Thus, in certain contexts, HAT inhibition may represent a novel approach to inhibit cancer growth and/or survival. Although there has been a dramatic increase in interest in HDACs as targets for anti-cancer drug development, there are few examples in the literature of small molecule HAT inhibitors.

Both HDACs and HATs comprise multi-protein families [5]. HATs can be divided into several clusters, of which PCAF/GCN5 and p300/CBP proteins are the most well-studied. Unlike the HDACs, for which there are now six structurally diverse classes of inhibitors and at least nine inhibitors in clinical trial, there are only three HAT inhibitors reported and none in clinical trial. Two peptide-coenzyme A conjugates, Lysyl CoA (Lys-CoA) and H3-CoA-20, which inhibit the HAT activity of p300 and PCAF respectively, have been described [6,7]. Both compounds are cell-impermeant and therefore cannot be used as anticancer therapeutics. One small molecule HAT inhibitor has been reported, the natural product anacardic acid, which inhibits the HAT activity of both p300 and PCAF [8]. Identification or design of additional small molecule HAT inhibitors, especially those that can distinguish among the various HAT proteins, will allow the exploration of this novel molecular target.

Curcumin is a component of turmeric, a yellow spice widely used as a food flavoring and coloring agent. The medicinal properties of curcumin have long been recognized in India and Southeast Asia, and experimental studies have shown the molecule to have anti-inflammatory properties, to prevent tumorigenesis, and to inhibit angiogenesis [9,10]. The anti-oxidant and anti-tumor effects of the drug have increased interest in its use in cancer prevention, especially since it does not appear to possess significant toxicity [11].

In this study, we identify curcumin as a specific inhibitor of the p300/CBP family of HAT proteins, with no activity toward the PCAF/GCN5 HATs. We found curcumin to directly inhibit the enzymatic activity of p300 *in vitro*, while promoting its proteasome-mediated degradation in intact cells. Finally, we have examined curcumin's mechanism of action, and provide evidence that the HAT-inhibitory activity of curcumin both *in vitro* and *in vivo*, requires its covalent association with p300 and CBP, and furthermore that this association is mediated by its Michael reaction acceptor functionality. Identification of curcumin as a specific p300/

*Address correspondence to this author at the Urologic Oncology Branch and Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA; Tel: 301-496-5899; Fax: 301-402-0922; E-mail: neckers@nih.gov

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CBP HAT inhibitor provides both a useful pharmacologic tool for investigating the role of p300/CBP in cancer, and a novel therapeutic lead compound with possible clinical potential.

RESULTS

Curcumin Inhibits Histone Acetylation *In Vivo*

We monitored histone H3 acetylation in untreated PC3-M cells and in cells treated with the HDAC inhibitor MS-275 [12] and/or curcumin (Fig. **1A**). The histone hyperacetylation readily observed following a 6 h exposure to MS-275, was almost fully abrogated in the presence of curcumin (Fig. **1A**, bar graph).

We next examined the effects of curcumin on histone acetylation in peripheral blood lymphocytes (PBL). In PBL, histone H3 acetylation was induced more than 7-fold by 6 h exposure to MS-275. Similar to the data obtained using PC3-M cells, simultaneous incubation with curcumin reduced MS-275-induced histone hyperacetylation by approximately 50 percent (Fig. **1B**).

Curcumin Regulates p300/CBP Proteins Post-Translationally by Promoting Their Proteasome-Dependent Degradation

Given the apparent inhibitory effect of curcumin on histone acetylation, we next examined curcumin effects on the steady-state level of several endogenous HATs including p300, CBP, PCAF and GCN5. SKBr3 cells were exposed to increasing concentrations of curcumin for 8 h, after which time protein lysates were analyzed by western blotting (Fig. **2A**, **B**). The data show that while the steady-state levels of

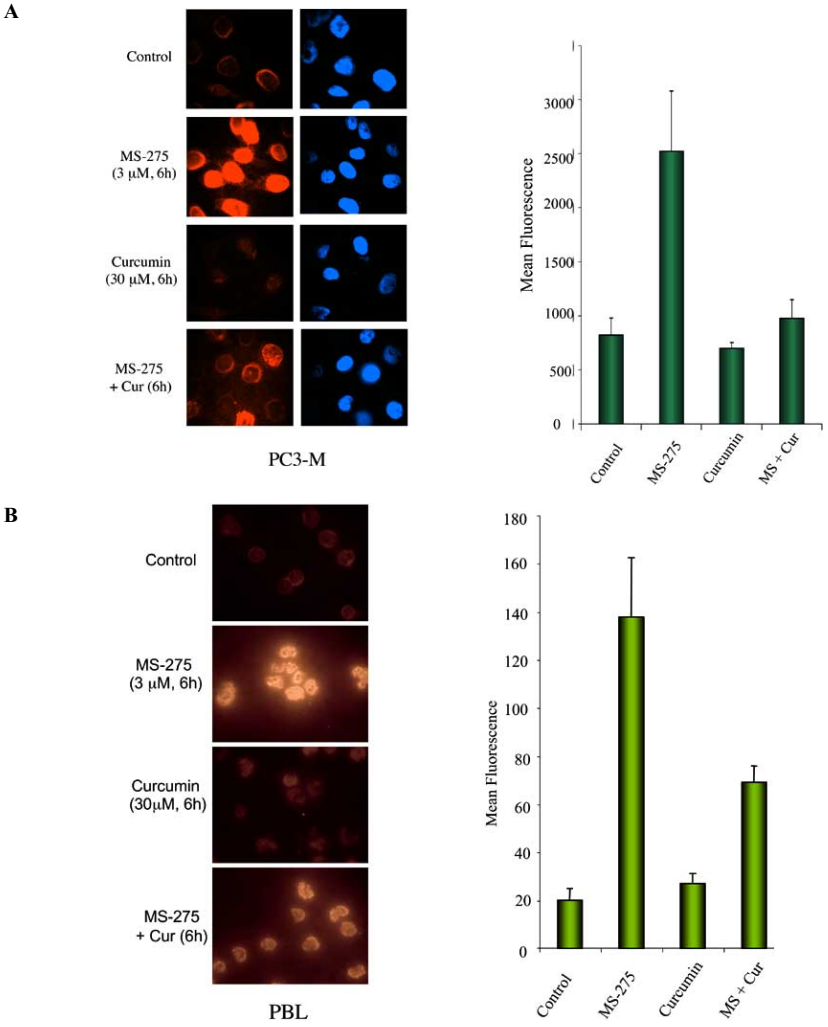

Fig. (1). Curcumin prevents histone hyperacetylation in response to HDAC inhibitor in normal and tumor cells *in vitro*. A. PC3-M prostate cancer cells were exposed to the HDAC inhibitor MS-275 alone or together with curcumin for 6 h, and acetylated histones were visualized by immunocytochemistry. B. Peripheral blood lymphocytes were treated and analyzed as in A.

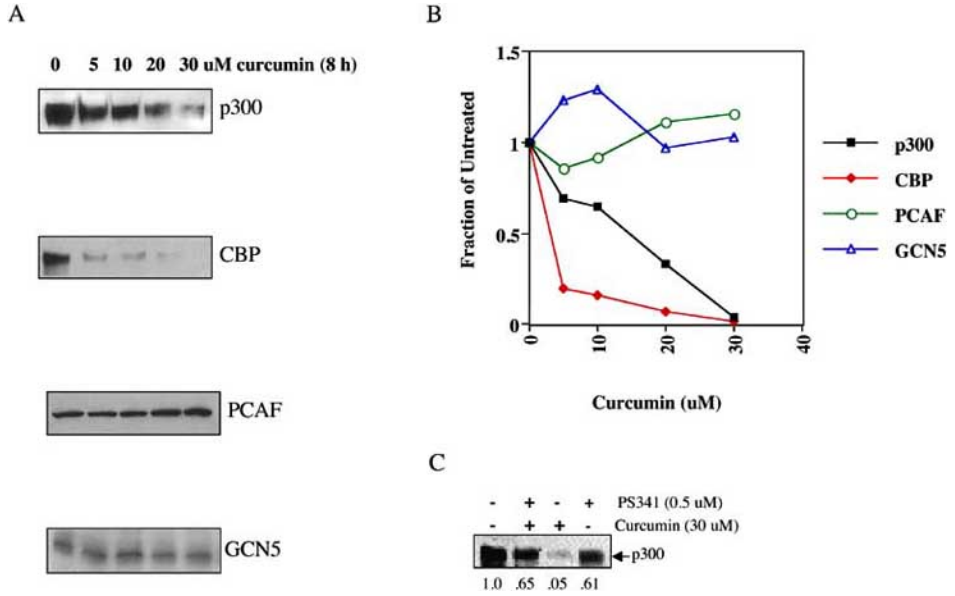

Fig. (2). Curcumin promotes proteasome-dependent degradation of p300/CBP. A. SKBR3 breast cancer cells were exposed to various curcumin concentrations for 8 h, at which time cells were lysed and equivalent amounts of cellular protein analyzed by western blotting for p300, CBP, PCAF, and GCN5 expression. B. A graphical plot of the data in panel A. C. Co-incubation of SKBR3 cells with curcumin and the proteasome inhibitor PS341 antagonizes the effect of curcumin alone on steady-state level of p300 protein.

p300 and CBP declined in response to curcumin in a dose-dependent manner, neither PCAF nor GCN5 levels were affected, at curcumin concentrations up to 30 μM.

These data demonstrate that within 8 h of exposure to 30 μM curcumin, both p300 and CBP proteins were reduced to undetectable levels. Meanwhile, real time RT-PCR analysis revealed a uniform and minimal effect on the mRNA level of all 4 HATs after 8 h exposure to 30 μM curcumin (data not shown). Given these data, we suspected that the loss of p300 and CBP proteins in response to curcumin was not transcription-dependent. Since p300 has been shown to be targeted to the proteasome in response to the HDAC inhibitor sodium butyrate [13], we tested whether the effects of curcumin were similarly proteasome-dependent. Indeed, as shown in Fig. **2C**, the presence of the proteasome inhibitor PS-341 significantly attenuated the loss of p300 in response to curcumin.

Curcumin Inhibits HAT Activity of Purified p300 *In Vitro*

Taken together, these data suggest that, when added to intact cells, curcumin is able to inhibit histone H3 acetylation by promoting proteasome-dependent degradation of p300 and CBP. Examination of curcumin structure suggested that the α, β unsaturated carbonyl group in the curcumin side chain may function as a Michael reaction site, and thus that curcumin may bind to available cysteines in p300/CBP and directly inhibit its HAT activity [14]. In order to determine whether curcumin directly inhibits p300 HAT activity, we carried out *in vitro* acetylation reactions using purified p300 as enzyme source and either purified histones or recombinant p53 as protein substrate. In some cases, p300 was pre-incubated for 30 minutes with either increasing concentrations of curcumin (10 – 100 μM) or with the specific p300 inhibitor Lys-CoA. After 1 h incubation, reaction products were separated by SDS-PAGE and [^{14}C]acetyl-histone or [^{14}C]acetyl-p53 were detected by autoradiography of dried gels (Fig. **3A**). The data show a dose-dependent inhibition of both histone and p53 acetylation, with maximal inhibition occurring between 40 and 100 μM curcumin. For comparison, 20 μM Lys-CoA was maximally inhibitory in this assay. Given these findings, we examined the effect of pre-incubating purified p300 with 80 μM curcumin on the rate of [^{14}C]acetyl-histone formation, using a scintillation-based filter binding assay (Fig. **3B**). Based on a comparison of the slopes of the 2 curves, pre-incubation of p300 protein with 80 μM curcumin inhibited the rate of histone acetylation by nearly 70 percent.

Curcumin Covalently Binds to CBP and p300 Proteins

The data thus far suggest that curcumin can directly inhibit p300 HAT activity *in vitro* while targeting p300 and CBP (but not PCAF or GCN5) proteins to the proteasome in intact cells. In light of these observations, and since curcumin contains 2 Michael acceptor motifs (see Fig. **4B**), we speculated that curcumin may covalently associate with p300 and CBP, causing enzyme inhibition *in vitro* and promoting recognition by proteasome targeting enzymes *in vivo*. To begin to test this hypothesis, we incubated [^{3}H]curcumin with purified p300, as well as with CBP and PCAF that had been immunoprecipitated from SKBr3 cells. After a 1 h incubation with labeled curcumin, the purified or immunoprecipitated HATs were separated by SDS-PAGE and the presence of radiolabel was detected by autoradiography (Fig. **4A**). A radioactive band corresponding to both p300 and CBP was readily detectable, while we were unable to detect radioactive labeling of immunoprecipitated PCAF protein, even though PCAF and CBP are expressed at

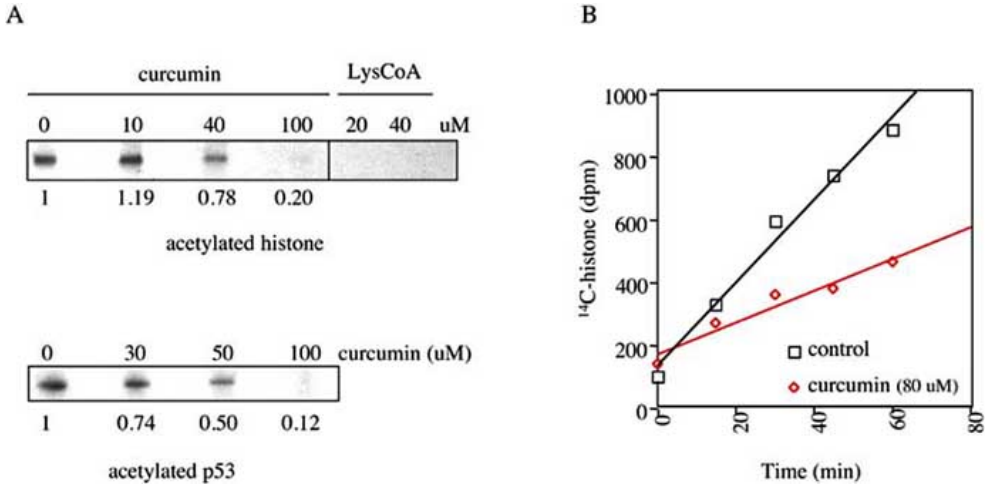

Fig. (3). Curcumin inhibits the acetyltransferase activity of purified p300 protein. A. Incubation of purified p300 protein with increasing concentrations of curcumin inhibits the acetylation of both purified histones and p53. The specific p300 inhibitor Lys-CoA is included as a positive control for acetyltransferase inhibition. B. Inhibition of p300-dependent histone acetylation upon pre-incubation of p300 with 80 μM curcumin.

approximately equivalent levels in SKBr3 cells (see Fig. **2A**). Thus, we were able to establish a direct correlation between the ability of curcumin to inhibit HAT activity *in vitro* and promote HAT degradation *in vivo*, and the drug's ability to associate with the enzyme *in vitro*.

Given these data, we wished to test whether the Michael acceptor activity of curcumin was required for its effects on p300. Curcumin contains 2 Michael reaction acceptor functionalities that are not present in tetrahydrocurcumin. For comparison the structures of curcumin and tetrahydrocurcumin are shown in Fig. **4B**. We examined whether the ability of curcumin to promote the degradation of p300 in SKBr3 cells depended on its Michael reaction acceptor activity by comparing the effects of tetrahydrocurcumin with those of curcumin on p300 steady-state level. The data in Fig. **4C** demonstrate that, indeed, tetrahydrocurcumin was unable to induce loss of p300 at concentrations at which curcumin itself was highly effective. Further, tetrahydrocurcumin, at concentrations up to 100 μM, did not affect the *in vitro* acetylation activity of purified p300 toward either histone or p53 (data not shown).

DISCUSSION

The data in this report identify curcumin, a medicinal natural product and major constituent of turmeric, as an inhibitor of the acetyltransferase activity of p300. While curcumin directly interacts with p300 and CBP proteins *in vitro* and promotes their proteasome-dependent degradation *in vivo*, the drug neither interacts with nor affects the stability of the HATs PCAF and GCN5. Nonetheless, curcumin is able to block HDAC inhibitor-induced histone hyperacetylation in both normal peripheral blood lymphocytes and PC3-M androgen-independent prostate cancer cells. The drug also effectively inhibits the ability of purified p300 to acetylate both histones and p53 *in vitro*. Our observation that tetrahydrocurcumin has no activity toward p300, either *in vitro* or *in vivo*, is consistent with the hypothesis that curcumin's Michael acceptor functionality mediates direct alkylation of p300, which is ultimately responsible both for inhibiting enzyme activity and marking the protein for proteasomal degradation. While this hypothesis is not yet proven, preliminary proteolysis studies with labeled drug suggest that curcumin covalently modifies multiple residues in p300. Positive identification of these interacting residues is continuing.

Our data identify curcumin as an exciting lead compound for development of novel, small molecule HAT inhibitors. While much emphasis has been placed on identification and characterization of HDAC inhibitors HATs have been somewhat overlooked in this regard. Nevertheless, HAT inhibitors may have contextual anti-cancer activity. Thus, over-expression of dominant-negative p300, or treatment of permeabilized cells with the specific p300 inhibitor Lys-CoA, down-regulates cyclin E, inhibits growth, and triggers a senescence checkpoint in melanoma cells [4]. Further, down-regulation of p300 with siRNA has been reported to inhibit androgen-independent activation of the androgen receptor [3], a key component of hormone-refractory prostate cancer. Intriguingly, our data demonstrate dramatic inhibition by curcumin of histone acetylation in the androgen-independent prostate cancer cell line PC3-M. Recently Horvath and colleagues demonstrated that interferon-induced gene expression is dependent on HDAC activity and is potently suppressed by HDAC inhibitors [15]. This is of particular interest in light of several studies that have shown that malignant transformation of normal prostatic epithelial cells and prostate cancer progression are associated with inhibition of interferon-activated gene expression. These data support the hypothesis that in certain settings HATs rather than HDACs may be an appropriate therapeutic target.

To our knowledge, anacardic acid is the only other non-peptide based, natural product inhibitor of p300 that has been described [8]. However, unlike curcumin, it does not discriminate between p300 and PCAF, inhibiting both HATs

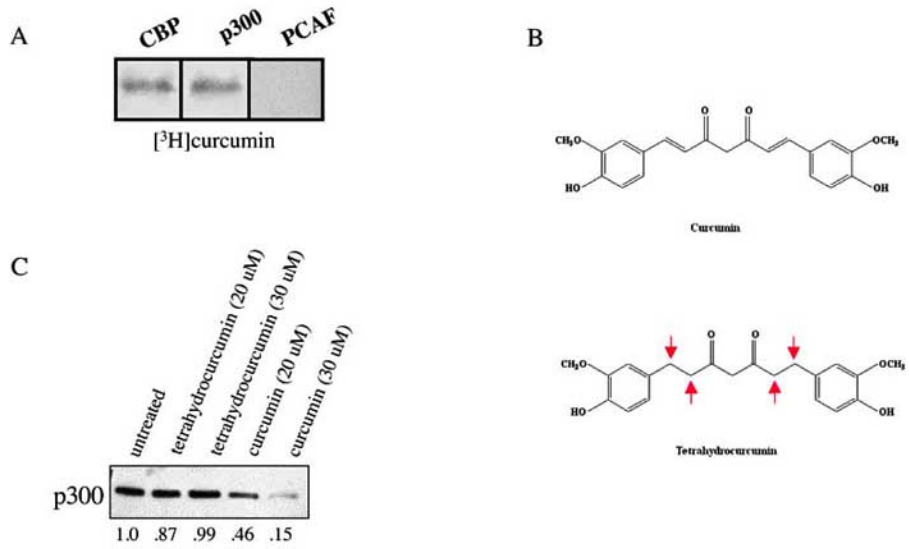

Fig. (4). Labeled curcumin binds covalently to p300 and CBP. A. Direct interaction of labeled curcumin with p300 and CBP, but not with PCAF, is demonstrated in panel A. Structures of curcumin and tetrahydrocurcumin are shown in panel B. The arrows identify sites of reduction in tetrahydrocurcumin. Tetrahydrocurcumin does not promote depletion of p300 when added to SKBR3 cells (panel C). In this experiment, curcumin is included as a positive control.

with equal potency (IC_{50} = 5-10 μM). Thus, curcumin and its congeners should prove to be useful reagents for the specific, pharmacologic manipulation of p300 activity in tumor cells. The micromolar potency of the parent compound, coupled with its lack of toxicity *in vivo* and the importance of its Michael acceptor functionality, suggest that more active, well-tolerated p300 inhibitors can be synthesized.

MATERIALS AND METHODS

Cell Culture

SKBr3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, and 1% Fungizone (Bio Whittaker, Walkersville, MD). For immunoprecipitation studies and Western analysis, cells were lysed with cold TNESV buffer (50 mM TRIS-HCl, pH 7.4, 1% Nonidet P-40, 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate) or HEPES buffer (10 mM HEPES, 150 mM potassium acetate, 1 mM EDTA, 0.1 % NP40) containing protease inhibitors. For nuclear extraction procedure, cells were washed with cold PBS (phosphate buffer saline) and lysed in cytosolic Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, protease inhibitors). After centrifugation, the pellets were resuspended in Buffer B (nuclear extract buffer: 20 mM Hepes, pH 7.9, 0.4 M NaCl; 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitors).

Western Blotting and Immunoprecipitations

Immunoprecipitation of p300 and CBP was performed by using specific antibodies (Pharmingen, Los Angeles, CA and Santa Cruz, CA) and Protein G beads (Invitrogen, CA). Briefly, soluble proteins were immunoprecipitated with 3 μg primary antibody, immune complexes were bound on protein G-Sepharose beads for 6 h at 4 °C (with rotation). The beads were washed 4 times with buffer, and bound protein was either subjected to binding experiments, or resolved by 5% SDS-PAGE and transferred to nitrocellulose membranes for western blot analysis. Western blotting was performed using standard procedures. Antigen-antibody complexes were detected by horseradish peroxidase-enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL).

Drugs and Chemicals

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl) –1,6-heptadiene-3,5-dione] was purchased from LKT (St. Paul, MN). PS-341 was obtained from Millenium Pharmaceuticals (Cambridge, MA). Lysine Coenzyme A (Lys-CoA) was a gift from Dr. P.A Cole, Johns Hopkins University, Baltimore, MD. Radiolabeled curcumin ([^{3}H], specific activity = 20 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA). To obtain tetrahydrocurcumin, curcumin (500 mg) and Pd/C powder (100 mg, palladium coated on carbon powder, Sigma, St. Louis, MO) were suspended in ethanol (50 ml) and allowed to react under hydrogen (50 psi) in a pressure reactor (model 4562, Parr Instrument Co., Moline, IL) for 1 hr. After filtration, the filtrate was evaporated to yield tetrahydrocurcumin. Formation of tetrahydrocurcumin was monitored by infrared spectroscopy (Bomem, Quebec, Canada) and H1-NMR (Varian, Inc., Palo Alto, CA).

In Vitro Histone Acetyltransferase (HAT) Assay

Purified calf thymus histones type III-SS were purchased from Sigma (St Louis, MO). The acetylation assay was performed in a total volume of 30 μl acetylation buffer (20 mM HEPES-NaOH, pH 7.4, 1 mM DTT, 10 mM sodium butyrate). 1-2 μg histones were acetylated in the presence of 0.1-0.2 μg purified p300 (baculovirus-expressed, ProteinOne, College Park, MD), 1 μl of [^{14}C] acetyl-Coenzyme A (60 mCi/mmol, Amersham, UK), with incubation for one hour at 37 °C. Curcumin (in DMSO) or Lys-CoA (in water) were

used for inhibition of the acetylation reaction, and were pre-incubated for 30 min with purified p300 protein. After one hour, labeled histones were resolved by SDS-PAGE, and gels were dried and exposed for autoradiography.

In Vitro p53 Acetylation

Purified p53 (1 μg, Protein One, College Park, MD) was incubated for one hour at 37 °C in acetylation buffer containing 0.2 μg purified p300 (ProteinOne, College Park, MD) and 1 μl of [^{14}C] acetyl-CoA (60mCi/mmol, Amersham, UK). Curcumin was pre-incubated for 30 min with p300, prior to initiation of the reaction. Acetylated p53 was analyzed by SDS-PAGE and autoradiography as above.

Curcumin-p300 /CBP Binding Assays

One μg of purified p300 (ProteinOne, College Park, MD) was incubated with 25 μCi [^{3}H] curcumin in acetylation buffer for one hour at room temperature. The reaction mixture was resolved by SDS-PAGE, and dried gels were exposed to x-ray film for visualization. In addition CBP and PCAF were immunoprecipitated from 50 x 10^6 SKBr3 cells, and processed as above.

Analysis of Histone Acetylation by Immunofluorescence

PC3-M prostate carcinoma cells were grown on sterile coverslips. Peripheral blood mononuclear cells were obtained from buffy coats provided anonymously as a byproduct of whole blood donations from paid healthy volunteer donors through an Institutional Review Board-approved protocol. Mononuclear cells were obtained by density centrifugation on Ficoll-Paque Plus (Amersham, Buckinghamshire, UK). Cells were incubated in the presence or absence of the histone deacetylase inhibitor MS-275 and/or curcumin, as indicated. After incubation for 6 hours, the cells were washed x 2 with PBS, fixed in 95% ethanol + 5% glacial acetic acid for 1 min, washed x 3 with PBS, incubated with blocking and permeabilizing solution (0.3% Triton X-100, 2% BSA in PBS) for 40 min, washed x 3 with PBS, incubated for 1 h with primary antibody (rabbit anti-acetylated histone H3, Upstate Biotechnology, Waltham, MA, dilution 1:500 in PBS). The coverslips were washed x 3 in PBS and incubated for 1 h with Cy3-conjugated goat anti-rabbit immunoglobulin (Molecular Probes, Eugene, OR, 1:200 dilution), washed x 3 with PBS, stained with DAPI for 10 min, washed x 1 with PBS, washed x 1 with H_20, air-dried, mounted using SlowFade (Molecular Probes), and imaged using a Zeiss Axioskop microscope interfaced with a CCD camera (Optronics Engineering, Goleta, CA) and analyzed using Openlab image analysis software (Improvision, Lexington, MA).

RNA Analysis

RNA was isolated using the RNeasy RNA Isolation Kit (Qiagen). For reverse transcription, 200 ng of RNA were used in a 20 μL reaction mixture containing 1X TaqMan RT buffer (TaqMan RT-PCR kit, Applied Biosystems), 5.5 mM magnesium chloride, 500 μM of each dNTP, 2.5 μM random hexamer, 0.4 U/μL RNase inhibitor, and 1.25U/μL of Multiscribe Reverse Trasnscriptase. Reverse transcription was performed as previously described using a PE 9700 thermal cycler (Applied Biosystems) [16]. Real-time PCR primers were designed using Primer Express Applications (Applied Biosystems), and reactions were carried out in an ABI Prism7700 Sequence Detection System (Applied Biosystems) as previously described [17]. Data were analyzed using the Sequence Detection application. Beta-actin was used as a normalizing control.

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