Characterization of novel inhibitors of histone acetyltransferases

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Abstract
Modification of proteins by histone acetyltransferases (HAT) or histone deacetylases plays an important role in the control of gene expression, and its dysregulation has been linked to malignant transformation and other diseases. Although histone deacetylase inhibitors have been extensively studied and several are currently in clinical trials, there is little information available on inhibitors of HATs (HATi). Starting from the natural product lead HATi anacardic acid, a series of 28 analogues was synthesized and investigated for HAT-inhibitory properties and effects on cancer cell growth. The compounds inhibited up to 95% HAT activity in vitro, and there was a clear correlation between their inhibitory potency and cytotoxicity toward a broad panel of cancer cells. Interestingly, all tested compounds were relatively nontoxic to nonmalignant human cell lines. Western blot analysis of MCF7 breast carcinoma cells treated with HATi showed significant reduction in acetylation levels of histone H4. To directly show effect of the new compounds on HAT activity in vivo, MCF7 cells were cotransfected with the p21 promoter fused to firefly luciferase and a full-length p300 acetyltransferase, and luciferase activity was determined following treatment with HATi. Significant inhibition of p300 activity was detected after treatment with all tested compounds except one. Effects of the new HATi on protein acetylation and HAT activity in vivo make them a suitable tool for discovery of molecular targets of HATs and, potentially, for development of new anticancer therapeutics. [Mol Cancer Ther 2007;6(9):2391–8]

Introduction
Two classes of enzymes, histone acetyltransferases (HAT) and histone deacetylases (HDAC), are involved in chromatin structure modifications via acetylation and deacetylation of proteins, respectively. Although precise functions of these proteins in the cells remain to be defined, it is clear that they are involved in important signaling pathways, and their loss-of-function or gain-of-function can lead to severe consequences for the cell and the entire organism, including malignant transformation (for a recent review, see ref. 1). Acetylation and deacetylation of histones have emerged as the key mechanisms regulating transcriptional activity. HATs catalyze transfer of acetyl groups to NH2-terminal lysine residues in histones, which results in more open conformation of nucleosomes and increased accessibility of regulatory proteins to DNA, whereas HDAC activity causes chromatin condensation and transcriptional repression (2). The acetylation status of several non-histone proteins [p53, ataxia-telangiectasia mutant (ATM), heat shock protein 90, and α-tubulin] is intimately related to their functions. Reversible acetylation of α-tubulin marks stabilized microtubule structures and may contribute to regulating microtubule dynamics (3). Acetylation of lysines at the COOH terminus of p53 tumor suppressor protein by p300/CAMP-responsive element binding protein (CBP) and CBP-associated factor (PCAF) was linked to its transactivation potential and ability to regulate cell cycle arrest and apoptosis, as well as to its stability and subcellular localization (4–7). Recently, PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumor suppressor protein was shown to interact physically and functionally with PCAF (8). This interaction resulted in inhibition of lipid phosphatase activity of PTEN, essential for its ability to block the cell cycle in the G1 phase and consequently to suppress tumor formation and progression. Furthermore, several HATs were found to be genetically altered in a variety of cancers (reviewed in ref. 9). Therefore, both protein acetylation and deacetylation pathways present attractive targets for therapeutic intervention.

HDAC inhibitors have been extensively studied as potential anticancer treatment, several are currently in clinical trials (for a recent review, see ref. 10), and Vorinostat (suberoylanilide hydroxamic acid) has been approved by the Food and Drug Administration for the
Characterization of Novel HATi

Materials and Methods

Materials

Anacardic acid was purchased from EMD Biosciences, HeLa nuclear extract was obtained from BIOMOL, and trichostatin A was from Sigma. Antibodies that recognize acetylated α-tubulin were from Sigma, acetylated histone H4 was from Upstate Biotechnology, and glyceraldehyde-3-phosphate dehydrogenase was (GAPDH) from Trevigen. 3-phosphate dehydrogenase was (GAPDH) from Trevigen.

Synthesis

Schemes, methods of synthesis for the new compounds, and spectroscopic data for selected inhibitors are presented in the Supplementary Data. Purity and identity was established for all compounds using mass spectrometry, nuclear magnetic resonance spectrometry, and elemental analyses (for carboxylic acids).

Cell Culture

Cell lines were obtained from the American Type Culture Collection. MCF7, PC3, and Jurkat cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies/Invitrogen). HeLa cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37°C in an atmosphere of 5% CO2.

HAT Activity Assay In vitro

To measure HAT activity in vitro, HAT assay kit (Upstate Biotechnology) was used according to the manufacturer’s instructions. Briefly, 100 ng of biotinylated histone H4 peptide were added per well in streptavidin-coated 96-well microplates. After blocking with 3% bovine serum albumin, tested compounds diluted in DMSO were added to the wells at the indicated concentrations. Acetyl-coenzyme A (100 µmol/L) and HeLa nuclear extract (5 µL) were then added to a total assay volume of 50 µL. The plates were incubated at 30°C for ~8 min, then washed five times with TBS, and incubated with anti–acetyl-lysine antibodies for 1.5 h at room temperature. After incubation with horse-radish peroxidase–conjugated secondary antibodies, 100 µL of the substrate mixture were added to each well and incubated for 10 min at room temperature. The reaction was stopped with 50 µL of fresh 1 mol/L sulfuric acid, and the plates were read at 450 nm.

Cell Proliferation Assay

Cells were seeded at 5,000 per well (20,000 per well for Jurkat cells) in 80 µL of growing medium in 96-well tissue culture plates. At 24 h after seeding, diluted compounds or DMSO vehicle control were added to each well to a total volume of 100 µL (three replicates per concentration) and incubated for 48 h at 37°C. Growth inhibition was determined using One Aqueous Proliferation Assay [3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] (MTS) (Promega) according to the manufacturer’s instructions, and the absorbance was measured at 490 nm on a microplate reader. Data were plotted as percentage of DMSO-treated control against compound concentrations using GraphPad Prism 4.0. The 50% growth inhibition was calculated as the compound concentration required to reduce cell number by 50% compared with control.

Luciferase Reporter Assay

MCF7 cells were seeded at 6,000 per well in 70 µL phenol red–free growing medium in 96-well plates. At 24 h after seeding, cells were transfected using Lipofectin (Invitrogen), according to the manufacturer’s instructions. At 24 h after transfection, cells were treated with diluted compounds or DMSO control for indicated periods. Luciferase activity was determined using Bright-Glo Luciferase Assay (Promega) according to the manufacturer’s instructions. To normalize for the reduced cell numbers caused by drug treatment, MTS proliferation assay was conducted with the cells grown in the same plates. All the luciferase experiments were carried out at least thrice in triplicate.

Cell Extracts and Western Blot Analysis

Cells were seeded at 20,000 to 30,000 per well in 96-well tissue culture plates. At 24 h after seeding, cells were treated with diluted compounds or DMSO and incubated for 4 h at 37°C. Cells were washed once with fresh medium and treated with 200 nmol/L trichostatin A for an additional 1 h. Following this incubation, cells were washed twice with PBS and total protein extracts were prepared by adding loading buffer (Bio-Rad) directly to the wells. For Western blot analysis, proteins were separated in 4% to 20% SDS-PAGE gels and blotted onto Immobilon-P membrane (Millipore). Enhanced chemiluminescence detection (Amersham) was done according to the manufacturer’s instructions.
Results

Newly Synthesized Compounds Differentially Inhibit HAT Activity

Currently, a very limited number of drug-like HATi is available (17, 18), which also limits the evaluation of the biology of HATs. Prototypic inhibitors are garcinol (16), curcumin (19), isothiazolones such as 1 (11), and anacardic acid 2 (12). Starting from the natural product lead inhibitor 2, we synthesized a set of compounds that are composed of a series of substituted phenoxyacetic acid ethyl esters 3, their corresponding acids 4, the debenzylated derivatives 5, sulfonamide 6, amide 7, and analogues of 4 (Fig. 1). The synthesis is outlined in the Supplementary Data.

The structural analogues were derived from anacardic acid by introducing a second aromatic ring, changing the position of the alkyl chain and its length, and making various substitutions at the second aromatic ring. The ability to inhibit HAT activity was analyzed in vitro using HeLa nuclear extract as a source of HAT enzymatic activity. Commercially available HATi anacardic acid was used as

![Chemical structures of newly synthesized compounds](image-url)

**Figure 1.** Scheme of the compounds. The methods of synthesis for the new compounds and spectroscopic data for selected inhibitors were presented in the Supplementary Data. Purity and identity was established for all compounds using mass spectrometry, nuclear magnetic resonance spectrometry, and elemental analyses (for carboxylic acids).

For 3 and 4:
- a: \( R, R' = H, R'' = C_4H_9 \)
- b: \( R, R' = H, R'' = C_{10}H_{21} \)
- c: \( R = CH_3, R' = H, R'' = C_{10}H_{21} \)
- d: \( R = OCH_3, R' = H, R'' = C_{10}H_{21} \)
- e: \( R = Cl, R' = H, R'' = C_{10}H_{21} \)
- f: \( R, R' = Cl, R'' = C_{10}H_{21} \)
- g: \( R, R' = H, R'' = C_{15}H_{27} \)
- h: \( R, R' = H, R'' = C_{16}H_{33} \)
- i: \( R = Br, R' = H, R'' = C_{10}H_{21} \)
- j: \( R = Cl, R' = H, R'' = C_{15}H_{27} \)
- k: \( R, R' = Cl, R'' = C_{13}H_{27} \)
- l: \( R, R' = Cl, R'' = C_{14}H_{29} \)
- m: \( R, R' = Cl, R'' = C_{16}H_{33} \)
a positive control. Initially, we conducted a screening of all compounds at 60 mol/L, which was ~10-fold greater than IC_{50} for anacardic acid in this assay. The tested compounds displayed a wide range of HAT-inhibitory potency, with up to 95% maximum inhibition (Fig. 2). These data were confirmed by assaying in vitro dose dependence of the most efficient compounds (Table 1). Their IC_{50} varied from 6 to 42 mol/L and was comparable with IC_{50} obtained for anacardic acid.

Comparison of chemical structures shows that HAT-inhibitory potency of the compounds is significantly diminished in all ethyl esters tested compared with the corresponding carboxylic acids (e.g., compare 3e versus 4e, 3g versus 4g, or 3o versus 4o). The introduction of a benzyloxy group led to more potent compounds compared with the free phenol (4b versus 5b). Relative position of oxygens on the first phenyl ring seems to affect HAT inhibition, with meta-position corresponding to the maximum potency (compare 4b versus 4n and 4o). Regarding a substitution on the aromatic ring of the benzyloxy group, better results were obtained for methyl than for a methoxy group (compare 4c versus 4d). An effect of other substitutions on the second phenyl ring is less clear-cut. For example, substitution of chlorine for methyl group slightly increases the potency in 4e versus 4c. Change in the length of the hydrocarbon chain from 13 to 16 carbon atoms only slightly affects the potency of the compounds. Generally, these compounds suffer from a very high lipophilicity and low solubility in aqueous medium. Attempts to substitute the methyleneoxy linker between the two aromatic rings led to the sulfonamide 6 and the benzamide 7. Although enzyme-inhibitory properties are retained and lipophilicity is lowered, the cellular activity (see below) was not increased much. Altogether, wide variability of inhibitory efficacy in structurally closely related compounds provides valuable information that could facilitate design of more potent HATi for future studies.

**HATi Suppress Cancer Cell Growth and Protein Acetylation**

We used several human cancer cell lines Jurkat (T-cell leukemia), HeLa (cervical carcinoma), MCF7 (breast carcinoma), and PC3 (prostate adenocarcinoma) for evaluating the ability of the compounds that were most efficient at inhibiting HAT activity in vitro to affect cancer cell growth. Concentrations of drugs causing 50% growth inhibition were determined by proliferation assay after 48 h of treatment with the inhibitors (Table 2). Of the compounds

Figure 2. Effect of HATi on total HAT activity in HeLa nuclear extracts. HeLa nuclear extracts were used as a source of HAT activity to determine acetylation of histone H4 in the presence or absence of the indicated compounds as described in Materials and Methods. Anacardic acid (AA) was used as a control inhibitor. Experiments were repeated thrice. Columns, relative HAT activity; bars, SD.

<table>
<thead>
<tr>
<th>Compound ID</th>
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<tbody>
<tr>
<td>4c</td>
<td>28</td>
</tr>
<tr>
<td>4d</td>
<td>37</td>
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<td>4e</td>
<td>18</td>
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<td>6</td>
<td>42</td>
</tr>
<tr>
<td>Anacardic acid</td>
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Abbreviation: NE, nuclear extract.
Table 2. Effects of HATi on cancer cells (50% growth inhibition at μmol/L, 48 h)

<table>
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<tr>
<th>Compounds</th>
<th>Jurkat</th>
<th>HeLa</th>
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<th>PC3</th>
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<td>92</td>
<td>110</td>
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<td>&gt;&gt;100</td>
</tr>
<tr>
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<td>93</td>
<td>122</td>
<td>119</td>
<td>&gt;&gt;100</td>
<td>&gt;&gt;100</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

tested, 4l was most toxic to all cells tested, followed by 4k, 4g, and 4f. Anacardic acid, used as a control, was relatively more toxic to Jurkat cells than to other cell lines tested. MCF7 and PC3 cells were relatively more sensitive to 4m than other cell lines. Two estrogen receptor α–negative cell lines, BT-20 and MDA-MB-231, treated with various inhibitors were more resistant to all drugs than estrogen receptor α–positive MCF7 cells (data not shown). To examine possible correlation between growth-inhibitory potency of the compounds and their effect on HAT activity, we treated MCF7 cells with 3e and 3g. Consistent with their low efficiency as HATi, these compounds did not produce toxic effects at up to 200 μmol/L, suggesting that their cytotoxicity is related to HAT-inhibitory properties. There was also statistically significant correlation between in vitro inhibition of HAT activity and cytotoxicity of the more efficient HATi (Fig. 3). Significantly, all tested compounds were relatively nontoxic to nonmalignant human fibroblast cell lines, NHP-5 and Hs-68.

To determine whether treatment with HATi causes changes in protein acetylation in vivo, we prepared protein extracts from MCF7 cells treated with several newly synthesized compounds or anacardic acid and analyzed changes in acetylation of histone H4 and α-tubulin by Western blotting. Because the level of acetylated proteins in untreated cells is normally low, we briefly treated cells with HDAC inhibitor trichostatin A for 1 h, to increase protein acetylation level, after 4-h pretreatment with HATi or DMSO vehicle (Fig. 4). As expected, treatment with trichostatin A caused significant increase in acetylated histone H4 and α-tubulin. However, pretreatment with 90 μmol/L HATi resulted in decreased acetylation level of histone H4 and, to a lesser degree, of α-tubulin compared with cells pretreated with DMSO. Acetylation of histone H4 was most strongly suppressed after pretreatment with 4f, 4g, and 4k, which also efficiently inhibited HAT activity in vitro. Effect of all tested HATi on α-tubulin acetylation was less significant. Interestingly, 4l that efficiently suppressed HAT activity in vitro and exhibited high cytotoxicity in proliferation assays had no negative effect on acetylation of histone H4 and only a slight effect on acetylation of α-tubulin. It is possible that weaker inhibition of α-tubulin acetylation compared with histone H4 is due to differential effects of the tested compounds on HATs responsible for acetylation of these proteins. Taken together, these data suggest that the new compounds not only inhibit HAT activity in vitro but also can negatively affect level of protein acetylation in vivo.

**Effect of HATi on p300 Activity in vivo**

To directly show that newly identified HATi can affect the activity of HATi in vivo, MCF7 cells were cotransfected with plasmids containing p21 promoter (snm fragment) fused to firefly luciferase reporter gene and the full-length p300 acetyltransferase and treated with the new compounds for 24 h. Reporter assays showed significant reduction of p300-induced activation of the p21 promoter in the presence of the HATi (data not shown). However, prolonged treatment with these inhibitors caused high cytotoxicity and complicated normalization of the assay data. Therefore, we determined kinetics of reporter activity after treating cells for shorter periods (Fig. 5). Treatment with anacardic acid or 4g caused time-dependent decrease in p300 activity, as measured by activation of p21 promoter, with the inhibitory effect for anacardic acid observed as early as 2 h after the addition of the drug. Subsequently, we did luciferase assay treating transfected MCF7 cells with new HATi at 90 μmol/L for 6 h (Fig. 6). Significant inhibition of p300 activity, although to a lower degree than with anacardic acid, was detected after treatment with all tested compounds except 4m. These data support the notion that these compounds can inhibit HAT activity in vitro. In conclusion, the results of this study indicate that newly designed compounds suppress HAT activity both in vitro and in vivo and can inhibit the growth of cancer cells.

**Discussion**

There is a growing body of evidence supporting importance of acetylation in regulating diverse cellular processes. Besides the well-established role of reversible histone acetylation in chromatin modifications and gene expression, several non-histone proteins were found to be regulated by acetylation. Recently, it was shown that DNA damage induces rapid acetylation of ATM. This acetylation depends on the Tip60 HAT. Suppression of Tip60 blocks the activation of ataxia telangiectasia mutant protein kinase activity and prevents ATM–dependent phosphorylation of p53 and Chk2 (20). Tip60 was also shown to be constitutively associated with DNA-PKcs and crucial for the activation of DNA-PKcs by DNA damage (21).

Alteration of HAT activity is common in cancer and other diseases. Rubinstein-Taybi syndrome has been found to be a result of mutations in the HAT domain of CAMP-responsive element binding protein–binding protein (22, 23). Overexpression of CBP was sufficient to induce hypertrophy in...
cardiac cells (24). Examples of deregulation of HATs in cancer include amplification and overexpression of AIB-1 in breast, ovarian, and gastric cancers (25, 26); translocation of HAT genes in acute myelogenous leukemia (27, 28); and mutations of p300 in human colorectal, gastric, breast, and pancreatic cancers (29, 30).

Given important role of acetylation as a regulatory mechanism, it is natural that inhibitors of both HDACs and HATs should be a focus of many studies. Although there are many publications devoted to HDAC inhibitors, relatively little is known about HATi. Lau et al. (31) designed and synthesized two peptide coenzyme A conjugates that were found to be potent selective inhibitors of p300 and PCAF. However, these inhibitors could not permeate the cells and showed poor pharmacokinetic properties (32). Several naturally occurring HATi (anacardic acid, garcinol, and curcumin) were characterized lately (12, 16, 19). Interestingly, they all were isolated during screen of the plant extracts known to possess anticancer properties. Using a highly purified recombinant HAT assay system, anacardic acid was shown to be a potent inhibitor of both p300 and PCAF (IC50, 8.5 and 5 μmol/L, respectively). Specific inhibitor of CBP (IC50, 25 μmol/L), curcumin, derived from the spice turmeric, inhibited proliferation of HIV-1 as well as the acetylation of HIV-Tat protein in vitro (19). Garcinol, a potent inhibitor of PCAF and p300 (IC50, 5 and 7 μmol/L, respectively), isolated from Garcinia indica fruit rind, was found to induce apoptosis and alter global gene expression in HeLa cells (16). None of these inhibitors had effect on HDAC activity or on histone-free DNA transcription. Anacardic acid was shown recently to inhibit Tip60 HAT in vitro and block Tip60-dependent activation of ATM and DNA-PK protein kinases by DNA damage in vitro (33). Treatment of human cancer cells with anacardic acid increased their sensitivity to ionizing radiation providing a novel therapeutic approach to radiosensitization of tumor cells.

The structures of the compounds used in our study were based on anacardic acid. To increase their biological activity, some modifications were introduced, including addition of a second aromatic ring, changes in the length and position of the alkyl chain, and introduction of polar groups in the spacer between the two aromatic rings. To determine the ability of these derivatives to inhibit HAT activity in vitro, we used HeLa nuclear extract as a source of multiple HAT activities. We detected a wide variability in HAT-inhibitory potency of the compounds, from total loss of inhibition to the effect similar to anacardic acid, although none of the analogues was more efficient.
than anacardic acid in inhibiting total HAT activity under these conditions. Conversion of the ethyl esters to the corresponding carboxylic acids was the single most efficient modification, increasing HAT-inhibitory potency up to 10-fold. *Meta*-position of oxygens at the first aromatic ring increased inhibitory potency of the compounds, although to a considerably smaller degree. The effect of the substitutions at the second aromatic ring was less clear-cut, although substitution of methyl for methoxy group in the 4-position slightly increased the potency of inhibition.

To study physiologic effects of the new compounds, we determined their ability to arrest growth of several human cancer cell lines. We found that their cytotoxicity was similar to that of anacardic acid or slightly higher. Relatively high concentrations required to achieve 50% growth inhibition are probably due to high hydrophobicity of the compounds that affects their solubility in aqueous media and effective intracellular concentration. Significantly, none of the tested compounds was toxic to normal human cells Hs-68 and NHP-5 at the concentrations sufficient to kill cancer cells.

Because anacardic acid was shown to inhibit other enzymes besides HATs, although with much lower efficiency (34, 35), we could not exclude the possibility of nonspecific cytotoxicity. However, we found a clear correlation between HAT-inhibitory potency of the compounds and their effects on cell proliferation. For instance, **3e** and **3g** that were not efficient in inhibiting HAT activity *in vitro* did not produce cytotoxic effects in the cells. Correlation of HAT inhibition *in vitro* and cytotoxicity *in vivo* in structurally similar compounds suggest cause-effect relationship. Variation in cytotoxicity of the compounds with similar *in vitro* inhibitory properties is possibly explained by their different ability to penetrate into the cells. Kubo et al. (15) examined correlation between structure of anacardic acid derivatives and their antibacterial activities. They found that even a small variation in the length of the alkyl side chain (e.g., C_{12} versus C_{15}) in anacardic acid analogues can have a dramatic effect on toxicity of the compound (up to 800-fold difference for some microorganisms). They suggested that this effect may

![Figure 5](image)

**Figure 5.** Effect of HATi on p300 activity in MCF7 cells: MCF7 cells were transiently transfected with p21 promoter and full-length p300 and treated with anacardic acid or 4g (90 μmol/L) for indicated times. The cells were then analyzed for luciferase activity using Bright-Glo Luciferase Assay System (Promega). To control for toxicity exhibited by HATi, the obtained values were normalized by cell numbers. Experiments were repeated three times. **Column**, relative luciferase activity; **bars**, SD. Representative experiment in triplicates.

![Figure 6](image)

**Figure 6.** HATi suppress p300 activity in MCF7 cells: transient transfection of p21 and p300 was carried out as indicated in Materials and Methods. After recovery overnight, the cells were treated with indicated compounds (90 μmol/L) or DMSO for 6 h. The cells were then analyzed for luciferase activity as described in Fig. 5.
be the result of the differences in hydrophobic interactions between the compounds and the lipids in the cell membrane. In our study, we found that 4m had the longest alkyl side chain (C14) was significantly less toxic to all cell lines tested than 4k and 4l (C13 and C14, respectively), although their in vitro anti-HAT effects were similar. 4m was also the least efficient in suppressing p300 HAT activity in luciferase assay. There is also a possibility of differences in intracellular trafficking of the compounds because 4l, which was the most toxic of all the tested compounds and presumably penetrated into the cells, did not affect acetylation of histone H4.

A recent study of a new group of synthetic HATi derived from isothiazolone suggested a relatively low hit rate for identifying HATi by the high-throughput screening (three hits out of a diverse compound library of 69,000 compounds; ref. 11). Therefore, identification of a new group of synthetic compounds possessing HAT-inhibitory properties presents both practical and theoretical interest. Analysis of structurally similar compounds displaying a wide range of potencies provides information necessary to design more efficient inhibitors as well as a tool for studying effects of HATs on target molecules. Work is currently under way to improve the solubility of the compounds and to improve their penetration into the cells. This could increase their anticancer effects and potential for drug development, either individually or in combination with other chemotherapeutic agents.

Acknowledgments

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Characterization of novel inhibitors of histone acetyltransferases

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