Isothiazolones as inhibitors of PCAF and p300 histone acetyltransferase activity

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Abstract

Histone acetylation plays an important role in regulating the chromatin structure and is tightly regulated by two classes of enzyme, histone acetyltransferases (HAT) and histone deacetylases (HDAC). Deregulated HAT and HDAC activity plays a role in the development of a range of cancers. Consequently, inhibitors of these enzymes have potential as anticancer agents. Several HDAC inhibitors have been described; however, few inhibitors of HATs have been disclosed. Following a FlashPlate high-throughput screen, we identified a series of isothiazolone-based HAT inhibitors. Thirty-five N-substituted analogues inhibited both p300/cyclic AMP–responsive element binding protein–binding protein–associated factor (PCAF) and p300 (1 to >50 μmol/L, respectively) and the growth of a panel of human tumor cell lines (50% growth inhibition, 0.8 to >50 μmol/L). CCT077791 and CCT077792 decreased cellular acetylation in a time-dependent manner (2–48 hours of exposure) and a concentration-dependent manner (one to five times, 72 hours, 50% growth inhibition) in HCT116 and HT29 human colon tumor cell lines. CCT077791 reduced total acetylation of histones H3 and H4, levels of specific acetylated lysine marks, and acetylation of α-tubulin. Four and 24 hours of exposure to the compounds produced the same extent of growth inhibition as 72 hours of continuous exposure, suggesting that growth arrest was an early event. Chemical reactivity of these compounds, as measured by covalent protein binding and loss of HAT inhibition in the presence of DTT, indicated that reaction with thiol groups might be important in their mechanism of action. As one of the first series of small-molecule inhibitors of HAT activity, further analogue synthesis is being pursued to examine the potential scope for reducing chemical reactivity while maintaining HAT inhibition. [Mol Cancer Ther 2005;4(10):1521–32]

Introduction

Histone acetylation and deacetylation are essential elements along with phosphorylation and methylation of an intricate ''histone code'' that regulates gene transcription (1–3). The reversible process of histone acetylation is controlled by two classes of enzyme, histone acetyltransferases (HAT) and histone deacetylases (HDAC), which catalyze the addition to and removal, respectively, of acetyl groups on lysine residues in protein. Hyperacetylation of histones has been associated with transcriptional activation of genes as chromatin becomes accessible to a number of transcription factors and coactivator complexes (4). Conversely, HDACs induce transcriptional repression by removing these acetyl groups, leading to chromatin condensation (5). In addition to histones, both HATs and HDACs target nonhistone protein substrates, such as transcription factors (e.g., p53; ref. 6) and structural proteins (e.g., α-tubulin; ref. 7).

HDAC family members are divided into three classes (i.e., class I, II, and III; ref. 8). There are six families of HATs, including the GCN5-related *N*-acetyltransferase family, which includes p300/cyclic AMP–responsive element binding protein–binding protein (CBP)–associated factor (PCAF); the MYST (MOZ, Yb2/Sas3, and TIP60) family; and the p300/CBP family (9).

The antitumor effects of HDAC inhibitors are well documented. Several drugs are in clinical evaluation (10). The validation of HATs as chemotherapeutic targets is, however, less complete. *HAT* genes have been shown to be genetically altered, with gene amplifications, mutations, and translocations reported in a variety of solid tumors and hematologic malignancies (11–14). Examples of deregulated HATs during oncogenesis include amplification and overexpression of AIB-1 (amplified in breast cancer-1 also known as ACTR), a nuclear hormone receptor coactivator with HAT activity (15), in breast, ovarian, and gastric cancers (16, 17). *HAT* genes are translocated in acute myeloid leukemia with several fusion proteins being

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formed between p300/CBP enzymes and MYST family HATs [e.g., t(8:13) MOZ:CBP (18) and (10;16) MORF:CBP (19)]. Interestingly, within all of the fusion proteins reported, the HAT domain of each enzyme has been retained, suggesting that mistargeted and deregulated HAT activity is important in the evolution of leukemic transformation. Mutated p300 has also been found in human colorectal, breast, and pancreatic cancers (20–23) and are also associated with the proliferation of prostate cancer both *in vitro* and *in vivo* (24).

In view of the increasing evidence that associates HAT function with cancer causation and progression, these enzymes are appealing as drug targets for the development of small-molecule inhibitors as anticancer agents. It may seem paradoxical to seek inhibitors of both HATs and HDACs when these enzymes have opposing catalytic reactions. However, the biology of HATs and HDACs is complex and still being elucidated, and it is unlikely that a simple ''on-off'' acetylation model will apply to gene transcription and cancer development. Small-molecule inhibitors of HATs would therefore be useful pharmacologic tools to enhance our understanding of histone acetylation and are also potential candidates for the development of anticancer agents.

The first report of HAT inhibitors involved the design and synthesis of peptides conjugated with acetyl-CoA, including Lys-CoA that selectively inhibits p300 and H3-CoA-20 that is selective for PCAF (25). The use of these peptides showed the potential for selective HAT inhibition. More recently, anacardic acid, isolated from cashew nut shell liquid, has been identified as a noncompetitive inhibitor of both p300 and PCAF HAT activity (26); however, due to poor cell permeability, this compound and its derivatives have yet to be associated with cellular activity (27). A polyprenylated benzophenone known as garcinol has been isolated from *Garcinia indica* fruit rind and is also identified as an inhibitor of p300 and PCAF HAT activity both *in vitro* and *in vivo* (27). These and other natural products (28) will provide valuable probes for investigating HAT functions, although their potential for development as clinical drug candidates remains to be determined.

To identify and develop novel, small-molecular-weight HAT inhibitors, a high-throughput screening FlashPlate assay using scintillating microplates to measure PCAF HAT activity was used to screen a library of diverse compounds (29). The activity of hit compounds was confirmed using a filter-based HAT enzyme assay (30, 31). The aim of this article is to show the ability of one hit series to inhibit PCAF and p300 HAT activity as well as to illustrate their effects on cellular acetylation and proliferation in cancer cell lines.

Materials and Methods

Materials

Basic FlashPlates (SMP200) and [acetyl-^{3}H]acetyl-CoA (NET290) were obtained from Perkin-Elmer Life Sciences (Buckinghamshire, United Kingdom). Lysine-rich histone fraction, type III-S from calf thymus, was purchased from Sigma-Aldrich Chemical Co. (Dorset, United Kingdom). Whatman P81 chromatography paper (cation exchanger) was obtained from Fisher Scientific Chemicals (Loughborough, United Kingdom). Antibodies that recognize specific acetylated lysine residues in histone H3 (K9 and K14) or H4 (K5, K8, and K12) and acetylated histones H3 and H4 were obtained from Upstate (Milton Keynes, United Kingdom). The antiacetylated protein antibody was from Abcam (Cambridge, United Kingdom), whereas the antiacetylated α-tubulin antibody was from Sigma-Aldrich Chemical.

The library of 35 N-substituted isothiazolone compounds (Table 1) was synthesized by Ultrafine (UFC Ltd., Manchester, United Kingdom). Compounds CCT129182, CCT129183, and CCT129184 were synthesized at the Cancer Research UK Centre for Cancer Therapeutics at the Institute for Cancer Research (Sutton, Surrey, United Kingdom).

Cell Culture

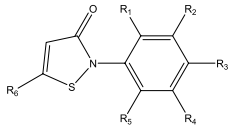
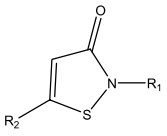
A range of human tumor cell lines was used. These cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were *Mycoplasma*-free before use as determined by the American Type Culture Collection *Mycoplasma* detection kit. Both cell lines were maintained in DMEM (Life Technologies/Invitrogen, Paisley, United Kingdom) supplemented with 10% fetal bovine serum (PAA Laboratories, Somerset, United Kingdom) and 1% penicillin/streptomycin (Life Technologies/Invitrogen) at 37°C in an atmosphere of 5% CO_2.

Biochemical Assays for Measuring HAT Activity

Enzyme Preparation. The recombinant HAT domains of PCAF (32) and p300 (residues 1,096-1,721) were expressed as glutathione *S*-transferase fusion proteins in *Escherichia coli* and supplied on glutathione-coated beads. These were briefly centrifuged at 12,000 rpm, the supernatant was removed, and the pelleted beads were resuspended in 1 mL of 50 mmol/L reduced glutathione in 100 mmol/L Tris-HCl (pH 8.0) to elute the enzyme. Following incubation at room temperature for 15 minutes and centrifugation, the supernatant was collected. Washing of the beads was repeated twice and the collected supernatants were placed separately in dialysis tubing and dialyzed for 24 hours at 4°C against 2 L of 20 mmol/L Tris-HCl, 0.5 mmol/L EDTA, 100 mmol/L KCl, and 10% glycerol (pH 8.0). After dialysis, samples were removed for enzyme assay and protein determination. Samples with the highest specific activity were stored in aliquots at −80°C and used for all subsequent enzyme assays.

FlashPlate Assay. The FlashPlate assay was carried out in 96-well scintillating microplates as described previously (29). In brief, the substrate concentrations were 0.4 μmol/L acetyl-CoA (0.02 μCi tritiated acetyl-CoA) and 2.5 μg histone in assay buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 10% glycerol (pH 8.0)]. For IC_{50} determinations, a range of stock concentrations of the compounds in DMSO was prepared and a 1-μL aliquot of each solution was transferred to the assay wells. Four or five concentrations around an estimated IC_{50} were generally used to generate

Table 1. Chemical structures and PCAF-inhibitory properties of a library of 35 N-substituted isothiazolone-based compounds

Compound	R_1	R_2	R_3	R_4	R_5	R_6	PCAF IC_{50}, μmol/L
A. Parent structure							
1	NO_2	H	H	H	H	H	14.4
2	H	NO_2	H	H	H	H	9.2
3	H	H	NO_2	H	H	H	9.6
4	H	H	NO_2	H	H	Cl	7.3
5	H	NO_2	Cl	H	H	H	1.1
6	OMe	H	H	H	H	H	100
7	H	H	OMe	H	H	H	11.0
8	Cl	H	H	H	H	H	9.1
9	H	Cl	H	H	H	H	7.8
10	H	H	Cl	H	H	H	7.3
11	Cl	H	H	Cl	H	H	10.2
12	Cl	H	H	Cl	H	Cl	31.1
13	Cl	H	Cl	H	H	H	11.0
14	Cl	CL	H	H	H	H	7.6
15	CH_3	H	H	H	H	H	17.1
16	H	CH_3	H	H	H	H	11.0
17	H	H	CH_3	H	H	H	8.4
18	CO_2Et	H	H	H	H	H	18.3
19	H	CO_2Et	H	H	H	H	5.0
20	H	H	CO_2Et	H	H	H	6.6
21	CF_3	H	H	H	H	H	10.2
22	H	CF_3	H	H	H	H	11.0
23	H	CF_3	H	H	H	Cl	6.5
24	H	CF_3	F	H	H	H	8.7
25	H	CF_3	F	H	H	Cl	5.4
26	H	Cl	CH_3	H	H	H	8.1
27	H	OPh	H	H	H	H	12.6
B. Parent structure							
28	iPr	H					50.0
29	Cyclopropyl	H					29.5
30	Benzyl	H					13.5
31	Et	H					44.6
32	Et	Cl					18.7
33	Decyl	H					10.6
34	CH_2CH_2OPh	H					15.0
35	CH_2CH_2OPh	Cl					25.0

the experimental values. Between 0.5 and 2.0 μg recombinant p300 or PCAF enzyme was then added to give a total assay volume of 50 μL. The plate was incubated at room temperature for 20 minutes and the enzyme reaction was stopped by the addition of excess assay buffer (150 μL). The signal was detected in a microplate scintillation counter (TopCount, Perkin-Elmer Life Sciences). Enzyme activity was linear with time (up to 30 minutes) and protein concentration (up to 5 μg/well).

HAT Filter Assay. The filter assay was run as described previously (31) in microcentrifuge tubes using substrate concentrations of 2 μmol/L acetyl-CoA (0.055 μCi radiolabeled cofactor) and 2.5 μg histone in assay buffer [50 mmol/L Tris-HCl, 0.5 mmol/L EDTA, 10% glycerol (pH 8.0)]. Recombinant enzymes were added (0.02-0.2 μg/tube) to give a total volume of 25 μL. Assay tubes were placed in a water bath at 30°C for 10 minutes before spotting the assay volume on a 2 × 2 cm square of P81

chromatography paper to terminate the reactions. The chromatography paper was placed in a Buchner funnel and washed with 400 mL of 50 mmol/L sodium carbonate buffer (pH 9.2) followed by 200 mL acetone. The squares were dried with hot air and counted in 5 mL liquid scintillation fluid (Ultima Gold, Perkin-Elmer Life Sciences). Enzyme activities were linear with time (up to 20 minutes) and protein (up to 0.5 μg/tube). IC_{50} values were produced as described for the FlashPlate assay.

Cell Proliferation Assay. Cells were seeded at 1,000 per well in a 200 μL volume of DMEM in 96-well tissue culture plates (Falcon, supplied by Marathon Laboratory Supplies, London, United Kingdom). At 24 hours after seeding, compound or DMSO vehicle control (5 μL) was then added to each well (four- or eight-well replicates per concentration) for the duration required (4, 24, 72, or 96 hours). To determine 4- and 24-hour IC_{50} concentrations, compounds were removed at these times and cells were washed once in 200 μL DMEM. The wells were refilled with 200 μL fresh DMEM and reincubated for the remainder of the assay. Growth inhibition was determined using the sulforhodamine B assay as described (33) and the absorbance was measured at 570 nm on a Wallac Victor2 microplate reader (Perkin-Elmer Life Sciences). Data were then plotted as percentage of DMSO control against compound concentrations using GraphPad Prism 3.0 software. The 50% growth inhibition (GI_{50}) was calculated as the compound concentration required to reduce cell number by 50% compared with DMSO control.

Time-Resolved Fluorescent Cell-Based Immunosorbent Assay. The assay has been briefly described previously (31). Human tumor cell lines were seeded at 8,000 cells/well in 96-well tissue culture plates (Falcon). At chosen time points, medium was removed and cells were fixed and permeabilized (0.25% glutaraldehyde, 3% paraformaldehyde, 0.25% Triton X-100) for 30 minutes at 37°C. The cells were washed once with PBS using an automated Wellwash 5000 plate washer (Denley Instruments, Inc., supplied by Thermo Life Sciences, Basingstoke, Hampshire, United Kingdom). At this stage, plates could be stored at 4°C for up to 2 weeks for later batch processing.

Each plate was blocked with 5% milk diluted in PBS (100 μL/well) for 30 minutes at 37°C and then again washed once with PBS as before. The cells were then incubated with a primary antibody diluted in PBS (100 μL/well) for 1 hour at 37°C and washed thrice with cell-based immunosorbent assay wash (0.1% Tween 20 in PBS). A europium-labeled secondary antibody (100 μL/well) diluted in DELFIA assay buffer (Perkin-Elmer Life Sciences) was then added for 1 hour at 37°C before the cells were washed thrice with cell-based immunosorbent assay wash. DELFIA enhancement solution (100 μL/well; Perkin-Elmer Life Sciences) was added to the cells, plates were shaken for 1 minute on a shaker, and time-resolved fluorescence was measured at 615 nm in time-resolved mode using a Wallac Victor2 microplate reader (Perkin-Elmer Life Sciences). The plates were washed with PBS as before and protein concentration was determined using the Pierce BCA assay (Pierce, Cramlington, Northumberland, United Kingdom) The fluorescent signal obtained in the time-resolved fluorescent cell-based immunosorbent assay was corrected (normalized) by dividing europium counts by the absorbance of the protein concentration.

Protein-Binding Assay. Compounds were prepared as 10 mmol/L stock solutions in DMSO and diluted with distilled water to 50 and 500 μmol/L. Each compound, at final concentrations of 5 and 50 μmol/L, was incubated for 1 minute with a 4.5 g/L solution of bovine serum albumin (BSA) at room temperature. Proteins were precipitated by the addition of 3 volumes of methanol. Samples were centrifuged at 2,800 × *g* for 10 minutes and the supernatant was removed for analysis. Control samples were prepared by adding a methanol-precipitated BSA solution. The liquid chromatography (LC)/UV system consisted of a SpectraSYSTEM AS3000 autosampler, P4000 pump, and UV6000 detector (ThermoFinnigan, Hemel Hempstead, United Kingdom). Detection was by UV absorbance at 270 nm. The sample injection volume was 10 μL. Chromatography was done using a Supelcosil ABZ column, 5 cm × 4.6 mm ID, 5 μm particle size (Supelco, Gillingham, Dorset, United Kingdom). The flow rate was 0.6 mL/min and the mobile phase consisted of methanol (A) and 10 mmol/L ammonium acetate (B). Analytes were eluted using the following gradient: time 0 minutes, 35% A and 65% B; time 3 minutes, 75% A and 25% B; time 6 minutes, 100% A and 0% B; time 7 minutes, 100% A and 0% B; time 8 minutes, 35% A and 65% B; and time 12 minutes, 35% A and 65% B. The percentage of the control signal remaining following incubation was calculated as 100 × sample peak area / control peak area. The percentage of compound bound to BSA was calculated as 100 − percentage of control signal.

Protein binding was also determined using a TSQ 700 triple quadrupole mass spectrometer equipped with an electrospray ionization source (ThermoFinnigan) coupled to a 600 mass spectrometry (MS) pump and 717 autosampler (Waters Ltd., Elstree, United Kingdom). The mass spectrometer was operated in positive ion mode (capillary temperature 250°C, spray voltage 4.5 kV). Spectra were acquired in full scan mode over the *m/z* range of 50 to 1,000. Samples (25 μL) prepared as for LC/UV were injected. The mobile phase consisted of methanol (A) and 0.1% formic acid in water (B; 1.0 mL/min) using a gradient over 15 minutes (ratios of A/B: 0–0.5 minutes 10:90, 0.5–6.0 minutes 90:10, 6.0–10 minutes 90:10, and 10–15 minutes 10:90).

Results

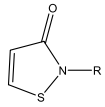
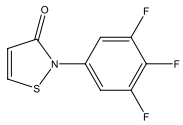
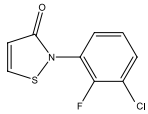
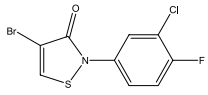
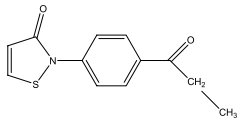
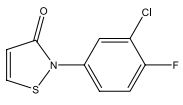
Identification of Isothiazolones as Inhibitors of HAT Activity by a High-Throughput Screening

The development of the biochemical screening assays (FlashPlate and filter) has been described previously (29, 31). To further validate these assays, IC_{50} values were measured for the two peptide conjugates, Lys-CoA and H3-CoA-20, against the PCAF and p300 HAT enzymes. The compounds displayed similar potencies to those described in the literature (25). Lys-CoA inhibited p300 (filter IC_{50} of

0.31 ± 0.11 μmol/L; FlashPlate IC_{50} of 0.42 ± 0.08 μmol/L) but not PCAF (both formats IC_{50} >100 μmol/L). Conversely, PCAF was inhibited by H3-CoA-20 (filter IC_{50} of 8 ± 2.5 μmol/L; FlashPlate IC_{50} of 2.9 ± 0.72 μmol/L) to a much greater extent than p300 (filter IC_{50} of 64.0 ± 17.0 μmol/L; FlashPlate IC_{50} of 34 ± 10 μmol/L).

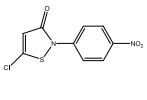
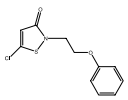
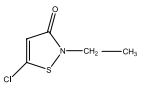
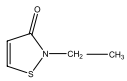
The FlashPlate assay was used to screen a diverse compound library of 69,000 compounds against PCAF. A series based on the isothiazolone structure (Table 2) was identified. Three compounds were identified as hits in the primary screen: CCT004464 and CCT004466 (PCAF enzyme IC_{50}, 3.1 and 5.4 μmol/L, respectively) and the less potent hit CCT004467 (PCAF enzyme IC_{50}, 12.4 μmol/L). A subsequent search for commercially available compounds with similar structures to the hit compounds identified CCT004463 and CCT004465 (Maybridge, Cornwall, United Kingdom), which were both found to display similar levels of PCAF inhibition as the original hits. CCT004464, CCT004466, CCT004467, CCT004463, and CCT004465 also caused growth inhibition of two colon cancer cell lines, HCT116 and HT29, with GI_{50} values between 5.8 and >50 μmol/L (Table 2). During the evaluation of the hits, it was realized that the HAT activity for this class of compounds may be due to a covalent binding to the enzyme via disulfide bond formation. It has been reported that the S-N bond of isothiazolones is reactive toward thiol groups as proposed for the mechanism of inhibition of $p56^{lck}$ kinase (34). With this in mind, 35 analogues (27 *N*-aryl and 8 *N*-alkyl isothiazolones) were designed, prepared, and evaluated for HAT inhibition. In particular, it was interesting to investigate if the reactivity of the S-N bond could be modulated by changing the electronegativity of the N-substituent. For this reason, strong

Table 2. Characteristics of N-aryl isothiazolones identified in the primary screen and by substructure search

Compound	Structure	PCAF IC_{50}, μmol/L	96-h GI_{50}, μmol/L	
		FlashPlate	HCT116	HT29
Isothiazolone				
CCT004464 (KM04537)*		3.1*	18.5*	5.8*
CCT004466 (KM04550)*		5.4*	19.5*	14.5*
CCT004467 (KM04564)*		12.4*	>50*	>50*
CCT004463 (KM04416)		2.5	21.0	9.1
CCT004465 (KM04549)		2.8	20.0	13.0

*Noted compounds were hits in the high-throughput screen.

Table 3. Enzyme and growth-inhibitory properties of four compounds selected from the library of 35 N-substituted isothiazolones as PCAF and p300 inhibitors

	Compound			
	CCT077791	CCT077792	CCT077796	CCT079769
Structure				
PCAF IC_{50}, μmol/L				
FlashPlate	7.3	15.0	18.7	54.7
Filter assay	2.2 ± 0.3	2.7 ± 0.7	20.2	ND
p300 (% inhibition at 35 μmol/L)	95	90	84	30
GI_{50}, μmol/L				
HCT116	3.0 ± 2.0	0.4 ± 0.1	1.4 ± 0.4	10.5 ± 4.9
HT29	2.1 ± 0.3	0.4 ± 0.1	2.2 ± 0.5	11.0 ± 4.2
Ratio of GI_{50}*/PCAF IC_{50}	1:0.8	1:6.8	1:11.2	1:4.7

NOTE: Data are mean ± SD. ND, not determined.
*Because of similar GI_{50} concentrations between these cell lines, an average has been taken to calculate the PCAF enzyme to GI_{50} ratio.

electron withdrawing groups, such as nitro and carboxylic ester, were introduced into the phenyl ring. The electronegativity of the R3 group seems to have no influence on inhibiting the enzyme because compounds 3, 7, 10, 17, and 20 displayed similar PCAF IC_{50} values (Table 1). However, the position of the substituent on the phenyl ring seems to play a role in inhibiting the enzyme; compounds 1, 6, 15, and 18 were less potent than their *para*-substituted counterparts, suggesting that steric effects may reduce the reactivity of the S-N bond, leading to a decreased inhibition of the enzyme.

This collection of 35 compounds inhibited PCAF HAT activity (IC_{50} range, 2.0 to >50 μmol/L) and caused growth inhibition of a panel of human colon (HCT116, HT29, and KM12) and ovarian (A2780, cisplatin-resistant A2780, CH1, 41M, and SKOV-3) tumor cell lines (GI_{50} range, 0.8 to >50 μmol/L).

As the 35 isothiazolones gave similar GI_{50} ranges in the panel of cell lines tested, HCT116 and HT29 cell lines were selected as representative cell lines for use in future experiments. These two cell lines express comparable levels of PCAF protein; however, they are interesting in that HCT116 cells contain a mutated *p300* gene (22, 23).

Isothiazolones Inhibit Cell Proliferation and Decrease Global Cellular Acetylation in Human Cancer Cell Lines

Four of the N-substituted isothiazolone compounds were selected for further investigation as examples of *N*-aryl and *N*-alkyl isothiazolones with a range of potencies for inhibiting HAT activity (Table 3). The GI_{50} concentrations in both HCT116 and HT29 cell lines are shown in Table 3. There was an overall 25-fold difference in GI_{50} concentrations of these four compounds. CCT077792 was the most potent (GI_{50}, 0.4 ± 0.1 μmol/L in both cell lines) and CCT079769 was the least potent compound (GI_{50}, 10.5 ± 4.9 μmol/L in HCT116 and 11.0 ± 4.2 μmol/L in HT29 cells; Table 3).

To assess their effects on global cellular acetylation, HCT116 and HT29 cell lines were treated with CCT077791, CCT077792, CCT077796, CCT079769 (10 μmol/L), or DMSO vehicle control for 24 hours and analyzed by the time-resolved fluorescent cell-based immunosorbent assay with an antiacetylated protein antibody (Fig. 1). In the absence of a commercially available positive control compound for

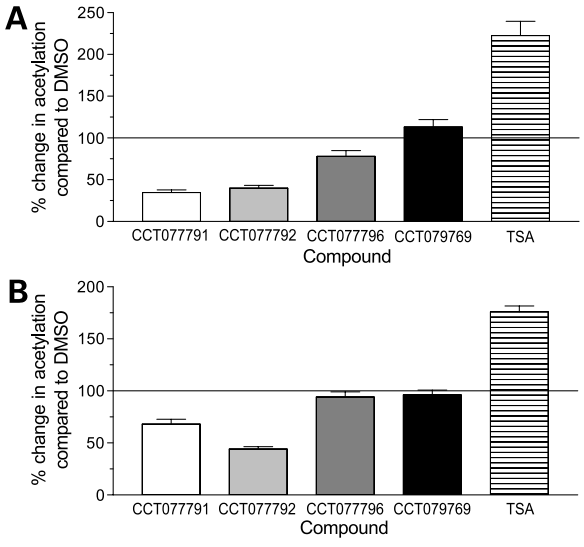


Figure 1. Effect of four isothiazolones on normalized acetylation for HCT116 (**A**) and HT29 (**B**) cells following treatment (10 μmol/L) for 24 h. The time-resolved fluorescent cell-based immunosorbent assay was conducted using an acetylated protein antibody. The fluorescent signal obtained in the time-resolved fluorescent cell-based immunosorbent assay was corrected (normalized) by dividing europium counts by the absorbance of the protein concentration. Data are normalized acetylation as a percentage change from DMSO control. *Columns,* mean of eight replicates for one typical experiment; *bars,* SD. Cells were also exposed to trichostatin A (*TSA*; 66 nmol/L) for 24 h in the assay as a positive control. *Columns,* mean of three replicates; *bars,* SD.

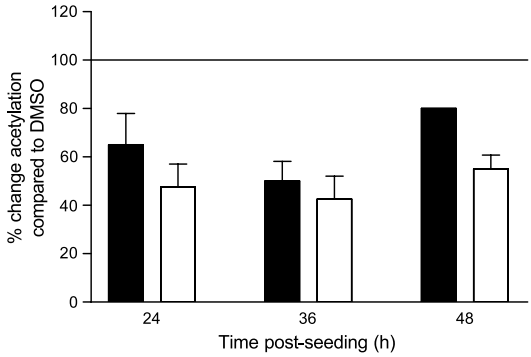


Figure 2. Effects of CCT077791 (*filled columns*) and CCT077792 (*open columns*) on normalized acetylation of HCT116 cells following 2 h of compound exposure. HCT116 cells were treated with compounds (10 μmol/L) at 22 or 46 h after seeding for 2 h (i.e., cells were fixed at 24 and 48 h after seeding, respectively). Cells were analyzed in the time-resolved fluorescent cell-based immunosorbent assay using an acetylated protein antibody. Data are percentage change from DMSO control. *Columns,* mean of four replicates of one experiment; *bars,* SD. Cells exposed to trichostatin A (66 nmol/L) for 2 h were also analyzed. Cellular acetylation was increased by 157.1 ± 11.9% (mean ± SD for three replicates) compared with control.

this assay, a standard HDAC inhibitor (trichostatin A) was used to determine the ability of the assay to detect changes in cellular acetylation (Fig. 1). CCT077791 and CCT077792 both induced a marked reduction in cellular acetylation following exposure for 24 hours (e.g., 65 ± 3.1% and 60 ± 3.4% decrease, respectively) in HCT116 cells. CCT077796 caused a reduction in acetylation of 22 ± 6.9% in HCT116 cells but did not markedly reduce acetylation in HT29 cells. CCT079769 had no effect on cellular acetylation in either cell line. Similar effects on cellular acetylation were also observed following 48 hours of exposure to the compounds (data not shown).

To address whether isothiazolones alter global acetylation following shorter periods of exposure, a time-resolved fluorescent cell-based immunosorbent assay was carried out following exposure of cells to CT077791 and CCT077792 at 22 or 46 hours after seeding for 2 hours (i.e., cells were fixed at 24 and 48 hours after seeding, respectively). CCT077796 and CCT079769 were not assayed as they had not markedly inhibited cellular acetylation in the previous continuous exposure (24–48 hours) experiment. Maximum reductions in cellular acetylation were 50.0 ± 8.2% for CCT077791 and 57.5 ± 9.6% for CCT077792 in HCT116 cells (Fig. 2), slightly less than the reductions observed at 24 hours (65% and 60%, respectively).

Time- and concentration-dependent effects of CCT077791 and CCT077792 in the time-resolved fluorescent cell-based immunosorbent assay were investigated using equipotent concentrations based on GI_{50} values (Table 3). Figure 3 shows that at low concentrations [CCT077791 (1× GI_{50}) and CCT077792 (1× GI_{50} and 2× GI_{50})] acetylation was not reduced compared with vehicle control. Increasing concentrations of CCT077791 (2× GI_{50}), however, led to a reduction in acetylation at 24 hours (22.3 ± 12.0% at 12 hours and 21.8 ± 6.0% at 24 hours) but not at 48 hours. In the presence of 5× GI_{50} concentrations, reduced acetylation was observed at both 24 and 48 hours. Similar results were obtained in HT29 cells (data not shown).

CCT077791 Reduces Acetylation of Histones H3 and H4 and α-Tubulin in Cancer Cell Lines

As the most potent compound at reducing global cellular acetylation at equitoxic doses, the effects of CCT077791 on histones H3 and H4 acetylation were investigated. Figure 4 shows the acetylation results for antibodies that specifically recognize acetylation of histones H3 and H4. Reduced acetylation of histones H3 and H4 was detected at 5× GI_{50} CCT077791 compared with vehicle control-treated cells (Fig. 4). Reduced acetylation on specific lysine residues of histone H3 and H4 compared with the vehicle control was observed. The largest decrease in acetylation compared with DMSO-treated controls was observed on K8, histone H4 (55.5 ± 5.4% decrease), whereas acetylation on K9, histone H3, was reduced to a lesser extent (38.6 ± 13.4% decrease; Table 4).

Within cells, many proteins are acetylated in addition to histones (e.g., transcription factors and structural proteins; refs. 6, 35–37). Figure 4 shows a 34 ± 4.9% reduction in α-tubulin acetylation in the presence of CCT077791 (10 μmol/L) in HCT116 cells.

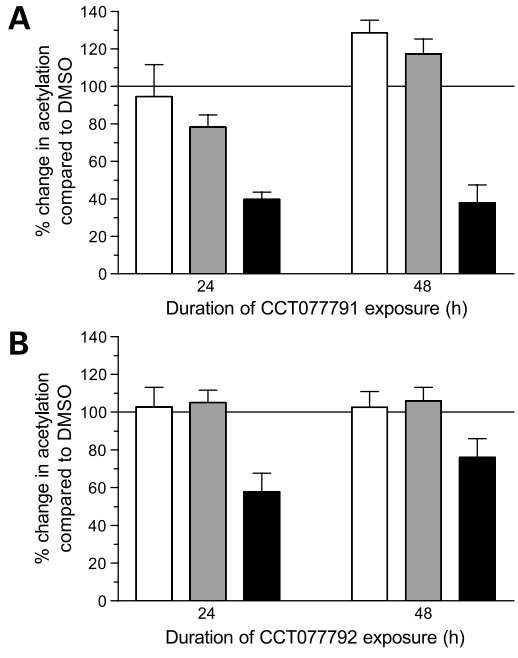


Figure 3. Normalized acetylation following increasing concentrations of CCT077791 (**A**) and CCT077792 (**B**): *open columns,* 1× GI_{50}; *gray columns,* 2× GI_{50}; *filled columns,* 5× GI_{50}, in HCT116 cells for 24 and 48 h. Cells were analyzed by time-resolved fluorescent cell-based immunosorbent assay using an acetylated protein primary antibody. *Columns,* mean percentage change in normalized acetylation compared with DMSO control for four replicates of one experiment; *bars,* SD.

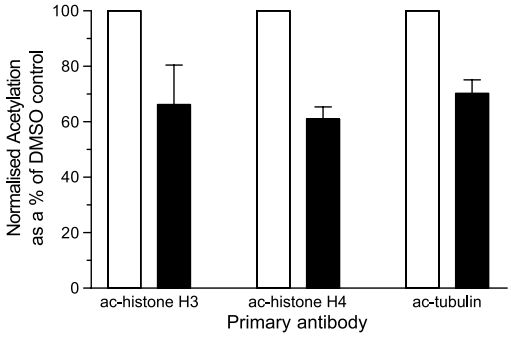


Figure 4. Effects of CCT077791 (*filled columns*) compared with DMSO control (*open columns*) on normalized acetylation of histones H3 and H4 and α-tubulin in HCT116 cells following 2 h of compound exposure. HCT116 cells were treated with CCT077791 (5× GI_{50}) for 2 h before each time point. *Columns,* mean percentage change from DMSO control for four replicates of one experiment; *bars,* SD. Following trichostatin A (200 nmol/L) treatment for 2 h, increases in acetylation compared with control were as follows: histone H3, 263.5 ± 9.5%; histone H4, 233.9 ± 9.8%; and tubulin, 144.6 ± 7.8% (mean ± SD for three replicates).

Isothiazolones Bind Irreversibly to Proteins via Thiol Interactions

An essential part of drug discovery is to establish the pharmacokinetic characteristics of novel compound series. However, during the development of a suitable chromatographic analytic method for pharmacokinetic studies, it was discovered that the original group of five compounds (Table 2) could not be measured in plasma following methanol precipitation.

To investigate the protein-binding characteristics of this compound series further, their ability to bind to BSA (a model plasma protein) was analyzed as described in Materials and Methods. The amount of each isothiazolone bound to protein at 5 and 50 μmol/L was compared with BSA added to methanol control. In general, the binding of the compounds to protein increased with increasing concentrations of BSA to reach a maximum at a concentration of 4.5 g BSA/L (10-fold less than the physiologic concentration of 45 g BSA/L). CCT077791, CCT077792, CCT077796, and CCT079769 were, respectively, 42.5%, 98.7%, 100.0%, and 84.1% bound to protein compared with control under these conditions. Using LC/UV analysis, only 5 of the 35 compounds in this set of isothiazolones were shown to bind BSA by <80% (at 5 and 50 μmol/L). The results for CCT077791, CCT077792, and CCT079769 are shown in Table 5 along with three additional isothiazolones (CCT129182, CCT129183, and CCT129184).

For example, using LC/MS, CCT129182, which originally seemed to have a relatively low degree of albumin binding (43.7%) by LC/UV, was shown to be completely bound to protein. For each compound, a peak corresponding to 2 mass units higher and eluting earlier than the parent compound was present but absent in the controls. It was hypothesized that this represented the open-ring conformation of the isothiazolone formed following its interaction with thiols and subsequent breakage of the covalent bond. This results in a compound with a similar chromophore to that of the parent and similar retention time, therefore explaining the results observed by LC/UV.

The demonstration that this series of isothiazolones bound irreversibly to proteins, potentially through a reactive S-N bond, prompted an investigation of the role of thiol interactions in the inhibitory activity of the compounds toward HAT enzymes. A HAT filter assay was conducted to determine the effect of thiol reagents on the IC_{50} values of CCT077791 and CCT077792 toward the PCAF enzyme. Without DTT, both compounds gave an IC_{50} concentrations of ~2 μmol/L; however, in the presence of 1 mmol/L DTT, which alone had no significant effect on control values, HAT-inhibitory activity was blocked. No inhibition was obtained in the presence of DTT with compound concentrations up to 100 μmol/L. Similar effects were observed in the presence of 50 μmol/L glutathione (data not shown).

The ability of the compounds to bind covalently to PCAF was also examined. PCAF enzyme (14.5 μg) was incubated with 50 μmol/L compounds and an aliquot was removed and stored at −80°C. The remaining samples were dialyzed for 24 hours with two changes of assay buffer (2 L) and a filter assay was conducted on dialyzed against nondialyzed samples. Incubation of the PCAF enzyme with 2 mmol/L *N*-ethylmaleimide, a thiol-reacting agent, was used as positive control. The inhibition of PCAF by the two isothiazolones could not be reversed by dialysis, suggesting that the compounds were binding covalently to the enzyme protein (Fig. 5).

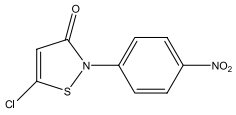
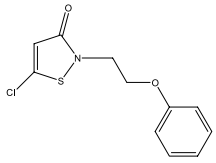
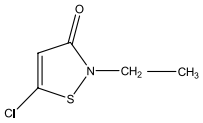
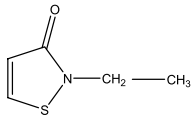
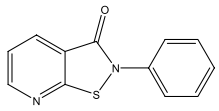
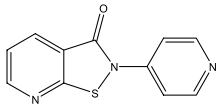
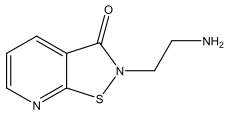
These findings may explain why rapid loss of cellular protein was observed in the cell-based assays. For example, in the time-resolved fluorescent cell-based immunosorbent assay, the BCA measurement of cellular protein, which is used to normalize europium counts to account for differences in protein levels between wells, showed a reduction of 26.2 ± 3.3% at 2 hours, 39.1 ± 0.3% at 12 hours, and 40.0 ± 1.2% at 24 hours

Table 4. CCT077791 reduces acetylation of individual lysine residues on histones H3 and H4

Acetylated lysine residue	HCT116 cells
K9 histone H3	38.6 ± 13.4
K14 histone H3	42.1 ± 3.0
K5 histone H4	43.8 ± 19.9
K8 histone H4	55.5 ± 5.4
K12 histone H4	43.2 ± 5.6

NOTE: Cells exposed to the respective GI_{50} concentrations for 2 hours were analyzed in the time-resolved fluorescent cell-based immunosorbent assay using acetylated lysine-specific antibodies (Upstate). Data represent percentage decrease in acetylation compared with control as mean ± SD for three replicates of one experiment.

Table 5. Protein binding of isothiazolones

Compound	Structure	% Bound (LC/UV)		% Bound (LC/MS)
		5 μmol/L	50 μmol/L	50 μmol/LCCT077791
CCT077791		49.2, 54.7	17.2, 67.8	ND
CCT077792		100, 96.0	100, 98.0	ND
CCT077796		100, 100	100, 100	ND
CCT079769		76.2, 87.1	83.1, 85.2	ND
CCT129182		ND	43.7	100
CCT129183		ND	18.1	100
CCT129184		ND	0	100

NOTE: BSA was incubated with selected isothiazolones (5 and 50 μmol/L) for 1 minute. Percentage of compound bound to protein was then determined using LC/UV and LC/MS (see Materials and Methods).

compared with DMSO controls following treatment with CCT077791 at 5× GI_{50}. Washout growth inhibition experiments were then conducted. Figure 6 shows that the growth-inhibitory effects of CCT077792 over 72 hours were similar whether cells were exposed to compound for 4 hours (GI_{50}, 0.8 μmol/L), 24 hours (GI_{50}, 1.3 μmol/L), or 72 hours (GI_{50}, 1.5 μmol/L).

Discussion

An understanding of the importance of histone and protein acetylation in fundamental cellular functions has led to an association between the expression and activity of HATs and disease, especially cancer (10, 14, 38). For example, aberrant HAT function has been identified in a number of epithelial and hematologic tumors due to a variety of

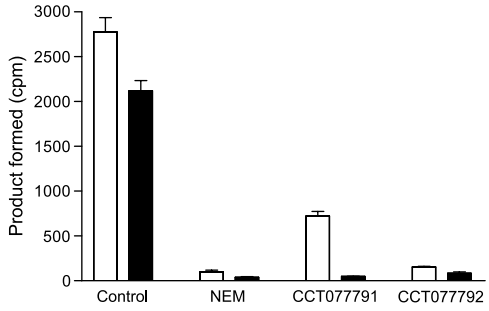


Figure 5. Isothiazolones inhibit PCAF HAT activity following dialysis. PCAF was incubated with CCT077791 or CCT077792 (50 μmol/L) for 10 min. DMSO and 2 mmol/L *N*-ethylmaleimide (*NEM*) were included as negative and positive controls, respectively. An aliquot of sample was then removed and stored at −80°C. The remaining sample was dialyzed as described in the text. A HAT filter assay was conducted to compare the activity of dialyzed (*filled columns*) versus nondialyzed (*open columns*) samples. *Columns,* mean; *bars,* SD.

genetic mutations, such as amplification, translocation, and point mutation of *HAT* genes (16, 19, 22, 39). In addition, inhibition of HATs has resulted in antiproliferative effects (24, 40, 41). These enzymes are therefore potential targets for cancer therapeutic intervention.

Currently, few HAT inhibitors have been described, with the exception of small acetyl-CoA peptides (25) and natural products, such as anacardic acid (26), garcinol (27), and curcumin (28). However, development of these agents as clinical candidates is likely to be hampered due to poor pharmacokinetic profiles, unclear cellular pharmacology, and potential for toxicity due to multiple cellular mechanisms. The availability of low molecular weight HAT inhibitors could prove valuable as laboratory tools to obtain further information on this class of enzyme, thereby providing information for target validation regardless of whether the compounds progress as development candidates for the treatment of cancer. With this goal in mind, a drug discovery project to identify inhibitors of HAT enzymes was established and a high-throughput screen (29) using recombinant PCAF enzyme was successfully completed.

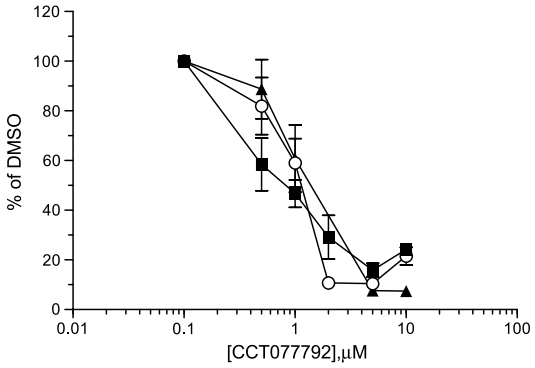


Figure 6. Short-term effects of CCT077792 on cell viability. HCT116 cells were exposed to CCT077792 (0.5–30 μmol/L) for 4 (■), 24 (○), or 72 (▲) hours before the medium was changed to contain no compound. Cells were reincubated until 72 h after compound addition and GI_{50} concentrations were determined. *Points,* mean of four replicates for one experiment; *bars,* SD.

Identification in the screen of a set of isothiazolone hitsed to the design and synthesis of 35 *N*-alkyl- and *N*-aryl-substituted isothiazolones. These compounds inhibited enzymatic activity of both PCAF (IC_{50} range, 2.5 to >50 μmol/L) and p300 (35–90% at 35 μmol/L) and also blocked cellular proliferation of a panel of human colon and ovarian tumor cell lines (96 hours GI_{50} range, 0.8 to >50 μmol/L). HDAC inhibitors cause rapid hyperacetylation of histones and other cellular proteins (42). HAT inhibition would be expected to lead to decreased acetylation of histones and other cellular proteins (27, 41). Four of the isothiazolones (Table 3) were selected for determination of their effects on cellular acetylation as determined by time-resolved fluorescent cell-based immunosorbent assay (31, 43). At an equimolar concentration (10 μmol/L for 24 or 48 hours), only two compounds (CCT077791 and CCT077792) showed the expected effect on cellular acetylation following a 24-hour exposure and to a similar extent after only 2-hour exposure. For the remaining two compounds (CCT077796 and CCT079769), the concentrations used (10 μmol/L) were approximately two and five times lower than their respective IC_{50} concentrations for enzyme inhibition; hence, the observed lack of hypoacetylation was not unexpected.

At low equitoxic doses (24 and 48 hours) of CCT077791 and CCT077792 (1× GI_{50} and 2× GI_{50}), neither compound significantly reduced cellular acetylation despite the appearance of an initial but nonsignificant effect in CCT077791-treated cells at 24 hours. Hypoacetylation of histones H3 and H4, of specific individual lysine residues on H3 and H4, and of α-tubulin was also shown. The relationship between the potency of experimental compounds on the putative target enzyme and that on the viability of cells can provide an insight into the contribution made by target inhibition to overall cellular effect. This comparison can highlight potential nonselectivity or ''off-target'' effects. In this limited series of compounds, the potency against the enzyme and cells is only comparable for CCT077791. For the other three compounds, the cellular GI_{50} values are significantly lower than the PCAF enzyme IC_{50} values (filter assay). For example, CCT077796 inhibits growth inhibition ~11 times more potently than the HAT activity of PCAF. In addition, CCT079769, which was only a weak inhibitor of the enzyme, did not show reduced cellular acetylation but showed relatively potent growth inhibition of 10 μmol/L (a concentration at least four to seven times less than its potency against the enzyme). These observations suggest that some of the isothiazolones may exert cytotoxic effects that are not associated with HAT inhibition.

During the course of these preliminary mechanistic studies, it was noted that the compounds showed rapid loss of cellular protein (20–50%). Subsequently, washout GI_{50}

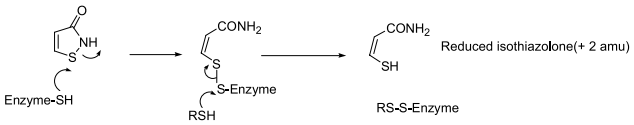


Figure 7. Proposed mechanism of action of isothiazolones (adapted from ref. 33).

experiments over 72 hours showed that cell growth was inhibited following only a 4-hour exposure and that the cells did not recover in the remaining incubation in drug-free medium.

Continuous exposure for 24 and 48 hours of CCT077791 and CCT077792 showed that at low equitoxic concentrations (1× GI_{50} and 2× GI_{50}) neither compound significantly affected acetylation compared with vehicle control despite the appearance of an initial but nonsignificant decrease in acetylation in CCT077791-treated cells at 24 hours. At 5× GI_{50} concentrations, however, both compounds significantly reduced acetylation at both time points.

A likely explanation for the rapid and irreversible loss of cell viability may be the chemical reactivity of isothiazolones. The reactivity of isothiazolones with cysteine residues has been described previously. Isothiazolones have growth-inhibitory activity toward *E. coli* and *Schizosaccharomyces pombe* (44). The addition of a thiol-reducing agent quenched this effect. Interaction of protein thiol groups has also been described for similar isothiazolones to those investigated here, which inhibit the interleukin-5 receptor (45), $p56^{lck}$ (34), and telomerase (46). Interestingly, however, the original isothiazolone hits did not markedly inhibit telomerase using the telomeric repeat amplification protocol assay.[3]

Consistent with these findings, HAT inhibition caused by this series of isothiazolones was abolished in the presence of the thiol-reducing agents DTT and glutathione. Furthermore, HAT activity was not restored in experiments involving the incubation of PCAF with both CCT077791 and CCT077792 followed by dialysis for 24 hours.

The inability to measure compounds in this series in extracts of plasma also highlighted their irreversible protein-binding properties. Using a LC/UV chromatographic method, >85% of the compounds in this series were highly bound (>85%) to protein (BSA). Subsequent analysis of three additional analogues using both LC/MS and LC/UV showed that compounds that seemed to have a low degree of binding to BSA when analyzed by LC/UV did so because the parent compound coeluted with its reduced ring-opened form. Closer examination of the LC/UV chromatograms consistently showed an earlier eluting peak in the incubated sample that was absent in the control. A chemical reaction scheme (Fig. 7) for the potential mechanism of isothiazolone reactivity has been proposed (34). It is likely that the covalent disulfide bridge of the drug-protein complex would be cleaved in the presence of excess thiol (e.g., cysteine and glutathione) to release the open-chain dihydro product as shown in Fig. 7.

To date, one key cysteine residue (Cys^{177}) has been identified as important for GCN5 HAT activity (47). Cys^{177} is believed to form hydrogen bonds that bind acetyl-CoA in close proximity with the histone substrate and to Glu^{173}, allowing catalysis of transfer of the acetyl group onto the lysine side chain of the histone (9, 48). Therefore, Cys^{177} is probably the amino acid whose side chain reacts with isothiazolones to form a new covalent bond with consequent loss of HAT catalytic activity. However, there is no direct evidence for this and an allosteric mechanism is also possible provided that it also involves covalent bonding.

In summary, the work described in this article has shown that a series of isothiazolones, identified from the high-throughput screening, inhibits HAT catalytic activity, are cell permeable, and can reduce global acetylation as well as acetylation of specific histones on H3 and H4 as well as the nonhistone protein, α-tubulin. In this limited series of aryl and alkyl N-substituted isothiazolones, protein binding of the compounds due to interaction with thiol groups runs in parallel with HAT inhibition. The compounds also seem to have considerable off-target effects, which may be attributable to their high chemical reactivity with thiol groups. This latter property also explains their extensive binding to plasma proteins.

The results from the high-throughput screening have suggested that the hit rate for identifying tractable HAT inhibitors is relatively low. It is interesting that in spite of great interest in HATs as therapeutic targets (26, 41, 49–51), few, if any, synthetic small-molecule (as opposed to natural product) inhibitors of these enzymes have been disclosed to date. The inhibitors identified here have significant potential liabilities that may restrict their use. However, the synthesis of further analogues in this series is ongoing to explore whether chemical reactivity can be minimized without compromising HAT catalytic inhibition.

Acknowledgments

We thank Chroma Therapeutics for valuable discussions.

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