A Novel Histone Acetyltransferase Inhibitor Modulating Gcn5 Network: Cyclopentylidene-[4-(4′-chlorophenyl)thiazol-2-yl)hydrazone

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Acetylation is a key modulator of genome accessibility through decondensation of the chromatin structure. The balance between acetylation and opposite deacetylation is, in fact, a prerequisite for several cell functions and differentiation. To find modulators of the histone acetyltransferase Gcn5p, we performed a phenotypic screening on a set of newly synthesized molecules derived from thiazole in budding yeast *Saccharomyces cerevisiae*. We selected compounds that induce growth inhibition in yeast strains deleted in genes encoding known histone acetyltransferases. A novel molecule CPTH2, cyclopentylidene-[4-(4′-chlorophenyl)thiazol-2-yl)hydrazone, was selected based on its inhibitory effect on the growth of a *gcn5*Δ strain. We demonstrated a specific chemical−genetic interaction between CPTH2 and HAT Gcn5p, indicating that CPTH2 inhibits the Gcn5p dependent functional network. CPTH2 inhibited an in vitro HAT reaction, which is reverted by increasing concentration of histone H3. In vivo, it decreased acetylation of bulk histone H3 at the specific H3-AcK14 site. On the whole, our results demonstrate that CPTH2 is a novel HAT inhibitor modulating Gcn5p network in vitro and in vivo.

Introduction

The main repeat unit of chromatin is the nucleosome. It is made up of an octameric histone core, bearing two copies of H2A, H2B, H3, and H4 with 145−147bp DNA wrapped around the central domain.[1,2] The degree of chromatin condensation determines the accessibility of genes to the transcriptional machinery. Several chromatin modifiers are responsible for adding different post-translational marks like acetylation, methylation, phosphorylation, and others on N-terminal histone tails and for dictating the degree of genomic compaction.[3−5] Histone acetyltransferases (HAT[a]) add the acetyl group on the specific lysine of histone H3 and H4 N-termini, and these signatures increase the accessibility of the underlying chromatin at specific genes or over vast regions of the genome.[6−9] Acetylation therefore determines a transcriptional active state of the genome. For this reason, HATs are directly engaged in transcription as coactivators.[10]

At least six distinct HAT families have been identified: GNAT (Gcn5-related *N*-acetyltransferase), which includes the Gcn5, Hat1, Elp3, Hpa2, MYST family, p300/CBP, Nuclear Receptor co activators, TBP-associated factor TAFII250, and TFIIIC.[11] Gcn5p, the prototype of histone acetyltransferases,[12] is the catalytic subunit of the two multiprotein complexes, ADA and SAGA, involved in remodeling the chromatin structure and acetylation of histone tails at specific lysines. Gcn5p is a chimeric protein made up of a number of functional domains; the HAT catalytic domain, the ADA domain, interacting with ADA and SAGA complexes, and the carboxy-terminal bromodomain, which has been shown to interact with histone at

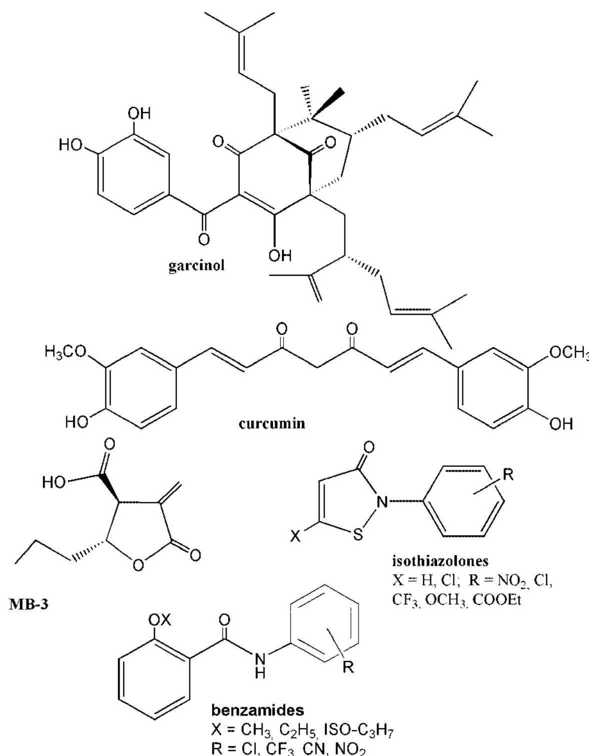

Figure 1. Known HAT inhibitors.

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[a] Abbreviations: HAT, histone acetyltransferase; GNAT, Gcn5-related *N*-acetyltransferase; HDAC, histone deacetylase; RTS, Rubinstein−Taybi syndrome; AIB-1, amplified in breast cancer-1; ADA, transcriptional adaptor; SAGA, Spt-Ada-Gcn5-acetyltransferase complex.

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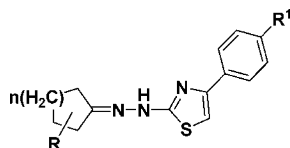
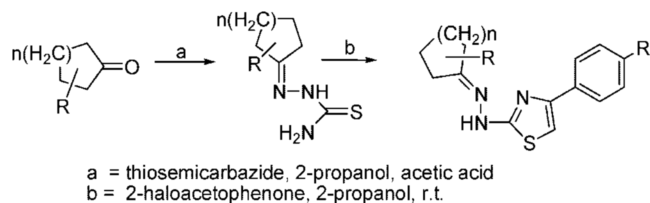


Figure 2. General chemical structure of synthesized thiazole compounds.

Table 1. Structures and Chemical Physical Data of Newly Synthesized Compounds **1−18**

compd	*n*	R	R[1]	mp °C	yield (%)	MW
1	1	H	OCH_3	196−197	66	287.38
2	1	H	Cl	190−191	99	291.80
3	1	2-CH_3	OCH_3	154−156	90	301.41
4	1	2-CH_3	Cl	175−177	99	305.83
5	1	3-CH_3	OCH_3	159−160	59	301.41
6	1	3-CH_3	Cl	184−186	83	305.83
7	2	H	OCH_3	140−142	57	301.41
8	2	H	Cl	174−175	89	305.83
9	2	2-CH_3	OCH_3	128−130	35	315.43
10	2	2-CH_3	Cl	187−188	99	319.90
11	2	3-CH_3	OCH_3	162−165	84	315.43
12	2	3-CH_3	Cl	195−199	84	319.90
13	2	(*R*)-(+)-3-CH_3	OCH_3	165−166	98	315.43
14	2	(*R*)-(+)-3-CH_3	Cl	201−203	99	319.90
15	2	4-CH_3	OCH_3	128−132	97	315.43
16	2	4-CH_3	Cl	177−179	92	319.90
17	3	H	OCH_3	175−176	99	315.43
18	3	H	Cl	194−196	98	319.85

Scheme 1. Synthesis of Compounds **1−18**



acetylated lysines.[13−15] The main targets for Gcn5p catalytic activity are mostly present on the histone H3 N-terminus, and one of the preferred substrates is lysine 14.[16] It has been shown recently that yeast Gcn5p is involved in the regulation of cell-cycle progression.[17,18] Accordingly, an unbalanced equilibrium between acetylation and deacetylation is often associated with cancer or leukemia.[19,20] In addition, misregulated HATs have been reported in solid tumors or in hematological malignancies.[21−23] For example, overexpression of AIB-1 (amplified in breast cancer-1),[24] a known HAT coactivator of a nuclear hormone receptor, is involved in breast, ovarian, and gastric cancer.[25,26] In addition, mutated HATs have also been found to be associated with genetic disorders like Rubinstein−Taybi syndrome (RTS).[27] Finally, they are also essential for HIV replication, and it has been reported that latent HIV is activated by treatment with HDAC inhibitors.[28] Collectively, a growing body of evidence demonstrates the relevance of histone acetyltransferases as novel targets for developing novel epigenetic drugs.[29,30]

Several compounds have been reported to inhibit HDACs and are undergoing clinical trials.[31,32] So far, however, few have been shown to inhibit HATs. It has been shown that among these, natural products like garcinol,[33] curcumin,[34] and anacardic acid[35] exhibit specific HAT activity. In particular, trifluoromethyl phenyl benzamides have been found to modulate p300[36] and isothiazolones as novel inhibitors of PCAF and p300 in cell cultures (Figure 1).[37]

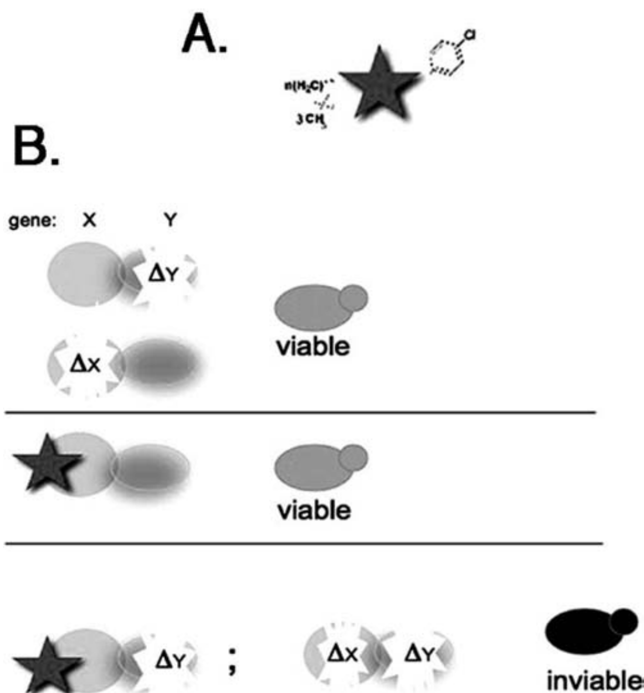


Figure 3. Chemical-genetic screening. In yeast *S.cerevisiae*, a chemiobiological approach may be used to identify drug (schematized as a star in A) secondary targets. The deletion of two genes (X and Y) that interact at a functional level produces a double-deleted strain showing a worse and sick phenotype (B). In this case, the new compound will produce a synthetic sick phenotype in which the deletion of a test gene (Y) interacts with a second gene (X) targeted by the new molecule.

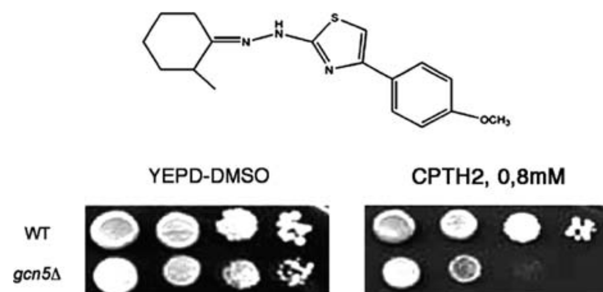


Figure 4. Yeast growth spot assay. 10-Fold serial dilutions of liquid yeast cell cultures were spotted on agar-solid medium. Compound **9** was added at a final concentration of 0.8 mM. Wild type, WT (S288c) and isogenic *gcn5*Δ strains were spotted and allowed to grow for 72 h at 28 °C.

Chemistry. Starting from these studies and continuing our research into the design and biological evaluation of new active molecules, we synthesized a new series of thiazole derivatives (**1−18**) (Figure 2 and Table 1).

Cycloalkylidene-(4-phenylthiazol-2-yl)hydrazone derivatives were synthesized as we reported in a previous communication (Scheme 1).[38]

The appropriate cyclic ketone reacted directly with thiosemicarbazide. The obtained cycloalkylidene-thiosemicarbazone subsequently reacted with suitable 2-haloacetophenones to yield the 4-substituted thiazole derivatives. In the synthesis of all compounds, 2-propanol proved to be the best solvent for our purpose. As a matter of fact, the reaction products precipitate and can be filtered and purified by crystallization from ethanol

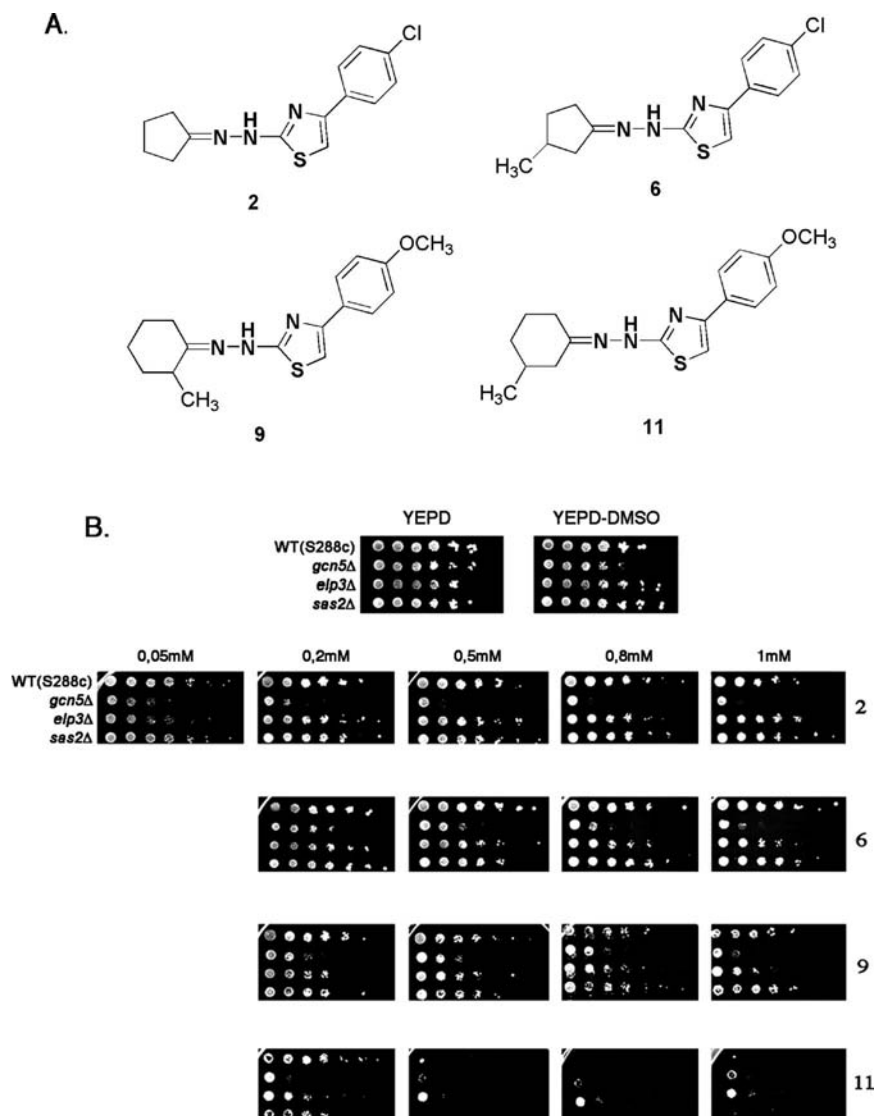


Figure 5. Selective growth inhibition of compounds **2**, **6**, **9**, and **11**. (A) Compounds subjected to cell growth spot assay. (B) Cell growth inhibitory effects of **2**, **6**, **9**, and **11** on WT (S288c) and isogenic strains deleted in HATs GCN5 (*gcn5*Δ), Elp3 (*elp3*Δ), and Sas2 (*sas2*Δ). Cells were serially diluted and grown for 72 h at 28 °C.

or ethanol/isopropanol. All the synthesized products were characterized by spectroscopic methods (see Supporting Information).

Results and Discussion

Cell Growth Inhibition in Wild Type and Strains Deleted in Gcn5, Elp3, and Sas2. Budding yeast is an ideal model system where fundamental cellular functions and machineries are highly conserved throughout evolution and, therefore, they may be finely dissected using several genetic and molecular tools. Up to about a decade ago, enzymes involved in regulatory signal-transduction pathways were described as independent entities. It has since become clear, however, that they act in interacting modules critical to a wide range of other biological processes.[39,40] In *Saccharomyces cerevisiae*, powerful functional genomics tools have been developed, thus revealing the importance of the interacting protein network. Genetics can be used to understand how mutations in two genes interact to modulate the resulting phenotype. Thus, by following functionally coherent genetic networks, it is possible to reveal the gene function. To gain information on global and highly complex biological processes, small molecules may be used as the chemical counterparts of genetic mutations, as schematized in Figure 3. We used a yeast-based drug-screening to identify novel HAT inhibitory compounds capable of reducing the growth of strains deleted in specific HATs like Gcn5p, Elp3, and Sas2.[41,42]

First, liquid cell cultures of wild type (S288c) strain and isogenic *gcn5*Δ lacking Gcn5p were serially diluted and spotted on agar solid medium (YEPD-DMSO) in the presence of the thiazole derivative **9** (Figure 4). Cells were incubated at 28 °C and allowed to grow for 72 h. The strain deleted in GCN5 showed a consistent inhibition of growth in the presence of the thiazole derivative (0.8 mM). This molecule was therefore taken as the lead compound for the synthesis of a novel set of the thiazole derivatives listed in Table 1. Compounds **1**−**18** were assayed for growth inhibition on wt and *gcn5*Δ strains and tested as shown in Figure 4 (data not reported). Compounds **2**, **6**, **9**, and **11** (Figure 5A) produced the strongest effect on *gcn5*Δ growth and, for this reason, were chosen to be tested not only on the Gcn5 deleted strain (*gcn5*Δ) but also on isogenic strains deleted in other HATs like Elp3 (*elp3*Δ) and Sas2 (*sas2*Δ). Growth spot assay (Figure 5B) showed that the closely related compounds **2**, **6**, and **9** produced similar inhibitory effects with marked selectivity for the *gcn5*Δ strain. Compound **11**, con-

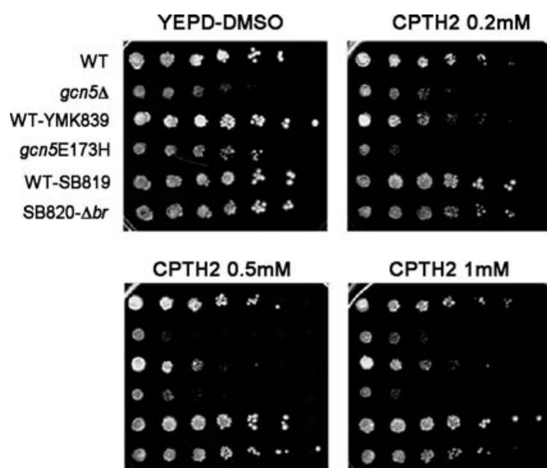


Figure 6. CPTH2 inhibits the growth of a GCN5 deleted strain and a single catalytic mutant E173H. YEPD-DMSO solid medium was supplemented with CPTH2 at the indicated, increasing concentrations. Yeast strains fully deleted *gcn5*Δ, HAT catalytic mutant *gcn5*E173H, and bromodomain deleted Δ*br* were compared with the growth of the respective isogenic wild type strains (WTS288c, WT-YMK839, and WT-SB819) listed in Table 2.

Table 2. *S. cerevisiae* Strains Used

strain	genotype	ref
S288C	*MAT alpha, SUC2, gal2, mal, mel, flo1 flo8−1, hap1*	50
*gcn5*Δ	*S288c background. Gcn5::KanMX*	T. Harkness
*sas2*Δ	*S288c background. Sas2::KanMX*	T. Harkness
*elp3*Δ	*S288c background. Elp3::KanMX*	T. Harkness
YMK839	*MATa, trp1, leu2−3, 112, ura3−52*	45
YMK986	*MATa, gcn5E173H, trp1, leu2−3, 112, ura3−5*	45
SB819	*MATalpha, gcn5::HIS3, (pRS306-P(ADH) -yGcn5 1−439 (URA3)), his3Δ200 leu2Δ1 ura3−52*	S. Berger
SB820ΔBro	*MATalpha, gcn5::HIS3, (pRS306-P(ADH) -yGcn5 1−350 (URA3)). his3Δ200, leu2Δ1 ura3−52*	S. Berger

versely, also showed a broader inhibitory effect on Elp3 and Sas2 deleted strains too. For this reason, it was not studied any further. We focused our analysis on compound **2**, cyclopentyl-idene-[4-(4′-chlorophenyl)thiazol-2-yl)hydrazone (CPTH2).

Growth of Gcn5 Catalytic Mutant in Invariant Glutamic Acid 173 is Inhibited by CPTH2. HAT reaction involves the formation of a ternary complex (histones, acetyl-CoA, and enzyme). In this reaction, glutamic acid 173 is a Gcn5p essential residue required to offer a base catalyst deprotonating the histone.[43,44] We decided to test whether the loss of the catalytic activity of Gcn5p or the acetyl-lysine binding bromodomain was correlated to CPTH2 sensitivity shown by the gcn5 deleted strain. Either these yeast mutants carry a single aminoacidic substitution E173H (*gcn5*E173H) in glutamic acid 173, which completely abolishes their HAT catalytic activity,[45] or they are deleted in the carboxyterminal bromodomain (SB820-Δ*br*).[46] They were spotted and assayed on plates additioned with CPTH2 at increasing concentrations. The growth patterns of the strains *gcn5*Δ, *gcn5*E173H, and SB0-Δ*br* are shown in Figure 6 Each mutant was compared with the respective isogenic wild type S288c, YMK986, and SB819. The result clearly demonstrates that while deletion of the bromodomain does not affect growth in the presence of CPTH2 even at a concentration of 1 mM, the growth of the catalytic mutant *gcn5*E173H is heavily inhibited by CPTH2 phenocopying deletion of the whole gene. This result indicates that the catalytic activity of Gcn5p is required for the inhibition exerted by compound CPTH2.

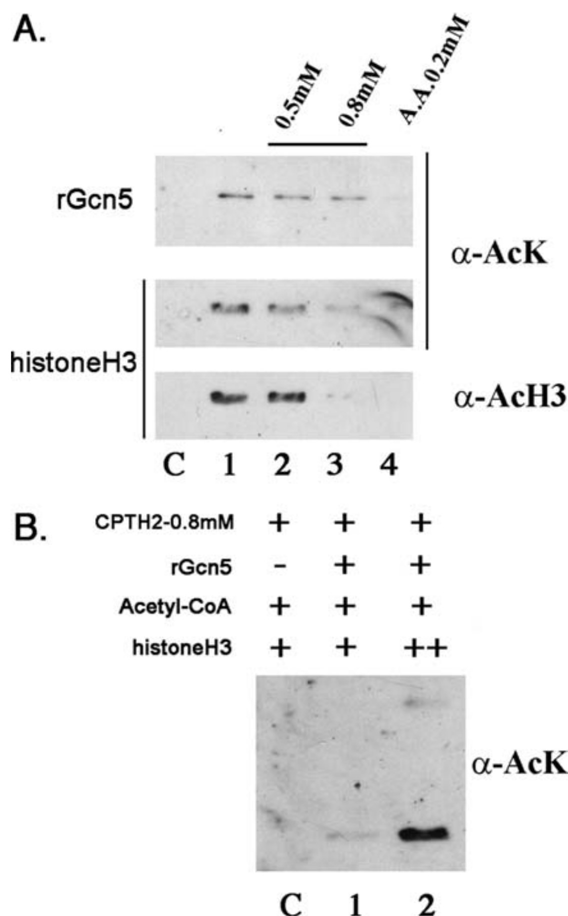


Figure 7. CPTH2 inhibits the HAT activity of recombinant Gcn5 in vitro bridging histone H3. (A) Effects exerted by CPTH2 on the in vitro HAT assay by recombinant Gcn5r. C, control reaction without Gcn5s, lane 1 without CPTH2, lane 2, and 3 in the presence of CPTH2 0.5 mM and 0.8 mM, respectively, lane 4 plus AA (anacardic acid) 0.2 mM. Western blot was hybridized first with the α-AcK antibody and a second time with α-AcH3. The (B) HAT assay was performed in the presence of CPTH2 0.8 mM at increasing concentrations of histone H3 substrate 1 (100 ng) and 2 (200 ng).

Inhibition of in Vitro Histone H3 Acetylation by Recombinant Human Gcn5. The in vivo specificity for Gcn5p encouraged us to test the inhibitory effect of this novel molecule directly on the in vitro histone acetyltransferase reaction. The catalytic reaction was run in microfuge tubes using acetyl-CoA, histone H3 (Roche), and human recombinant Gcn5r. Samples were incubated at 28 °C for 1 h, loaded on 15% SDS-PAGE, blotted, and serially hybridized first with ΔAc-Lys antibody (Santa Cruz) and then with a pan-acetylated Ac−H3 antibody (Upstate). The results are shown in Figure 7. The reaction was inhibited in the presence of CPTH2 (0.8 mM) and similarly that of anacardic acid (0.2 mM)[35] chosen as a bona fide reference drug. The reaction revealed autoacetylation on rGcn5, which was missing in the anacardic acid sample. This suggests that the two molecules inhibit the reaction in a profoundly different way. A drastic reduction in the acetylated histone H3 was obtained by addition of either CPTH2 or anacardic acid (Figure 7A). To understand how CPTH2 might inhibit the HAT reaction in vitro, we performed a substrate competition assay. A 2-fold increased concentration of histone H3 was sufficient to completely reverse the inhibition by CPTH2 on the HAT reaction (Figure 7B). We performed the same assay while increasing Acetyl-CoA with no effect (data not shown). To demonstrate once more the role of histone H3 in bridging CPTH2, it is

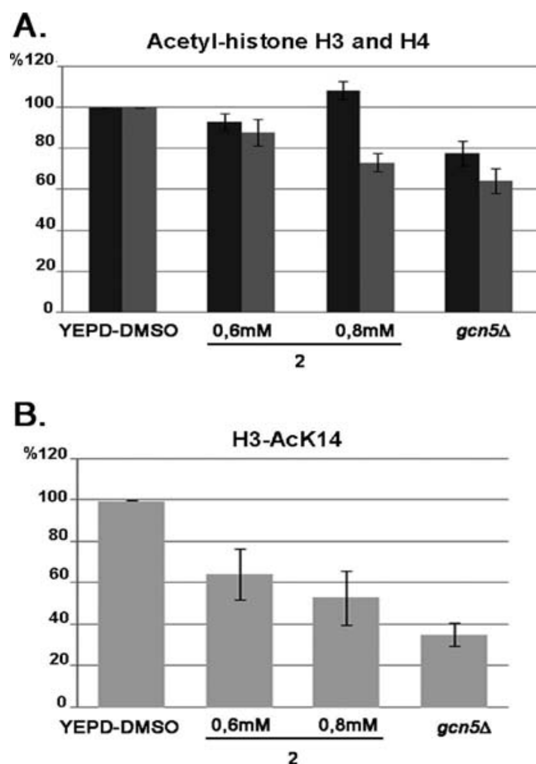


Figure 8. CPTH2 inhibits histone H3 acetylation in vivo. (A) Acetylation of histone H4 (black bar) and H3 (gray) of bulk histone preparation from wild type yeast cells grown in rich medium (YEPD-DMSO), or medium supplemented with CPTH2 (2) at 0.6 and 0.8 mM, as the control acetylation level in the disrupted *gcn5*Δ strain grown in YEPD-DMSO is reported. (B) Acetylation of histone H3 at specific lysine14, H3-AcK14 in the same samples shown in panel A.

significant that CPTH2 has no effect of on the auto acetylated band corresponding to rGcn5 (Figure 7A), which is inhibited by anacardic acid but not by CPTH2. This demonstrates the specific activity of CPTH2, which, by bridging histone H3, might interfere directly with Gcn5p catalytic activity.

Inhibition of Global Histone H3 Acetylation in Vivo. To assess the effect of CPTH2 on global cellular histone acetylation, we tested yeast cell cultures grown in the presence of CPTH2 0.8 M for 24 h at 28 °C. Global acetylation of histone H3 was analyzed by hybridization with primary antibody recognizing histone H3 and H4 global acetylation or directed toward specific lysine H3−K14Ac. The hybridization signals were normalized to a constitutively expressed yeast protein, Ada2p, as internal reference of protein loading onto the gel. The results are shown in the histogram in Figure 8. Panel A shows the decrease in global acetylation of histone H3. After treatment of cells for 24 h with CPTH2 0.8 mM, we obtained a 30% decrease in histone H3 bulk acetylation, as compared to *gcn5*Δ strain. Conversely no reduction in acetylation of histone H4 was obtained. The same experiment was used to trace acetylation of histone H3 at the single lysine 14 (Figure 8B), which is one of the preferred targets of Gcn5p. The result showed a greater decrease in H3K14Ac acetylation, which was fully comparable to that obtained in the deleted GCN5 strain. We therefore conclude that acetylation in vivo by CPTH2-treated cells of bulk histones is more significantly reduced at histone H3-lys14, which is one of the main targets for Gcn5p HAT activity.

Conclusion

HATs and HDACs belong to a complex network of proteins, which act as modifiers in the epigenetic control of histones.[3] Acetylation and deacetylation, along with other post-translational modifications of histone tails like phosphorylation and methylation, are essential elements of the combinatorial histone "code".[4,5] The balanced equilibrium between acetylation and reverse deacetylation is the key mechanism in regulating transcriptional activity and gene expression during differentiation or normal cell division.[17,19] Alteration of this fine equilibrium, as in the case of mutations and translocations in HAT genes, is common in cancer and other diseases.[21−23] Furthermore, there is also a growing body of evidence supporting the importance of acetylation in regulating nonhistone proteins that act in several cellular processes.[11]

Given the important role of acetylation, the focus of many studies in recent years has been on development inhibitors of both HAT and HDAC as promising classes of anticancer agents. Generally speaking, the potential of HDACs inhibitors are well-documented and several molecules are undergoing clinical trials as chemotherapeutic agents.[31,47] On the contrary, very little is known about HATs and their related specific inhibitors. In this study, with the aim of identifying a specific HAT inhibitor related to Gcn5p, we decided to perform a yeast-based growth-inhibition screening. From a wide selection of molecules, a thiazole derivative, capable of inhibiting growth of a *gcn5*Δ strain, was identified and a subset of closely related derivative molecules **1**−**18** were synthesized. After a preliminary screening, CPTH2 was chosen for its highly selective inhibition on the growth of a *gcn5*Δ strain at 0.8 mM. In fact, CPTH2 has no effect on the growth of yeast strains deleted in other HATs like Elongator Elp3 and Sas2. This evidence indicates that CPTH2 targets the Gcn5p functional network through an interacting protein.[40]

Furthermore, we showed, that CPTH2 inhibits growth not only in a fully deleted strain of GCN5 but also in a mutant carrying a single amino acid substitution in the catalytic residue *gcn5*E173H, thus demonstrating that its inhibitory effect is directed at HAT catalytic activity.

We next investigated the mechanism of action of CPTH2 on the in vitro HAT assay by recombinant Gcn5r. We showed that CPTH2 inhibits the in vitro acetylation of histone H3 in a similar way to anacardic acid, used as a control inhibitor.[35]

The growth results suggested that CPTH2 was selective for a Gcn5p and that it might target an interacting partner. In parallel, in vitro data showed a direct inhibition on the acetylation reaction. We therefore wanted to test the pharmacokinetic characteristics of this novel compound by performing a substrate competition assay. We found that, in vitro, a 2-fold increase in histone H3 was sufficient to reverse the HAT-inhibitory activity of CPTH2, leading us to conclude that CPTH2 may act on Gcn5p via bridge-interaction with histone H3, which in turn inhibits HAT catalytic activity. To date, this novel molecule and its high selectivity provide a novel discriminating compound for targeting the Gcn5p specific protein network.

In the presented analysis, we have provided experimental data demonstrating that histone H3 is responsible for the targeted inhibition of CPTH2 on Gcn5 in vitro. In vivo, we propose that CPTH2 might interfere with additional, unidentified histonic and/or non histonic Gcn5p substrates, resulting in a more severe growth defect. We have shown that CPTH2 is highly selective for the Gcn5p functional network, providing a tool for targeting Gcn5p defective cells, which are recurrent in many types of solid and hematological tumors.

Because genetic mutations and translocations in HAT genes have been found in a number of epithelial and hematological tumors,[24,48] we believe that CPTH2 and future derived com-

pounds will be of great interest, taking into account the selectivity toward the Gcn5p functional network here demonstrated in yeast. Our preliminary mechanistic studies will be improved in the future in order to gain a better understanding of the mechanism of CPTH2 inhibition. The CPTH2 growth inhibition of yeast Gcn5 deleted cells may be useful for screening selected human cell cultures corresponding to specific pathological states where Gcn5 is mutated or not fully functional.

Experimental Section

Chemistry. Melting points are uncorrected and were determined on a Reichert Kofler thermopan apparatus. ^{1}H NMR spectra were recorded on a Bruker AMX (300 MHz) in DMSO or $CDCl_3$ as solvents (chemical shifts in δ values, J in Hz) referring to the solvent peak. Elemental analyses for C, H, and N were performed on a Perkin-Elmer 240 B microanalyzer, and the analytical results were within ±0.4% of the theoretical values.

General Procedure for the Preparation of Cycloalkylidene-(4-phenylthiazol-2-yl)hydrazone Derivatives (1−18). The appropriate cyclic ketone (50 mmol) was dissolved in 100 mL of 2-propanol and refluxed under magnetic stirring with an equimolar amount of thiosemicarbazide for 24 h. The thiosemicarbazone, precipitated from the reaction mixture, was filtered and crystallized from $EtOH/CH_2Cl_2$. Equimolar amounts of the thiosemicarbazone and of the haloacetophenone, both dissolved in 2-propanol, were reacted at room temperature under stirring for 2 h. The white precipitate was filtered and crystallized from ethanol or ethanol/2-propanol to give compounds **1**−**18**. For all compounds **1**−**18**, we report the chemical and physical properties in Table 1 and in the Supporting Information.

Yeast Strains and Growth. All yeast strains used in this study are listed in Table 1. The *gcn5*Δ, *elp3*Δ, and *sas2*Δ strains (from Dr. T. Harkness) were disrupted in wild type S288c background. Catalytic mutant gcn5E173H (strain YMK986) was obtained from M. H. Kuo and bromodomain deleted (SB820Δbro) from S. Berger laboratory. Yeast cells were grown at 28 °C in YEPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) liquid or solid (plus 2% agar). YEPD was supplemented with solvent DMSO (1%). To test the sensitivity of the derivatives, 0.2 OD/mL of yeast cells growing exponentially were serially diluted (1/5) and spotted on solid medium containing each compound. CPTH2 was dissolved in DMSO (100 mM stock solution) and added to the medium at the indicated concentrations.

HAT Assay. The HAT assay tube reaction was run for 1 h at 28 °C in the presence of human Gcn5r (50 ng, Active Motif), Acetyl-CoA (1 mM) and histone H3 (100, 200 ng) (Roche) in assay buffer (Tris-HCl pH 8.0 50 mM, EDTA pH 8.0 0.1 mM, KCl 50 mM, DTT1 mM, glycerol 5%), with or without CPTH2 as indicated in the text. Western blot filters were serially hybridized with acetylated anti-AcH3 (Upstate) and anti-AcLys (Santa-Cruz) primary antibodies.

Protein Extraction for Histone Bulk Preparations. Protein extraction from yeast was performed using an alkaline protocol.[49] Extracted proteins were run on 15% SDS-PAGE gels and blotted onto Hybond membranes (Amersham). Histones H3−Ac, H4−Ac, and H3-AcK14 (Upstate) were detected using primary histone or antiAc-Lys antibodies, acetylated signals were normalized with the anti-Ada2 (Santa-Cruz) antibody, revealing a constitutively expressed yeast protein. The signals were quantified by Optiquant densitometric scanning, and the respective values are reported in the histogram shown in the Figure 8. HRP-labeled IgG secondary antibodies were diluted 1:10000. Protein signals were visualized using an Enhanced Chemio-Luminescence (ECL) system (Amersham).

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Supporting Information Available: Analytical and spectral data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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