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A novel Gcn5p inhibitor represses cell growth, gene transcription and histone acetylation in budding yeast

Prisca Ornaghi [a], Dante Rotili [b], Gianluca Sbardella [c], Antonello Mai [b], Patrizia Filetici [d,*]

[a] *Dipartimento Genetica e Biologia Molecolare, Università degli Studi di Roma "La Sapienza", P.le A. Moro 5, I-00185 Roma, Italy*
[b] *Istituto Pasteur-Fondazione Cenci Bolognetti, Dipartimento di Studi Farmaceutici, Università degli Studi di Roma "La Sapienza", P.le A. Moro 5, I-00185 Roma, Italy*
[c] *Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte don Melillo, I-84084 Fisciano (SA), Italy*
[d] *Istituto di Biologia e Patologia Molecolari CNR, Dip. Genetica e Biologia Molecolare, Università degli Studi di Roma "La Sapienza", P.le A. Moro 5, I-00185 Roma, Italy*

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Abstract

Histone acetyltransferases are key chromatin regulators responsible for transcriptional activation and cell cycle progression. We propose a simple yeast-based assay to determine the specificity and targets of novel Gcn5p inhibitors. Here, we report the finding of a novel, small molecule, MC1626, which is able to inhibit yeast cell growth, Gcn5p-dependent gene transcription and acetylation of the histone H3 N-terminal tail in vivo. Because HATs misregulation is invariably associated with human diseases, the identification of MC1626 as a novel cell-permeable Gcn5p inhibitor suggests that it may be a very useful starting tool for the further development of new molecules to be applied to expression profiling of genes regulated by histone H3 acetylation. In addition, our results demonstrate that MC1626 is a Gcn5p-dependent yeast growth inhibitor.

Keywords: Gcn5p; HAT inhibitor; Histone acetyltransferase; MC1626; Chromatin; Yeast

1. Introduction

Chromatin acetylation induces conformational changes in the nucleosome; subsequently, modification of chromatin structure makes the nucleosome accessible to transcriptional machinery. In the cell, the dynamic equilibrium between acetylation and deacetylation is maintained by the activity of histone acetyltransferases (HATs) and deacetylases (HDACs), which thus regulate the expression of the whole genome [1]. Acetylation of the ε-amino group of lysines is important not only in determining the epigenetic histone code but also in the post-translational modification of an increasing number of other proteins, including DNA-binding proteins [2], general transcription factor-like TFIIB [3], DNA metabolic enzymes and viral effectors like Tat [4–6], E1A [7] and polyoma virus large T antigen. Aberrant lysine acetylation is implicated in oncogenesis. Very often, mutations and translocations lead to the production of chimeric activators like MLL-p300 and MLL-CBP, which are recurrent in malignancy [8]. This provides evidence that mistargeted HATs are correlated to leukemogenesis. Acetylation of non-canonical target genes and misregulation of HATs are recurrent in cancer [9] and viral infection [10]. For example, C-MYC, one of the most frequently overexpressed oncogenes in human cancer [11], is a substrate for hGCN5/PCAF, and its acetylation increases c-Myc stability [12]. This may be pathologically relevant, since a mere two-fold change in its expression has been shown to affect cell cycle progress. Similarly, acetylation of androgen receptors enhances coactivator binding and promotes prostate cancer growth [13]. HAT hGcn5 is also involved in viral infection. Latent HIV [14] is activated by acetylation of the Tat transactivator. This brief summary shows how GCN5 and HATs proteins [15,16] are crucial players in several diseases, however, HATs-specific

* Corresponding author. Tel.: +39 06 49912241; fax: +39 06 4440812.
E-mail address: patrizia.filetici@uniromal.it (P. Filetici).

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inhibitors and their possible application to medical therapy have been poorly investigated so far [17].

Here, we present experimental data showing the specific activity of a novel small cell-permeable molecule, MC1626 (2-methyl-3-carbethoxyquinoline) Fig. 1, as a histone acetyltransferase Gcn5p inhibitor in yeast. This compound was found in the attempt to design novel chinolic analogs of p300 inhibitor anacardic acid. We demonstrated that treatment of yeast cells with MC1626 inhibits cell growth in a Gcn5p-dependent way. Basal, more drastically activated transcription of the HIS3-lacZGcn5p-regulated promoter [18] is inhibited by MC1626, whereas the constitutive Gcn5p-independent GAL10-CYC1-lacZ (Guarente) is not. These results provide strong evidence for a Gcn5p-specific transcriptional inhibition not due to a broad, unspecific effect. Moreover, we have shown that the level of histone H3 acetylation is drastically diminished after incubation with the inhibitor, again providing evidence that this small molecule can significantly diminish Gcn5p-dependent H3 acetylation in vivo.

The development and the study of new small HATs inhibitors [19] are valuable not only for long-term application but also for selective transcriptome analysis of Gcn5p-regulated genes. Very few HATs inhibitors can permeate the cell membrane [20]; to date only anacardic acid, garcinol [21] and curcumin [22] have been reported. Here we show that the MC1626 molecule is able to inhibit yeast cell growth, Gcn5p-dependent transcriptional activation and histone acetylation in vivo. Future work will be needed to analyze in more detail the selectivity of this compound for other HATs and responsive genes in a wide genomic analysis. Going forward, our interest will focus on the design of more potent MC1626-related compounds.

2. Materials and methods

2.1. Materials

Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. ^{1}H NMR (sciogliere la sigla; scrivi il termine prima per esteso, poi tra parentesi inserire la sigla) spectra were recorded at 400 MHz on a Bruker AC 400 spectrometer; chemical shifts are reported in δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). All compounds were routinely checked by TLC (sciogliere la sigla) and ^{1}H NMR. TLC was performed on aluminium-backed silica gel plates (Merck DC, Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light. All solvents were reagent grade and were purified and dried by standard methods when necessary. Concentration of solutions after reactions and extractions was carried out using a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulphate. Analytical results are within 0.40% of the theoretical values. All chemicals were purchased from Aldrich Chimica (Milan, Italy) or from Lancaster Synthesis GmbH (Milan, Italy), and were of the highest purity.

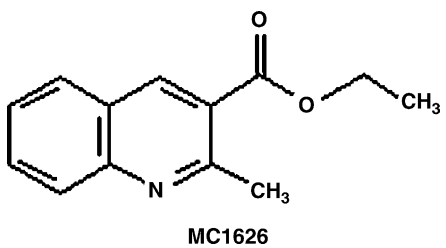

Fig. 1. Structure of MC1626.

2.2. Synthesis of 2-methyl-3-carbethoxyquinoline, MC1626

A 250-mL round-bottom flask equipped with a stir bar and reflux condenser was flame dried under an atmosphere of N_2. *o*-Nitrobenzaldehyde (1.00 g, 6.6 mmol) and ethyl acetoacetate (1.03 g, 7.9 mmol) were added, followed by 40 mL of anhydrous ethanol. Anhydrous $SnCl_2$ (6.270 g, 33.0 mmol, 5 equiv.), $ZnCl_2$ (4.50 g, 33.0 mmol, 5 equiv.), and approximately 1.0 g of 4 Å molecular sieves were added to the solution. This mixture was then heated at 70 °C under an atmosphere of N_2 for 3 h. The reaction was cooled to room temperature and made basic (pH 8) with 100 mL of 10% $NaHCO_3$. The mixture was then transferred to a separatory funnel and extracted with 3 × 40 mL of ethyl acetate. Organics were combined and washed thoroughly with saturated NaCl, and dried over Na_2SO_4. Following vacuum evaporation of the solvent, the remaining material was subjected to chromatography on silica gel column eluting with an ethyl acetate:chloroform (1:10) mixture to give MC1626 as a white solid. Yield: 75%; mp: 68–70 °C; [23] recrystallization solvent: cyclohexane; ^{1}H NMR ($CDCl_3$) δ 1.46 (t, 3H, CH_2CH_3), 3.00 (s, 3H, CH_3), 4.44 (q, 2H, CH_2CH_3), 7.54 (m, 1H, *H* C_6), 7.78 (m, 1H, *H* C_7), 7.86 (dd, 1H, *H* C_5), 8.04 (dd, 1H, *H* C_8), 8.74 (s, 1H, *H* C_4). Anal. ($C_{13}H_{13}NO_2$): calculated C, 72.54%; H, 6.09%; N, 6.51%; found C, 72.83%; H, 6.12%; N, 6.27%.

2.3. Yeast strains and growth

All yeast strains used in this study are listed in Table 1. The yPO4 and yPO13 strains were produced by gene disruption using a polymerase chain reaction (PCR) cassette carrying kanMX4 [24] or the HIS3 gene. Cells were grown at 28 °C in YPD rich medium (1% yeast extract, 2% bactopeptone, 2% glucose) or in SD minimal medium (0.67% YNB (yeast nitrogen base) 2% glucose), SG minimal medium (0.67% YNB, 2% galactose) both supplemented with 0.01% of requested amino acids. For amino acid deprivation experiments, the yPO14 strain

Table 1
S. cerevisiae strains

Name	Genotype	Reference
W303	*MATa, ade2-1, trp1-1, leu2-3, 112 his3-11,15, ura3, can1-100, ssd1*	
yPO4	*MATa, gcn5::KanMX4, ade2-1, trp1-1, leu2-3, 112 his3-11,15 ura3 can1-100, ssd1*	This work
yPO13	*MATa, ade::HIS3 ade2-1, trp1-1, leu2-3, 112 his3-11,15, ura3, can1-100, ssd1*	This work
yPO14	*MATa, ade::HIS3 ade2-1, trp1-1, leu2-3, 112 his3-11,15, ura3, can1-100, ssd1 (YCp87-LEU2, his3-LacZ)*	This work
yPO15	*MATa, ade::HIS3 ade2-1, trp1-1, leu2-3, 112 his3-11,15, ura3, can1-100, ssd1 (pLGSD5-URA3, GAL10-CYC1-LacZ)*	This work
yMK839	*MATa, trp1, leu2-3,112, ura3-52*	[30]
yMK984	*MATa, trp1, leu2-3,112, ura3-52 gcn5F221A*	Kuo

was inoculated into 20 mL of SD and incubated at 28 °C for 16 h, then 25 mM 3-aminotriazole (3-AT) were added for 4 h at 28 °C with shaking. Cells were harvested and used for β-galactosidase determinations. For galactose induction experiments, the yPO15 [25] strain was inoculated into 20 mL of SD and incubated at 28 °C for 16 h; the cells were then washed and grown in SG for 4 h at 28 °C. Microscopic observations were performed using a fluorescence microscope (Axioskop Zeiss) equipped with oil immersion objective 100× and 10× ocular.

2.4. Determination of β-Gal activities

Protein extracts were prepared from 0.8 OD_{600} harvested yeast cultures. Cells were washed twice with H_2O and once with extraction buffer (100 mM Tris–HCl; 1 mM DTT; 20% glycerol). Soluble extracts were prepared by resuspending whole cells in extraction buffer and grinding them with glass beads in a Vortex mixer. Protein concentration was determined by the Bradford method [26]. β-Gal activities were determined as previously described [27]. One unit of β-galactosidase corresponds to μmol of *o*-nitrophenol produced per minute.

2.5. Protein extraction and Western blot analysis

Protein extraction from yeast was performed using an alkaline protocol [28]. Extracted proteins were run on 15% SDS-PAGE gels and blotted onto Hybond membranes (Amersham Histones H3, H3-Ac and H4-Ac were detected using primary histone antibodies (Upstate Biotechnology), and HRP-labeled IgG secondary antibody, diluted 1:10000. Detected proteins signals were visualized using an enhanced chemiluminescence (ECL) system.

3. Results

3.1. Growth inhibition of yeast cells in the presence of MC1626

Yeast strains lacking Gcn5p (gcn5Δ) showed slow growth and accumulation in G2 phase [29]. Accordingly, we sought compounds that could be added to the medium to slow cell growth and thus mimic the effects produced by the absence of Gcn5p. We found that the small molecule MC1626 was cell permeable, as demonstrated by direct microscopy visualization of fluorescent MC1626 present inside the treated yeast cells (Fig. 2A). The picture shows that the molecule is similarly located inside wild-type and null *gcn5*Δ disrupted yeast cells, which accounts for the same cell permeability of both strains; the lower fluorescent signal in the bud is related to the cell cycle stage of the emerging bud. Yeast growth of a wild-type strain was assayed in liquid culture in the presence of MC1626 at a concentration of 0.6 and 1.0 mM. Compared with the growth of the untreated strain, the growth curves of the treated strain slowed increasingly as MC1626 was added (Fig. 2B). To assay for Gcn5p selectivity, we tested the growth of a disrupted *gcn*5Δ strain, which, because it lacked Gcn5p, was expected to be less sensitive to inhibitor treatment. In the presence of MC1626, wild-type cell growth was impaired, while null *gcn*5Δ was remarkably less sensitive to the inhibitor and showed persistent growth (Fig. 2C left panel). Point mutation F221A in the HAT minimal catalytic domain of yGcn5p has been reported to abolish both promoter-directed histone acetylation and Gcn5p-mediated transcriptional activation [30]. To determine whether there was a direct link between MC1626 activity and the catalytic activity of Gcn5p, we tested the effect of MC1626 on growth in the mutant F221A and compared it with the respective isogenic wild-type strain (yMK839) A comparable effect of MC1626 on both the fully disrupted and the catalytic mutant strains provided evidence that inhibition is exerted on the catalytic activity but not on the whole protein (Fig. 2C, right panel). To further validate the spot assay, we followed growth in liquid culture in the presence or absence of 1 mM MC1626 in the disrupted *gcn*5Δ (Fig. 2C, bottom-left) and in the catalytic F221A mutant (Fig. 2C, bottomright). While the growth of the wild-type strain was reduced by about 50% in the presence of MC1626, the inhibitor proved to be significantly less effective on the gcn5Δ strain and the F221A catalytic mutant, reducing growth by around only 25%. The residual reduction of growth in the gcn5Δ and F221A strains may be related to the activity of other acetyltransferases that we intend to investigate using a similar approach. Taken together, these results demonstrate that the inhibitory activity of MC1626 on growth rate is Gcn5p-specific and correlates to its HAT catalytic activity.

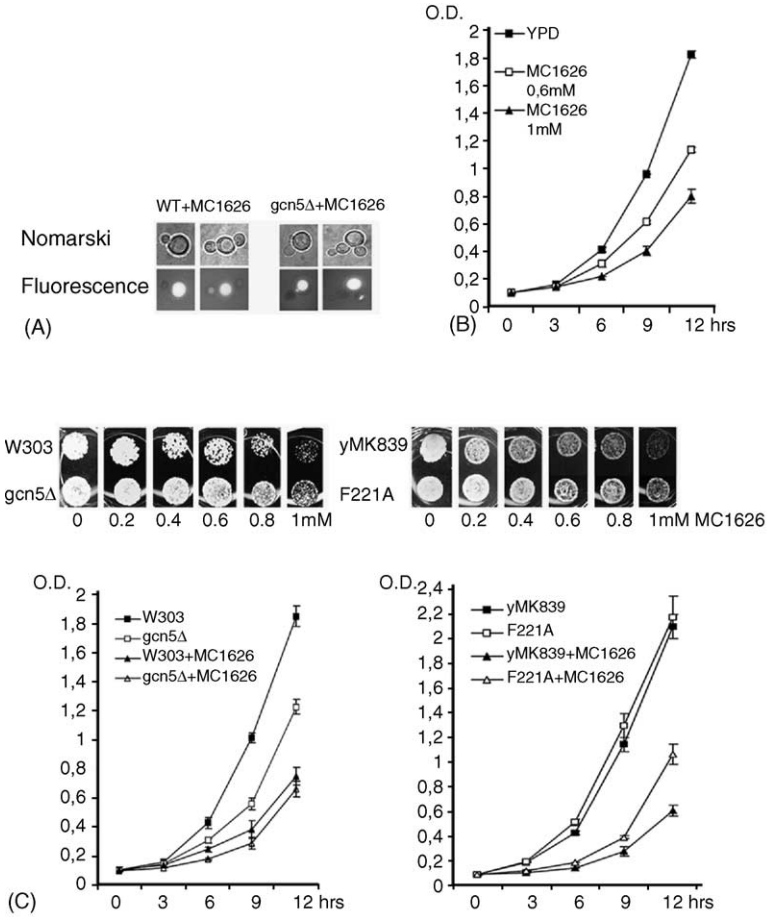

Fig. 2. MC1626 inhibits growth of yeast cells. (A) MC1626 is a fluorescent molecule and is cell permeable. After incubation of cells at 28 °C in YPD supplemented with 0.6 mM MC1626, the cells were observed under fluorescence microscopy, and the MC1626 was localized in wild type (W303) and *gcn*5Δ (yPO4) cells. (B) Growth of W303 in YPD media (closed square) or in the presence of 0.6 mM (open square) and 1 mM (triangle) MC1626 was followed for 12 h at 28 °C; standard deviation was calculated for each point. (C) Yeast growth spot assay of 1:100 dilution of 0.8 $O.D._{600}$ culture of wild-type, W303 and yMK839 strains, respectively, and null gcn5Δ (yPO4) and HAT catalytic mutant F221A strains on solid medium at increasing concentrations of MC1626 incubated for 48 h at 28 °C (upper panel). Yeast growth assay of wild type, W303 and yMK839 strains, in YPD (closed square) or in YPD with 1 mM MC1626 (closed triangle) and null gcn5Δ (yPO4) and HAT catalytic mutant F221A strains in YPD (open square) or in the presence of 1 mM MC1626 (open triangle) (lower panel).

That MC1626 appears to be less effective when Gcn5p is missing (*gcn*5Δ) or is catalytically inactive (F221A) suggests that alternative pathways may override Gcn5p-alleviating growth defects. To determine whether MC1626 induced cell death, we assayed cell viability upon drug addition by following single colony duplication on a glass slide. The results obtained in different experiments clearly pointed to a cytostatic effect, which slowed cell duplication without cell death.

3.2. Inhibition of Gcn5p-dependent transcription

Histone acetyltransferase activity of Gcn5p is required for transcriptional activation of target genes in vivo [29]. In yeast, Gcn5p was first described as a transcriptional coactivator of amino acid biosynthetic genes, of which HIS3 is one of the most affected [18]. To measure the effect of MC1626 on Gcn5p-specific transcriptional inhibition, we assayed a HIS3-lacZ reporter gene fusion under conditions of basal (SD) and activated (SD + 3AT) transcription in a wild-type strain. HIS3-lacZ was properly induced in the wild-type strain grown in amino acid deprivation (+3AT) (Fig. 3A). In contrast, reporter activity was drastically reduced in the presence of 0.6 mM MC1626 in basal and, more drastically, in activated transcription. To avoid unspecific broad inhibition on global transcription, we comparatively tested a constitutively expressed promoter not responding to Gcn5p regulation carrying a GAL10-CYC1 promoter fused to a LacZ reporter. This reporter was clearly unaffected by inhibitor treatment, and the basal and the activated transcription (SG) remained unaltered (Fig. 3B). This demonstrates that MC1626 is effective in inhibiting Gcn5p-dependent gene transcription and that the inhibitory effect is particularly exerted under conditions of gene-specific activation where the transcriptional coactivator activity of Gcn5p is maximally required. This assay also highlights the potential of this simple yeast-based system as a useful tool to analyze Gcn5p-driven transcription-related effects of inhibitory molecules in vivo.

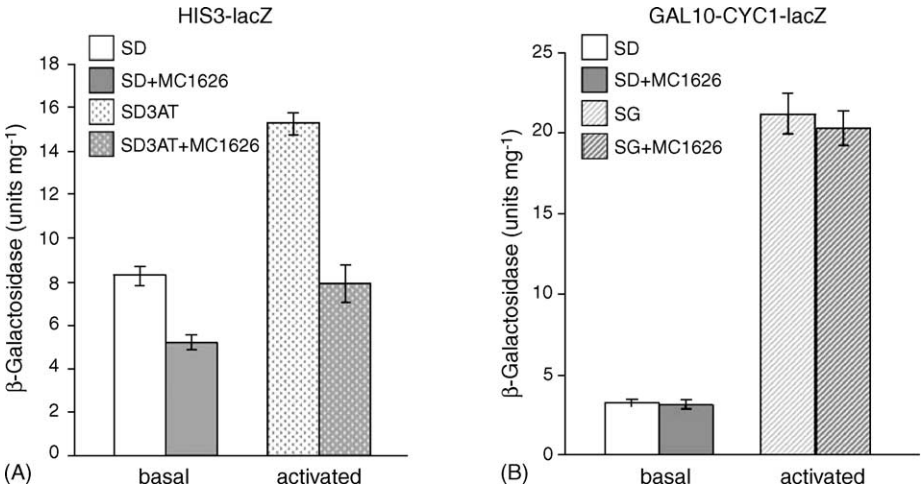

Fig. 3. Gcn5p-dependent HIS3-lacZ transcription is inhibited by MC1626. β-Gal activity was determined in extracts obtained from the wild-type strain (yPO14, yPO15) harvested after growth for 16 h in SD (white bars) or in the presence of 0.6 mM MC1626 (grey bars). (A) To test the effect of MC1626 on HIS3 activated transcription (dotted bars), yeast cultures were grown for 4 h in amino acid deprivation (SD + 3AT) and β-Gal activity was determined. (B) To test the effect of MC1626 on Gcn5p-independent GAL10-CYC1-lacZ activated transcription (dashed bars), cells were grown for 4 h in SG.

3.3. MC1626 inhibits Gcn5p histone acetylation in vivo

Histone acetyltransferase activity is required for targeted acetylation and for transcriptional activation of specific genes in vivo [30]. Since our results demonstrated the inhibitory effect of MC1626, we next wanted to see whether the acetylation state of histone H3 and the H4 N-terminal tails [31,32] was altered in order to demonstrate that acetylation itself is the primary target for MC1626 inhibitory activity. To do this, we measured the acetylation of histone H3 and H4 tails in vivo using histone-specific antibodies: anti-acetyl H3 and anti-acetyl H4. Western blot analysis of histones extracted from mid-log growing cells treated with or without MC1626 for 16 h was performed and protein level normalized with antibody anti-ada2, an housekeeping protein control. Fig. 4A shows that under steady-state conditions the H3 acetylation level was drastically reduced by inhibitor treatment at 0.6 mM MC1626.

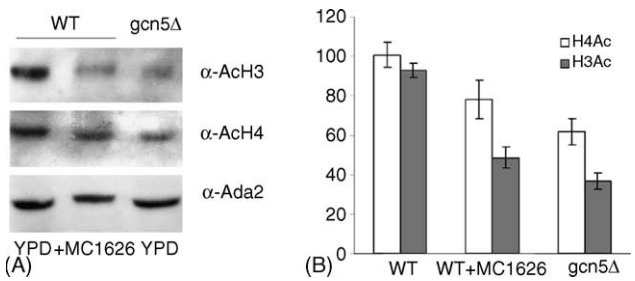

Fig. 4. MC1626 reduces in vivo histone H3 and H4 N-terminal acetylation. (A) We evaluated the modification status of histone H3 and H4 tails in vivo by Western blot analysis of protein extracts from WT (W303) and gcn5Δ (yPO4) cells grown for 16 h in YPD and in the presence of 0.6 mM MC1626. We used anti-acetyl-H3 (α-AcH3) and anti-acetyl-H4 (α-AcH4) primary antibodies. Normalization of gel loading was performed with anti-Ada2 (a-Ada2) antibody (bottom line). (B) Quantification of acetylation of histone H3 and H4 was performed, values are shown in comparison with the steady state acetylation level in the gcn5Δ (yPO4) strain.

Quantification of the results was performed and standard deviation calculated, histones H3 and H4 acetylation was compared with a gcn5Δ strain (Fig. 4B). Acetylation of histone H4 was also reduced albeit at a lesser extent, in particular, the acetylation of histone H3 is of the same level of gcn5Δ, while the effect on acetylation of histone H4 is weaker according to Gcn5p specificity toward H3 tail substrate. This result showed that H3 histone is mostly deacetylated in the presence of MC1626 and that the resulting hypoacetylation of H3 and H4 are effectively involved in the inhibition of gene specific and cell growth.

4. Discussion

Acetylation represents a crucial step in gene activation; its misregulation is primarily involved in many diseases. Biochemical and molecular studies on the lysine acetyltransferases family [33] and the mechanisms of lysine acetylation [31] have underscored their key role at the single protein level or as catalytic subunits of large multiprotein complexes [34]. It is becoming clear that not only histone tails but also a growing number of transcription factors, signal regulators, viral proteins and activators represent specific targets for HAT catalytic activity. In leukaemia, for example, HATs are often translocated, and aberrant mislocalization can lead to pathologies [35]. In addition, acetylated chromatin domains are recognized by specific protein modules such as the acetyl–lysine interacting bromodomain [36,37]. Acetylated chromatin interactors may also be mistargeted when an aberrant acetylation of the genome is present. As with bromodomain and chromatin, an anomalous interaction of motives may further amplify the negative effects of genomic aberrant acetylation. To find small molecules that inhibit HATs, we set up a simple yeast-based model system for screening

novel HATs OFF compounds. Yeast is, in fact, an ideal system, which couples to the evolutionary conservation of the fundamental mechanisms of gene regulation the peculiar use of a variety of single gene disrupted and mutant strains that can be easily tested in comparison with the respective wild-type isogenic background. The search for Gcn5p inhibitors capable of reproducing slow growth in *gcn*5Δ was what initially motivated us to assay novel, small molecules in yeast. Here, we provide evidence for an inhibitory effect the cell-permeable MC1626 specifically exerted on wild type cells. Inhibition was significantly diminished in a null *gcn*5Δ and a HAT catalytic mutant strain (F221A). This result was obtained in different strain backgrounds, proving that in the absence of Gcn5p the main target of the inhibitor alternative pathways may override the absence of Gcn5p. We also have demonstrated that MC1626 inhibited Gcn5p-dependent transcriptional activation but was ineffective toward a Gcn5p not regulated gene. Consistently, acetylation of the histone H3 tail was drastically reduced and provided a background level of acetylated histone H3 similar to *gcn*5Δ. Following this yeast-based method, we discovered that the quinolone compound MC1626 is a selective inhibitor of Gcn5p in vivo. Building on these results, we plan to investigate the inhibitory activity of MC1626 in a wider spectrum of cases. The compound may also be applied at the genomic level to profile genomic expression upon addition of MC1626. For profiling we will use the growth of *gcn*5 null strains and their resistance to MC1626 treatment as a potential screening tool to define additional related target genes because Gcn5p, being a global epigenetic regulator, may be involved in the expression of acetylation-dependent gene families. This approach may also be valuable for assessing gene expression profiling in different external conditions and upon addition of specific factors. Further tests will clarify the mechanism of action of this very promising new inhibitor, considering its high cell permeability, simple structure, and high Gcn5p specificity tested in vivo. We conclude by underlining the general deliverable of this yeast-based method for wide, easy screening of HATs OFF compounds.

Acknowledgments

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