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Panspecies small-molecule disruptors of heterochromatin-mediated transcriptional gene silencing

Citation for published version:

Digital Object Identifier (DOI):
10.1128/MCB.01102-14

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Molecular and Cellular Biology

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Heterochromatin underpins gene repression, genome integrity, and chromosome segregation. In the fission yeast *Schizosaccharomyces pombe*, conserved protein complexes effect heterochromatin formation via RNA interference-mediated recruitment of a histone H3 lysine 9 methyltransferase to cognate chromatin regions. To identify small molecules that inhibit heterochromatin formation, we performed an *in vivo* screen for loss of silencing of a dominant selectable *kanMX* reporter gene embedded within fission yeast centromeric heterochromatin. Two structurally unrelated compounds, HMS-11 and HMS-12, alleviated *kanMX* silencing and decreased repressive H3K9 methylation levels at the transgene. The decrease in methylation caused by HMS-11 and HMS-12 was observed at all loci regulated by histone methylation, including centromeric repeats, telomeric regions, and the mating-type locus, consistent with inhibition of the histone deacetylases (HDACs) Clr3 and/or Sir2. Chemical-genetic epistasis and expression profiles revealed that both compounds affect the activity of the Clr3-containing Snf2/HDAC repressor complex (SHREC). *In vitro* HDAC assays revealed that HMS-11 and HMS-12 inhibit Clr3 HDAC activity. HMS-11 also alleviated transgene reporter silencing by heterochromatin in *Arabidopsis* and a mouse cell line, suggesting a conserved mechanism of action. HMS-11 and HMS-12 bear no resemblance to known inhibitors of chromatin-based activities and thus represent novel chemical probes for heterochromatin formation and function.
tional silencing (15, 16). SHREC consists of the HDAC Clr3, the SNF2-related chromatin remodeling factor Mit1, and two nonenzymatic components, Clr1 and Clr2 (15). The HDACs Clr6 and Sir2 also facilitate removal of histone acetylation to maintain heterochromatin integrity (17–19). SHREC resembles the vertebrate Mi-2/NuRD silencing complex, which contains HDAC1/2 and SNF2 ATPase Mi-2, among other proteins (20), although it remains unclear whether RNAi directly modulates chromatin modification in mammals (1). Related protein complexes are known to couple RNAi to DNA methylation, H3K9 methylation, and gene silencing in plants (21, 22). In Arabidopsis, RNAi also contributes to heterochromatin integrity by directing de novo DNA methylation to homologous sequences (23, 24), where it recruits Suv39 methyltransferase related proteins (25).

RNAi and heterochromatin components are not essential for viability of fission yeast. This has facilitated mechanistic dissection of the process initially through genetic screens and subsequently via mass spectrometric analysis of purified protein complexes (10, 15, 26–29). Deletion of individual RNAi or heterochromatin components disrupts silencing of reporter genes inserted within heterochromatin (10, 15, 28, 30). Small-molecule inhibitors provide an alternative means for probing biological pathways. In contrast to mutations, inhibitor effects are usually reversible and thereby enable precise determination of functional dependencies in complex pathways (31–33). For example, in vivo screens based on telomere position effect in budding yeast have previously allowed the identification of sirtinol and splitomicin, which inhibit Sir2 (34, 35). Fission yeast is dimorphic and splitomicin, which inhibit Sir2 (34, 35). Fission yeast is amenable to high throughput cell-based screens (36–38) and the integrity of its heterochromatin and associated gene silencing have been shown to be sensitive to the HDAC inhibitor trichostatin A (TSA) (39, 40). Unbiased small-molecule screens may thus identify novel compounds that inhibit the function of components of the RNAi-directed chromatin modification system in fission yeast, such as Dicer, Argonaute, Clr4 H3 lysine 9 methyltransferase and the various HDACs. Because small molecules identified from yeast screens may also inhibit conserved orthologs (41–44), inhibitors of fission yeast heterochromatin integrity may yield insights into related processes in higher eukaryotes, including humans. Small-molecule inhibitors of heterochromatin may be of therapeutic value in cancer and other diseases caused by aberrant gene regulation. For example, the HDAC inhibitors vorinostat and romidepsin, as well as the histone lysine methyltransferase inhibitor chaetocin, have antitumorigenic activity (45, 46).

We report here a cell-based screen for small-molecule inhibitors of fission yeast heterochromatin. Two novel compounds, called HMS-I1 and HMS-I2, were identified that disrupt heterochromatin integrity (17–19). SHREC resembles the vertebrate Mi-2/NuRD silencing complex, which contains HDAC1/2 and SNF2 ATPase Mi-2, among other proteins (20), although it remains unclear whether RNAi directly modulates chromatin modification in mammals (1). Related protein complexes are known to couple RNAi to DNA methylation, H3K9 methylation, and gene silencing in plants (21, 22). In Arabidopsis, RNAi also contributes to heterochromatin integrity by directing de novo DNA methylation to homologous sequences (23, 24), where it recruits Suv39 methyltransferase related proteins (25).

Materials and Methods

Fission yeast growth and chemical screens. Haploid Schizosaccharomyces pombe cells were grown in YES (yeast extract with supplements) medium at 32°C and assessed in log phase for all experiments. Cells and compounds were dispensed in 96-well microplates using a Biomek FX liquid handling robot (Beckman Coulter) and plates were read (optical density at 595 nm (OD$_{595}$)) every 15 min for 48 to 72 h at 32°C with continuous shaking in a Sunrise plate reader (Tecan). Growth curves generated for each compound were analyzed using in-house R scripts and the grofit R package to extract parameters for doubling time, lag time and saturation time. Table 1 contains a list of the strains used in the present study.

**Table 1** Fission yeast strains used in this study

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**Characterization of chemical compounds.** H$_1$ and 13C NMR, recorded at 400 and 100 MHz, respectively, were performed on a Varian 400-MR spectrometer. Assignments of H$_1$ and 13C NMR signals were carried out by correlation spectroscopy (COSY) and Heteronuclear Single Quantum Coherence (HSQC) experiments. Mass spectral (MS) and high-resolution mass spectrometry (HRMS) data were recorded on Agilent 6120 Quadrupole LC/MS and Agilent LC/MSD TOF (model 61969A) systems, respectively.

The data obtained for compound 2-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-6-methylimidazo[1,2-a]pyridine (HMS-11) were as follows: (i) H NMR (400 MHz, CDCl$_3$): 6 (ppm) 7.87 (s, 1H, MeCCHN), 7.66 (s, 1H, NHC$_2$), 7.50 (d, J = 9.90 Hz, 1H, N$_2$CCH), 7.41 to 7.47 (m, 2H, o-CH$_2$), 6.99 (d, J = 1.17, 9.39 Hz, 1H, MeCCH$_2$), 6.92 (d, J = 7.83 Hz, 1H, p-OC$_6$H$_4$), 4.30 (s, 4H, OCH$_2$), 2.31 (s, 3H, CH$_3$); (ii) 13C NMR (100 MHz, CDCl$_3$): 6 (ppm) 145.6, 144.8, 143.9, 143.6, 127.8, 127.7, 127.4, 122.0, 119.4, 117.6, 116.8, 115.0, 107.3, 64.6, 64.5, 18.2; (iii) 13C NMR (100 MHz, CDCl$_3$; CH$_2$ = 2) from HSQC: 6 (ppm) 127.7 (MeCCH$_2$), 123.4 (MeCCHN), 119.4 (p-OC$_6$H$_4$), 117.7 (p-OC$_6$H$_4$), 116.8 (N$_2$CCH$_2$), 115.1 (m-OC$_6$H$_4$), 107.3 (NHC$_2$), 64.5 (OCH$_2$), 18.2 (CH$_2$); (iv) HRMS (ESI-TOF) m/z: [M + H]$^+$ calculated for C$_{20}$H$_{20}$Cl$_2$N$_3$O$_2$: 367.1128; found 367.1137.

The data obtained for compound N-(benzo[b][1,4]dioxin-2-yl)-4-(2-chloro-6-fluorobenzyl)pyrazine-1-carboxamide (HMS-12) were as follows: (i) H NMR (400 MHz, CDCl$_3$): 6 (ppm) 7.72 (d, J = 7.92 Hz, 1H), 7.55 (d, J = 7.63 Hz, 1H), 7.13 to 7.32 (m, 4H), 6.96 to 7.06 (2H, Ar$_2$CH(NH)$_2$), 6.73 (s, 1H), 3.76 (d, J = 2.35 Hz, 2H, CH$_2$Ar), 3.45 to 3.59 (m, 4H, CH$_2$NCO), 2.59 to 2.66 (m, 4H, CH$_2$NCO); (ii) 13C NMR (100 MHz, CDCl$_3$; CH$_2$ = 2) from HSQC: 6 (ppm) 129.4 (CH$_2$), 125.6 (CH$_2$), 124.3 (CH$_2$), 122.4 (CH$_2$), 121.8 (CH$_2$), 121.5 (CH$_2$), 114.0 (CH), 105.2 (CH), 52.3 (CH$_2$Ar), 52.2 (CH$_2$NCO), 44.2 (CH$_2$NCO); (iii) LC-MS (APCI) m/z: [M + H]$^+$ calculated for C$_{20}$H$_{20}$ClF$_2$N$_3$OS: 404.1; found 404.1.
ChIP. Chromatin immunoprecipitation (ChIP) analysis was performed as previously described (47). Briefly, cells were grown at 32°C to log phase and fixed in 1% formaldehyde for 15 min at room temperature. Cells were lysed with a bead beater (Biospec Products) and sonicated using a BioRuptor sonicating water bath (Diagenode) for 15 min (30-s duty cycle). For immunoprecipitations, H3K9me2 (m5.1.1 (48)), H3K9ac (Active Motif, catalog no. 39137), H3K14ac (Millipore, catalog no. 07-353), Flag M2 (Sigma, catalog no. F1804), and c-Myc (Santa Cruz, catalog no. 9B11) antibodies were incubated overnight with the lysate. After bead washes, samples were treated with Chelex resin, followed by incubation with proteinase K. Samples were diluted and used in quantitative PCRs (qPCRs).

RNA analyses. Reverse transcription-PCR (RT-PCR) of centromeric outer repeat transcripts and Northern analysis of centromeric siRNAs were performed as previously described (47, 49, 50).

qPCRs. ChIP and RT-PCR samples were quantified by qPCRs carried out with SYBR green on a LightCycler 480 instrument (Roche). The data were analyzed using Light Cycler 480 software 1.5 (Roche). For ChIP, enrichment was calculated as the ratio of target sequence to control sequence (act1) in the immunoprecipitated sample over input lysate. For RT-PCR, quantification was relative to untreated dimethyl sulfoxide (DMSO) control using 

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Affymetrix microarray expression analysis. Total RNA was extracted from S. pombe cells using the hot phenol method (51). RNA was purified with an RNaseeasy mini-kit (Qiagen) and tested for integrity on a 2200 Tapestation (Agilent). 150 nanograms of RNA was used for labeling and array hybridization using GeneChip S. pombe tiling 1.0FRI microarrays according to the GeneChip Whole Target labeling and hybridization protocol (Affymetrix). The data were normalized with quantile normalization plus scaling using Tiling Analysis Software (TAS) 1.1 and imported into the Podbat browser program (52). Podbat was used to produce upregulated gene lists for different treatments using settings for various fold changes and a P value of <0.05. GeneSpring GX software (Agilent Technologies) was used to compare the gene lists obtained for compound treatments with the gene lists for various S. pombe mutants present in an in-house database in the Ekwall laboratory. Cutoffs of 1.28 and 1.5 were used for the HMSI-1 and HMSI-2 treatments, respectively, to recover a comparable number of genes for both treatments. Biological duplicates were prepared and analyzed for each condition.

Plant growth and histochemistry. The transgenic Arabidopsis thaliana L5 line has been described elsewhere (53). Seeds were surface sterilized and sown on MS agar plates with 15 g/liter sucrose. Plates were cold treated for 2 days at 4°C. Seedlings were grown at 20°C under fluorescence light (fluence rate of 40 to 60 μmol m⁻² s⁻¹) with a 16-h light/8-h dark photoperiod. At 12 days postgermination, seedlings were transferred to liquid MS in a 40-ml flask and, after 24 h, 10 μM HMSI-11, 10 μM HMSI-12, or DMSO was added. Compound treatment was repeated 3 days later, and samples were collected 2 days later. β-Glucuronidase (GUS) staining was performed as previously described (54) with minor modifications. Plant tissues were immersed in histochemical GUS staining buffer [100 mM NaPO₄, 0.5 mM K₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆], 20% methanol, 0.3% Triton X-100, and 1 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid; pH 7)], vacuum infiltrated under pressure for 10 min times three, and then incubated overnight at 37°C. Samples were washed several times with absolute ethanol until complete tissue clarification, incubated for 24 h in water, and stored in 50% glycerol. Images were taken using a Dotslide Olympus VS120.

Transcriptional silencing assay in MEL cells. Murine erythroleukemia (MEL) suspension cells were grown in Dulbecco modified Eagle medium (Gibco) with fetal bovine serum and penicillin-streptomycin at 37°C in the presence of each compound or DMSO solvent control. Fluorescence-activated cell sorting (FACS) analysis of enhanced green fluorescent protein (eGFP) expression was performed with a Becton Dickinson FACS analyzer. For ChIP analysis, MEL cells were grown for 3 days in the presence of compounds and then fixed with 1% formaldehyde for 5 min at room temperature. Immunoprecipitated DNA was obtained with antibodies to H3K9me2 (Uranos; Kimura 007) and H3K9me3 (Abcam; Kimura 007). Quantitation of ChIP was performed by qPCR as described above.

Recombinant Clr3 protein production. Using the MultiBac system (55), Clr3 was expressed in Sf9 insect cells as a N-terminal fusion of the codon optimized sequence to a multifunctional tag composed of OneStep-Sumostar (OSS) (36), T7 tag, and a TEV site. The OSS-Clr3 protein was purified on a StrepTrap column (GE Healthcare), cleaved from the tag using tobacco etch virus (TEV) protease, and purified to homogeneity by gel filtration on Superdex 200 (GE Healthcare) in 10
mM HEPES (pH 7.6), 200 mM KCl, 1 mM dithiothreitol, and 50 mM magnesium acetate.

**In vitro HDAC assays.** Clr3 assays were performed with an HDAC fluorometric assay kit from BioVision (K330-100) according to the manufacturer’s instructions. Recombinant human HDAC assays were performed by Reaction Biology (Malvern, PA). Compounds and purified recombinant HDACs were preincubated 20 min in assay buffer (50 mM Tris-HCl [pH 8.0], 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl₂, 1 mg/ml bovine serum albumin, 1% DMSO), before the addition of 50 μM fluorogenic substrate [Arg-His-Lys-Lys(Ac)-AMC]. Reaction mixtures were incubated for 2 h at 30°C. The deacetylated substrate was then cleaved by trypsin, which released the fluorescent 7-amino-4-methylcoumarin. Fluorescent signals were read with an EnVision multilabel plate reader (Perkin-Elmer).

**siRNA knockdown of HDAC6 and HDAC10 in MEL cells.** Cells were transfected with sense and antisense small interfering RNA (siRNA) at a final concentration of 100 nM (HDAC6 sense, GGAGGAAGAUGAAGUGGAAuu; HDAC10 sense, GGACAAGCCUCCACAAAuu) with DharmaFECT2 reagent. Cells were collected 48 h after transfection. Scrambled siRNAs were included as controls. Statistical significance was assessed by using the Student t test comparing HDAC6/10 siRNA with scrambled siRNA treatments.

**RESULTS**

**Small-molecule inhibitors of fission yeast centromeric heterochromatin integrity.** To screen for inhibitors of fission yeast heterochromatin integrity, we used a previously described approach (Gray et al., 2013). We identified small molecules that disrupt centromeric heterochromatin integrity in fission yeast. Two small molecules, HMS-I1 and HMS-I2, were found to disrupt centromeric heterochromatin integrity in fission yeast.

**FIG 1** HMS-I1 and HMS-I2 disrupt centromeric heterochromatin integrity in fission yeast. (A) Schematic diagram showing the position of the cen1 imr1R(NcoI):kanMX (cen1-kanMX for short) reporter gene in centromere 1 relative to the dg and db outer repeat (otr) elements, the innermost repeats (imr) and the central core (cnt). The position of heterochromatin and CENP-A<sup>cnp1</sup> chromatin domains is indicated. Locations of primers used in panel E below are indicated by the gray and black bars. cen1(imr) primers amplify a sequence present in all centromeres. (B) Plating assay of the cen1-kanMX strain on YES plates with 10 μg/ml G418 or without G418 (nonselective). (C) Growth curves of cen1-kanMX cells in YES media with 5 μM HMS-I1, 5 μM HMS-I2, and 10 mM nicotinamide (Nic), with 10 μg/ml G418 or without G418 (nonselective). (D) Chemical structures of HMS-I1 and HMS-I2. (E) qChIP analysis of H3K9me2 levels associated with the cen1-kanMX insertion or cen1(dg) repeats, relative to act1<sup>+</sup>. (F) qChIP analysis of H3K9ac and H3K14ac levels associated with the cen1-kanMX insertion, relative to act1<sup>+</sup>. In panels E and F, cells were grown in media containing 5 μM HMS-I1 and 5 μM HMS-I2, with 10 μg/ml G418. Error bars indicate standard deviations for three biological replicates. *, P < 0.05; **, P < 0.01; ***, P < 0.0001.

**siRNA knockdown of HDAC6 and HDAC10 in MEL cells.** Cells were transfected with sense and antisense small interfering RNA (siRNA) at a final concentration of 100 nM (HDAC6 sense, GGAGGAAGAUGAAGUGGAAuu; HDAC10 sense, GGACAAGCCUCCACAAAuu) with DharmaFECT2 reagent. Cells were collected 48 h after transfection. Scrambled siRNAs were included as controls. Statistical significance was assessed by using the Student t test comparing HDAC6/10 siRNA with scrambled siRNA treatments.

**GEO accession number.** All microarray data are available under GEO accession number GSE57207.
erochromatin integrity, we constructed a reporter strain harboring a dominant selectable kanamycin (\textit{kanMX}) reporter gene within the heterochromatin of centromere 1 (\textit{cen1}). The \textit{kanMX} gene was inserted in the innermost repeats (\textit{imr}) on the right side of \textit{cen1} [\textit{cen1-imr1R}(NcoI):\textit{kanMX}], abbreviated as \textit{cen1-kanMX} (Fig. 1A). Expression of \textit{kanMX} normally confers resistance to G418, but because in \textit{cen1-kanMX} cells the reporter gene is silenced by surrounding heterochromatin, these otherwise wild-type cells exhibit sensitivity and grow poorly on plates containing G418. Disruption of heterochromatin by deletion of the gene encoding Clr4 methyltransferase (\textit{clr4}/H9004) allowed expression of \textit{cen1-kanMX} and resistance to G418 and thus increased growth on G418 medium (Fig. 1B). Microwell-based liquid culture growth assays, performed with or without G418 on \textit{cen1-kanMX} cells, enabled the reproducible detection of G418 resistance in \textit{clr4}\Delta cells compared to wild-type (\textit{wt}) cells by continual monitoring of optical density. Addition of the known Sirtuin HDAC inhibitor nicotinamide also resulted in increased growth of wild-type cells in the presence of G418 (Fig. 1C). This validated \textit{cen1-kanMX} reporter strain was subsequently used to screen 1,570 compounds that had been previously selected for chemical diversity and bioactivity (57). Two compounds, 2-(2,3-dihydro-1,4-benzo[b][1,4]dioxin-6-yl)-6-methylimidazo[1,2-a]pyridine and N-(1-benzo[b]thiophen-2-yl)-4-(2-chloro-6-fluorobenzyl)piperazine-1-carboxamide, here referred to as HMS-I1 and HMS-I2, respectively (heterochromatin-mediated silencing inhibitors 1 and 2), were identified as reproducible hits that increased the resistance of wild-type \textit{cen1-kanMX} cells to G418 to a similar extent as nicotinamide (Fig. 1C and D).

To our knowledge, biological activity for HMS-I1 and HMS-I2 has not been reported previously. Although HMS-I1 and HMS-I2 could conceivably fit into a common pharmacophore model, their structural disparities precluded straightforward conclusions. For instance, the imidazopyridine and piperazine carboxamide cores of HMS-I1 and HMS-I2, respectively, feature different numbers and placement of hydrogen bond donors/acceptors, while their lipophilic flanking groups differ in size and shape. HMS-I1 and HMS-I2 lack compelling structural similarities to well-established inhibitors of chromatin-modifying enzymes such as HDAC inhibitors (58, 59). We note that HMS-I1 and HMS-I2 do not contain canonical zinc chelating moieties possessed by type I and II HDAC inhibitors: HMS-I1 cannot form a chelate due to a lack of heteroatoms disposed in a 1,4 relationship and the conceivable chelation modes for HMS-I2 are electronically and sterically suboptimal. Taken together, such structural considerations suggest that HMS-I1 and HMS-I2 may be acting via a novel mechanism.

A hallmark of centromeric heterochromatin is the presence of methylated histone H3K9 (H3K9me) chromatin over the centromeric repeats and inserted marker genes. Disruption of centromeric heterochromatin results in reduced levels of H3K9me and loss of silencing of inserted marker genes (28, 47, 60). Quantitative ChIP (qChIP) analyses on cells grown under G418 selection revealed that HMS-I1 and HMS-I2 lack canonical zinc chelating moieties possessed by these compounds. Instead, the observed effects are likely due to the specific interaction of HMS-I1 and HMS-I2 with centromeric heterochromatin.
elements (Fig. 1E). Importantly, the qChIP assay monitored H3K9me2 associated with the 18 copies of otr/dg centromere repeats distributed over the three centromeres. HMS-I1 and HMS-I2 thus altered the status of H3K9 methylation across all three centromeres, demonstrating that the compounds affect heterochromatin throughout the nucleus and not just at the selected cen1-kanMX locus. Neither compound induced a complete loss of H3K9me2, as observed in cells lacking Clr4 H3K9 methyltransferase (clr4/H9004) (Fig. 1E). Growth of cen1-kanMX cells in the presence of HMS-I1 or HMS-I2, but in the absence of G418 selection, had less dramatic effects since the H3K9me2 levels were lower on kanMX but not on the native outer repeat sequences (data not shown and see Fig. 3).

Histone hypoacetylation is also a hallmark of heterochromatin, and an increase in histone lysine acetylation is expected to accompany the loss of centromeric marker gene silencing. Consistently, we observed an increase in H3K9 and H3K14 acetylation on cen1-kanMX in cells treated with HMS-I1 and HMS-I2 grown in the presence of G418 (Fig. 1F). We conclude that HMS-I1 and HMS-I2 increase the G418 resistance of cen1-kanMX cells by general disruption of centromeric heterochromatin and concomitant elevated expression of the embedded kanMX resistance gene.

HMS-I1 and HMS-I2 inhibit events downstream of RNAi in chromatin modification and heterochromatin formation. RNAi is required for both the establishment and the maintenance of centromeric heterochromatin in fission yeast (61–63). In contrast, following RNAi-triggered establishment of heterochromatin domains over the mat2-mat3 region of the mating-type locus or adjacent to telomeres, RNAi is not continuously required to maintain heterochromatin since alternative pathways propagate heterochromatin at these locations (64–67). ChIP analyses showed that, as expected, H3K9me2 was lost at mat2-mat3 and telomeres in cells that lacked Clr4 H3K9 methyltransferase, or the HDACs Clr3 and Sir2, but was retained in cells that lack RNAi due to deletion of Dcr1. H3K9me2 levels were also consistently reduced at both mating-type and subtelomeric regions when cen1-kanMX cells were incubated with HMS-I1 or HMS-I2 and selected for G418 resistance (Fig. 2A and data not shown).
Heterochromatin-mediated silencing at the mating-type locus ensures that the silent mating-type cassettes encoding plus (P) or minus (M) information (mat2-P and mat3-M) cannot be expressed. These cassettes must normally be recombined into the active mat1 expression site to escape the powerful repressive heterochromatin environment of the silent loci and allow expression. Mutations in CLRC (Clr4-Rik1-Cul4) complex and HDAC components are known to alleviate transcriptional silencing at the mat2-P and mat3-M loci (26). Strikingly, treatment of a sterile yeast strain lacking the kanMX reporter gene with HMS-I1 and HMS-I2 resulted in the expression of Pc/Pi and Mc transcripts, respectively (Fig. 3). HMS-I1 and HMS-I2 disrupt the robust form of heterochromatin-mediated silencing that normally ensures that mat2-P or mat3-M are not transcribed. From these results we concluded that both HMS-I1 and HMS-I2 must interfere with heterochromatin formation downstream of RNAi.

In cells with defective RNAi or Clr4, noncoding outer repeat transcripts accumulate as they are no longer processed by RNAi to siRNA and transcriptional silencing is reduced. In contrast, cells that lack either Clr3 or Sir2 exhibit reduced H3K9 methylation levels on centromeric repeats but retain high siRNA levels with only a slight increase in centromeric transcripts (15, 16, 62). These phenotypic differences allow the mechanism of action of specific mutants or inhibitors to be distinguished. Despite the reduced levels of H3K9me2 detected on centromere repeats in wild-type cells grown in the presence of HMS-I1 or HMS-I2 and G418, these cells retained high levels of centromeric siRNA, while centromeric repeat transcripts remained low (Fig. 2C and D). Hence, HMS-I1 or HMS-I2 treated cells resemble clr3Δ and sir2Δ cells. These results demonstrate that HMS-I1 and HMS-I2 phenocopy the loss of Clr3 and Sir2 and suggest that both compounds might interfere with either Clr3- or Sir2-related functions.

The Clr3 HDAC is a putative target of HMS-I1 and HMS-I2. Epistasis analysis has shown that RNAi operates in a parallel pathway to the HDACs Clr3 and Sir2 to maintain heterochromatin integrity at centromeres (61, 62, 68). Cells devoid of an RNAi component such as Dcr1, or either the Clr3 or Sir2 HDACs, retain significant levels of H3K9me on centromeric repeats. However, the removal of both Dcr1 and Clr3 or Sir2 reduces H3K9me to much lower levels than that observed in dcr1Δ, clr3Δ and sir2Δ cells alone (62, 68). To determine whether HMS-I1 and HMS-I2 might exacerbate the phenotype of cells lacking Dcr1, Clr3 or Sir2, we compared the levels of H3K9me2 on centromeric repeats in wild-type, dcr1Δ, clr3Δ, or sir2Δ cells grown in the presence or absence of either compound, all in the absence of G418 selection. HMS-I1 and HMS-I2 exhibited strong chemical-genetic interactions with dcr1Δ and sir2Δ, since a greater reduction in the level of H3K9me2 associated with centromeric repeats was observed in the presence of each compound but not with clr3Δ (Fig. 3). HMS-I1 and HMS-I2 therefore behaved as chemical mimetics of the clr3Δ mutation in terms of interactions with either dcr1Δ or sir2Δ (62, 68). These chemical-genetic interactions, coupled with the observation that neither compound further reduced H3K9me2 levels on centromere repeats in clr3Δ cells, imply that both HMS-I1 and HMS-I2 inhibit Clr3 HDAC activity in vitro without affecting recruitment of SHREC at heterochromatin. (A and B) qChIP analysis of Clr3 (Clr3-3XFLAG) (A) and Mit1 (Mit1-13xmyc) (B) recruitment at centromeric repeats [cen(dg)] and at the silent mat2-mat3 region of the mating-type locus (mat), relative to act1Δ. Cells were grown in the presence of 5 μM HMS-I1 or HMS-I2. Error bars indicate the standard deviations of three biological replicates. *, P < 0.05; **, P < 0.01; ***, P < 0.0001. (C and D) Fluorescence-based deacetylation assays were used to detect inhibition of recombinant S. pombe Clr3 HDAC activity in vitro at the indicated concentrations of HMS-I1 (C) and HMS-I2 (D). Error bars indicate standard deviations for three replicates.
HMS-I2 interfere with the function of either the HDAC Clr3 and/or associated proteins in the SHREC complex.

HMS-I1 and HMS-I2 mimic the effect of SHREC mutations on gene expression. Previous genome-wide expression profiles showed that the Clr3 HDAC is required to repress expression of a broad cohort of genes (183 genes \( \pm 1.5 \)-fold upregulated in \( \text{clr3}^{-735} \) [69]). Clr3 has been shown to associate with Clr1, Clr2, and Mit1 within the repressive SHREC complex, and all four components contribute to heterochromatin integrity (15). If HMS-I1 and HMS-I2 interfere with Clr3 or SHREC function as predicted, then each compound should cause induction of a gene set that overlaps with that upregulated in SHREC mutant cells. In cells grown in the presence of compounds and G418, HMS-I1 and HMS-I2 increased the expression of 37 and 200 genes by \( \pm 1.5 \)-fold, respectively. Significant overlap was evident between the sets of genes affected by HMS-I1 and HMS-I2: 36 of the 37 genes upregulated 1.5-fold by HMS-I1 were also upregulated 1.5-fold by HMS-I2 (\( P = 3.28 \times 10^{-34} \)) and 131 of the 200 genes upregulated 1.28-fold by HMS-I1 were also upregulated 1.5-fold by HMS-I2 (\( P = 1.09 \times 10^{-75} \)) (Fig. 4A; see Data set S1 in the supplemental material).

Compared to expression profiles for \( \text{clr1}^{-5} \), \( \text{clr2} \), \( \text{clr3}^{-735} \), and \( \text{mit1} \) strains (69) (see also Data set S1 in the supplemental material), we observed significant overlap between genes derepressed in the SHREC mutants and HMS-I1 or HMS-I2 treated cells (Fig. 4B). No significant overlap was detected when we compared genes upregulated by HMS-I1 or HMS-I2 with those increased in cells that lack RNAi components (\( \text{ago1} \Delta \) and \( \text{dcr1} \Delta \); Fig. 4B). Previous analyses showed that Clr3 and Sir2 mediate the repression of an overlapping set of genes (18), which explains the few genes whose expression increases after exposure to HMS-I1 or HMS-I2 and that are also upregulated in \( \text{sir2} \Delta \) cells (Fig. 4B). However, the overlap between genes upregulated by HMS-I1 and HMS-I2 and those upregulated in \( \text{sir2} \Delta \) cells is not significant.

Based on this analysis of global gene expression, we conclude that the SHREC complex is a likely target for the inhibitory activity of both HMS-I1 and HMS-I2.

HMS-I1 and HMS-I2 reduce in vitro Clr3 HDAC activity. The genetic analyses described above indicate that HMS-I1 and HMS-I2 impede the activity of the fission yeast SHREC complex, which contains the type II HDAC Clr3. We first assessed whether HMS-I1 and HMS-I2 acted by preventing the recruitment of SHREC components at heterochromatin. ChIP analyses showed that neither HMS-I1 nor HMS-I2 affected the recruitment of either the Clr3 HDAC or the Mit1 chromatin remodeling components of SHREC to mating-type or centromeric heterochromatin (Fig. 5A and B). To determine whether these compounds exert their effect on SHREC function through inhibition of Clr3 catalytic activity, we performed in vitro fluorescence-based HDAC assays. HMS-I1 and HMS-I2 reproducibly reduced the activity of Clr3 by 40 and 80%, respectively (Fig. 5C and D). Under these assay conditions, HMS-I2 inhibits Clr3 to a greater extent than HMS-I1, a finding consistent with the greater reduction in H3K9 methylation observed in HMS-I2-treated \( \text{sir2} \Delta \) and \( \text{dcr1} \Delta \) cells and the greater increase in gene expression caused by HMS-I2 (see Fig. 3 and 4). Collectively, these data suggest that HMS-I1 and HMS-I2 are inhibitors of Clr3 HDAC activity.

HMS-I1 and HMS-I2 disrupt transgene silencing in multicellular eukaryotes. To assess whether HMS-I1 and HMS-I2 might also disrupt heterochromatin integrity in multicellular eukaryotes, we tested for the effects on gene silencing in plants and mammalian cells. We first tested whether HMS-I1 and HMS-I2 could alleviate reporter gene silencing in the \( \text{Arabidopsis thaliana} \) L5 transgenic line, which contains a transcriptionally silenced GUS transgene (53). HMS-I1 caused an increase in the GUS reporter activity.
expression in the root and leaf tissue, whereas HMS-I2 alleviated silencing in the leaf tissue only (Fig. 6). Transcriptional gene silencing mutants reactivate the expression of GUS, whereas RNAi mutants do not (70), indicating that, as in fission yeast, HMS-I1 and HMS-I2 mediate their effects in Arabidopsis independently of RNAi.

We next assessed the effect of the compounds on reporter gene silencing in MEL cells. A β-globin gene promoter-driven eGFP reporter is known to be silenced and coated in repressive H3K9 methylated chromatin at the RL5 locus in these MEL cells when vector backbone bacterial DNA is inserted upstream of the transgene (designated RL5/pYB) (Fig. 7A) (71). In addition, the inclusion of an array of human α-satellite repeats upstream of the reporter (designated RL5/pYB-αSat) (Fig. 7A) bolsters eGFP silencing and H3K9me levels across the transgene (71). We utilized both RL5/pYB and RL5/pYB-αSat MEL cell lines to determine whether HMS-I1 or HMS-I2 interfere with the integrity of heterochromatin at the reporter locus. FACS analysis of the RL5/pYB and RL5/pYB-αSat cell lines showed that eGFP expression increased over time with HMS-I1 but not with HMS-I2 (Fig. 7B). Thus, growth in the presence of HMS-I1 overcomes the strong silencing mediated by α-satellite repeats in MEL cells.

To determine whether HMS-I1 also alters H3K9 methylation levels associated with silent transgenes in multicellular eukaryotes, we performed ChIP analysis with the RL5/pYB-αSat cell line, for which the observed effect on silencing was the strongest. A substantial decrease in both H3K9me2 and H3K9me3 levels associated with the eGFP reporter occurred after 3 days of incubation with HMS-I1. Furthermore, a reduction in H3K9 methylation was also detected on the endogenous mouse amylase gene (Fig. 7C) that is normally repressed in MEL cells (72). Thus, HMS-I1 can disrupt heterochromatin associated with endogenous repressed genes, in addition to that formed by mammalian repetitive elements over the β-globin/eGFP reporter.

In S. pombe, our data suggested that the type II HDAC Clr3 is a target of HMS-I1. Although the identity of HMS-I1 target(s) in mammalian cells remains to be defined in vivo, an in vitro HDAC assay performed with a panel of human recombinant enzymes indicated that HMS-I1 reduced the activity of the type IIb HDACs, HDAC6 and HDAC10 (Fig. 8A). Consistent with inhibition of these HDACs, siRNA knockdown of either HDAC6 or HDAC10 expression resulted in increased β-globin–eGFP transcript levels in both RL5/pYB and RL5/pYB-αSat MEL cell lines compared to scrambled siRNA controls (Fig. 8B and C). Collectively, these results suggest that HMS-I1, and possibly also HMS-I2, derepress transcriptionally silent heterochromatin in evolutionarily distant species through inhibition of conserved HDACs.

DISCUSSION

Heterochromatin is tightly associated with transcriptional silencing in most eukaryotes and is particularly prevalent over repetitive elements, where it prevents inappropriate recombination events and genome rearrangements (3). The aberrant silencing of genes through unscheduled heterochromatin formation is known to contribute to several human diseases, including acute myeloid leukemia and Friedreich’s ataxia (73, 74). Moreover, the manipulation of transcriptional silencing has been used to improve crop plant produce and yields (75). Since RNAi-related mechanisms establish sequence-specific heterochromatin in many eukaryotes, we exploited the well-understood process of heterochromatin formation in fission yeast to develop a novel screen for small-molecule modulators of heterochromatin integrity. Collectively, our analyses indicate that HMS-I2 alleviates heterochromatin-mediated gene silencing in both fungal and plant systems but not MEL cells, whereas HMS-I1 abrogates silencing across the fungal, plant, and animal kingdoms. Both HMS-I1 and HMS-I2 were observed to inhibit the in vitro activity of fission yeast class II Clr3 HDAC,
whereas only HMS-I1 inhibited the human subclass IIb enzymes, HDAC6 and HDAC10. Phylogenetic analysis indicates that human HDAC6 and HDAC10 are closely related to Clr3 (76), with a similar domain architecture of the HDAC catalytic domain followed by a conserved C-terminal domain. Although the major substrate of HDAC6 has been reported to be acetylated tubulin (77), this enzyme has also been implicated in chromatin regulation and could play a direct role in histone deacetylation (78). HDAC10 possesses HDAC activity and has been implicated in autophagy and cancer (79, 80). Therefore, our cross-species comparisons suggest that type II HDAC enzymes may be conserved targets of HMS-I1 and potentially also HMS-I2.

Inspection of the chemical structure of HMS-I1 and HMS-I2 suggests that their mechanism of action is likely to differ from that of canonical HDAC inhibitors, particularly as neither HMS-I1 or HMS-I2 have obvious zinc-chelating characteristics. HMS-I1 and HMS-I2 may therefore represent a novel class of HDAC inhibitor.

FIG 8 HMS-I1 reduces the activity of mammalian HDAC6 and HDAC10 in vitro. (A) Fluorescence-based deacetylation assays were used to detect inhibition of recombinant human HDAC6 and HDAC10 activity in vitro at the indicated concentrations of HMS-I1. Error bars indicate the standard deviations from two replicates. (B) Quantification of eGFP transcripts relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcript levels after siRNA knockdown of HDAC6 and HDAC10 in MEL cells with a β-globin/eGFP reporter inserted with (RL5/pYB-αSat) or without (RL5/pYB) adjacent α-satellite arrays at the RL5 locus. Error bars indicate the standard deviations from three biological replicates. (C) Quantification of HDAC6 and HDAC10 transcripts relative to GAPDH transcript levels after siRNA knockdown of HDAC6 and HDAC10 in RL5/pYB and RL5/pYB-αSat MEL cells. Error bars indicate standard deviations for three biological replicates. *, P < 0.05.
HMS-I1 and HMS-I2 directly target HDAC activity, it remains formally possible that these compounds also affect heterochromatin integrity in vivo by interfering with other activities and/or protein interactions within HDAC complexes. A detailed structure-activity relationship (SAR) analysis will be required to improve potency and further delineate the mechanism of HMS-I1 and HMS-I2 action. Regardless, our discovery of HMS-I1 and HMS-I2 as panspecies inhibitors of heterochromatin-mediated transcriptional gene silencing demonstrates the striking functional conservation of the silencing machinery through eukaryotic evolution. HMS-I1 and HMS-I2 should serve as new chemical probes for dissection of silencing functions in various species and represent possible candidates for early stage drug development in diseases that result from inappropriate gene silencing events.

ACKNOWLEDGMENTS

We thank members of the Allshire lab, Ricardo Almeida, Aileen Greig, Fabio Pucci, and Leemor Joshua-Tor for support and Alison Pidoux for comments on the manuscript. We are grateful to Takeshi Urano and Hiroshi Kimura for the anti-H3K9me2 and anti-H3K9me3 antibodies, respectively, to Hervé Varcheret for the A. thaliana L5 line, to Vladimir L. Lariov for the MEL R5L cell lines and input on experiments with MEL cells, and to Shiv Grewal and Janet Partridge for strains and reagents. We also thank Peter Swenssen with help in preparing the GEO submission. E.C. was supported by a scholarship from the Natural Sciences and Engineering Research Council of Canada, and D.J.S.C. was supported by a Canadian Institutes of Health Research postdoctoral fellowship. R.C.A. was supported by the Wellcome Trust (065061 and 095021) and the European Commission Network of Excellence EpigenGes (HEALTH-F4-2010-257082). M.T. was supported by the Wellcome Trust (085178), the European Institutes of Health Research (MOP 119572), and an award from the Ministère de l’Enseignement Supérieur, de la Recherche, de la Science, et de la Technologie du Québec through Génome Québec and by a Canada Research Chair in Systems and Synthetic Biology. W.C.E. was supported by the Wellcome Trust (073915). The Wellcome Trust Centre for Cell Biology is supported by core funding from the Wellcome Trust (092076). A.K. is a Chancellor’s Fellow of the University of Edinburgh. The Spanish Ministry of Education, Ciencia, y Tecnologia (MEC, AGL2010-22287-C02-02) supported the work of A.G.C. K.E. was supported by grants from the Swedish Research Council (VR-M279 and VR-N1448) and the Swedish Cancer Society (CAN2012/238). T. Schalch was supported by the Swiss National Science Foundation (PP00P3_139137), University of Geneva, Fondation Ernst et Lucie Schmidheiny, Fonds Constantin Tolpadi, and Société Académique de Genève, and C. Brugger was supported by the University of Geneva.

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