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NOTE

Inhibition of histone H3K9 methyltransferases by gliotoxin and related epipolythiodioxopiperazines

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Histone lysine methyltransferases (HMTs) regulate transcriptional activity by writing epigenetic marks. Methylation of histone H3K9, a hallmark of silent chromatin,[1] is mainly regulated by two subgroups of HMTs, G9a/G9a-like protein[2] and Suv39h.[3] G9a and G9a-like protein induce mono- and di-methylation of histone H3K9 (H3K9me1 and H3K9me2) in the euchromatin region,[4] whereas Suv39h contributes to tri-methylation of histone H3K9 (H3K9me3) in the heterochromatin.[3] As methylation at histone H3K9, increased DNA methylation and reduced levels of activating chromatin modifications (e.g., histone acetylation) have been detected at promoter regions of aberrantly silenced tumor suppressor genes in cancer cells,[5,6] HMTs responsible for histone H3K9 methylation may represent promising targets for drug discovery. Indeed, overexpression of G9a is associated with several types of cancers and downregulation of G9a by RNAi inhibits tumor cell proliferation.[7–9] Chaetocin and BIX-01294 are small molecules that inhibit histone H3K9 HMTs. Chaetocin, which inhibit both G9a and Suv39h activities, is a member of the epipolythiodioxopiperazine (ETP) class of fungal metabolites.[10] On the other hand, BIX-01294 is a synthetic compound that selectively inhibits G9a but not Suv39h1[11] Based on a co-crystallization analysis of G9a with BIX-01294, BIX-01294-related molecules have been developed; these novel compounds are both more potent inhibitors and more membrane permeable than the parent compound.[12–15]

MATERIALS AND METHODS

Materials

Anti-mono- and di-methylated histone H3K9 antibodies were purchased from Merck Millipore (Billerica, MA, USA). pGEX4T-1-mG9a (706−stop amino acid (a.a.)), pGEX4T-3-histone H3 (1−57 a.a.) and pGEX4T-3-mSuv39h1-H320R (74−412 a.a.) were previously described.[2] The pET-28a(+)-Set7/9 was kindly provided by Dr Kenichi Nishioka (National Institute of Genetics).

Bacterial protein expression and purification

pGEX4T-1-mG9a (706−stop a.a.), pGEX4T-3-mSuv39h1-H320R (74−412 a.a.), pET-28a(+)-Set7/9, or pGEX4T-3-histone H3 (1−57 a.a.) was introduced into *Escherichia coli* BL21 (DE3). Expression of each recombinant protein was induced by 0.1 mM isopropyl-β-D-galactopyranosid at 18 °C for 24 h. Purification of GST-and $(His)_6$-fused proteins were carried out by either glutathione-affinity (GE Healthcare UK Ltd, Little Chalfont, UK) or nickel (Ni^{2+}) affinity (GE Healthcare UK Ltd).

In vitro HMT assay

Each purified recombinant HMT was pretreated in the presence or absence of a given compound in HMT buffer (50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 20 mM KCl, 10 mM 2-mercaptoethanol and 250 mM sucrose) containing 3 $\mu g\,ml^{-1}$ of BSA for 1 h. Next, 10 $\mu g\,ml^{-1}$ of SAM and 20 $\mu g\,ml^{-1}$ of GST-fused histone H3 (1−57 a.a.) were added into the reaction mixture and further incubated for 1 h at 37 °C. To detect histone methylation on western blots, samples were transferred onto Immobilon membranes (Merck Millipore), probed using appropriate primary antibodies, and visualized using horseradish peroxidase-linked secondary antibodies.

In order to identify small molecules that affect the function of histone H3K9 methyltransferases, we used enzyme-linked immunosorbent assay to screen a chemical library derived from microorganisms.[16] From this screen, we identified gliotoxin, an ETP secondary metabolite produced by fungal pathogens, as a G9a inhibitor (Figure 1a). To examine the inhibitory activity of gliotoxin against G9a in detail, we

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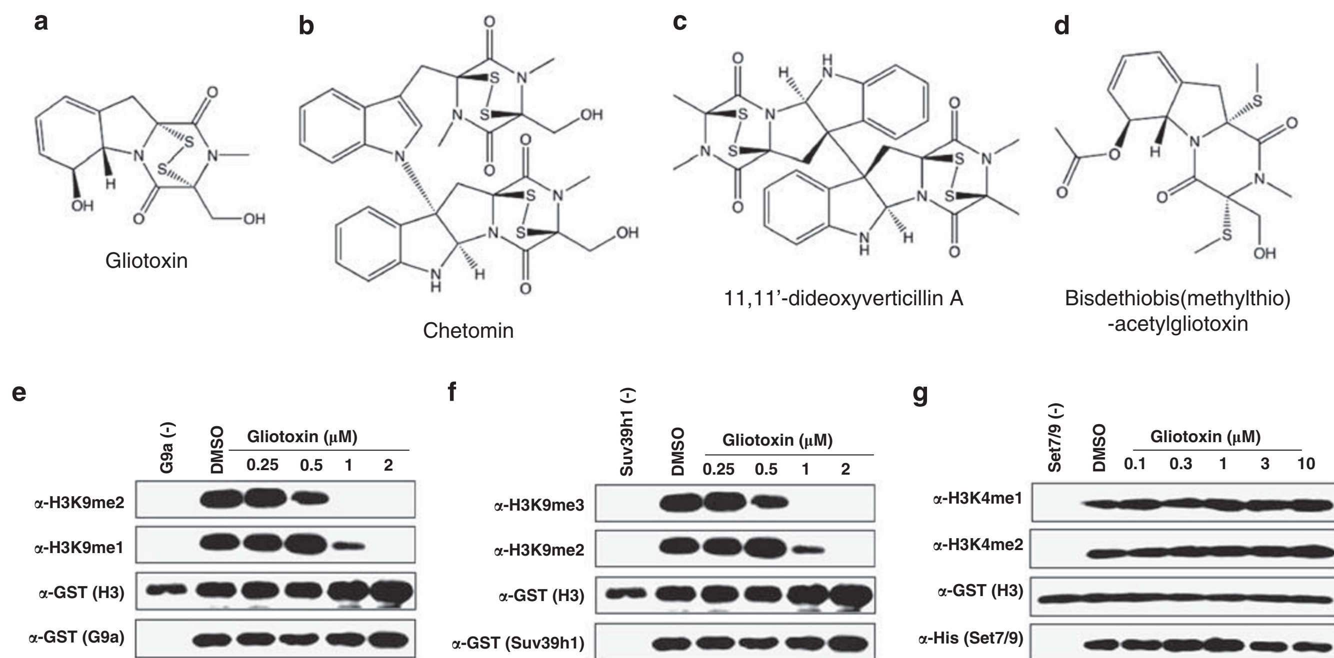

Figure 1 Gliotoxin inhibits methyltransferase activities of G9a and Suv39h1 *in vitro*. Structures of gliotoxin (**a**), chetomin (**b**), 11,11′-dideoxyverticillin A (**c**) and bisdethiobis(methylthio)-acetylgliotoxin (**d**). (**e**–**g**) Dose response of histone H3 methylation inhibition as a function of gliotoxin concentration. Indicated concentrations of gliotoxin were added to the methylation reaction mixture containing GST-fused histone H3 (1–57 a.a.), $10\,\mu g\,ml^{-1}$ of SAM and either GST-fused G9a (**e**), GST-fused Suv39h1-H320R (**f**) or His-tagged Set7/9 (**g**). GST-fused methylated histone H3 was detected by immunoblotting using an anti-H3K9me1, anti-H3K9me2 or anti-H3K9me3 antibody as indicated. Total levels of GST-fused histone H3 and methyltransferases were detected by immunoblotting using anti-GST and anti-His antibodies.

performed *in vitro* HMT assay by western blotting. In this assay, methylation of recombinant GST-fused histone H3 (1–57 a.a.) by G9a was measured using specific antibodies against methylated histone H3. As shown in Figure 1e, in the presence of recombinant GST-fused G9a together with SAM, G9a exhibited HMTase activity, producing mono- and di-methylated histone H3K9. However, the enzymatic activity of G9a was inhibited by gliotoxin in a dose-dependent manner. The IC_{50} value of gliotoxin for di-methylation of histone H3 was 0.53 μM (Table 1). Moreover, in almost the same concentration range, gliotoxin inhibited the HMTase activity of Suv39h1H320R (active form[3]) (Figure 1f and Table 1). On the other hand, gliotoxin did not inhibit the HMTase activity of Set7/9, another methyltransferase that methylates histone H3K4 *in vitro*[17] (Figure 1g and Table 1). These results suggested that gliotoxin selectively inhibits histone H3K9 methyltransferases.

In further analysis of the inhibitory activity of related ETP compounds, we selected chetomin (Figure 1b), 11-11′-dideoxyverticillin A (Figure 1c) and bisdethiobis-acetylgliotoxin (Figure 1d), and tested these compounds for their activity to inhibit histone H3K9 methyltransferases *in vitro*. Indeed, chetomin and 11-11′-dideoxyverticillin A could inhibit the HMTase activities of both G9a and Suv39h1H320R (Table 1). The capacity of chetomin to inhibit Suv39h1H320R was relatively strong (IC_{50}: 0.066 μM). Importantly, however, bisdethiobis-acetylgliotoxin, which does not contain the disulfide bridge, exhibited no inhibitory activity up to 10 μM (Table 1). This observation suggests that the disulfide group is essential for the inhibition of HMTs by ETPs. This is also consistent with the previous report of the activity of synthetic analogs of chaetocin lacking disulfide.[16] Because G9a and Suv39h1, but not Set7/9, possess pre- and post-SET domains, which bind zinc atoms with their cysteine-rich regions,[2] ETPs might interact with these domains through their own sulfur-containing functional groups.

Table 1 Selectivity of gliotoxin and other ETPs against inhibitory activities of HMTs

	IC_{50} (μM)		
Compound	*G9a*	*Suv39h1-H20R*	*Set7/9*
Gliotoxin	0.53 ± 0.13	0.26 ± 0.02	>10
Chetomin	0.17 ± 0.00	0.066 ± 0.008	ND
11,11'-dideoxyverticillin A	0.63 ± 0.06	0.53 ± 0.15	ND
bisdethiobis(methylthio) acetylgliotoxin	>10	>10	ND

Abbreviations: ETP, epipolythiodioxopiperazine; HMT, histone lysine methyltransferases.
The IC_{50} value was calculated by measuring the intensity of bands corresponding to the histone H3K9 di-methylation in western blotting. Means ± s.d. from three independent experiments are shown.

Gliotoxin and the other ETPs inhibit several other proteins, including creatine kinase, NF-κB, adenine nucleotide transporter and farnesyltransferase;[18] these compounds also exhibit antitumor and immunomodulatory activities. However, the structure–activity relationship of ETPs is poorly understood. In this study, we demonstrated that gliotoxin, chetomin and 11-11′-dideoxyverticillin A strongly inhibit H3K9 HMTs (Table 1) probably through their disulfide bonds, suggesting that the inhibition of H3K9 HMTs is a common activity of ETPs. Because accumulating evidence suggests that G9a and Suv39h1 are potential targets for cancer therapy, ETPs may serve as a drug seed. In particular, gliotoxin is one of the structurally simplest ETPs; derivatives of this compound may provide information that will be useful for development of anticancer small molecules that specifically inhibit HMTs but not other proteins.

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