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Inhibition of histone lysine methyltransferases G9a and GLP by ejection of structural Zn(II)

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ABSTRACT

Histone lysine methyltransferases G9a and GLP are validated targets for the development of new epigenetic drugs. Most, if not all, inhibitors of G9a and GLP target the histone substrate binding site or/and the S-adenosylmethionine cosubstrate binding site. Here, we report an alternative approach for inhibiting the methyltransferase activity of G9a and GLP. For proper folding and enzymatic activity, G9a and GLP contain structural zinc fingers, one of them being adjacent to the S-adenosylmethionine binding site. Our work demonstrates that targeting these labile zinc fingers with electrophilic small molecules results in ejection of structural zinc ions, and consequently inhibition of the methyltransferase activity. Very effective Zn(II) ejection and inhibition of G9a and GLP was observed with clinically used ebselen, disulfiram and cisplatin.

Histone posttranslational modifications (PTMs), including methylation, acetylation, phosphorylation and many others, play an important role in human gene regulation.[1] Methylation of lysine residues is catalyzed by members of histone lysine methyltransferases (KMTs) that transfer the methyl group from S-adenosylmethionine (SAM) to lysine residues on histone *N*-terminal tails, core histones and non-histone proteins. Recent structural, mutagenesis and molecular modelling studies provided basic mechanistic insight into histone methyltransferase catalysis.[2–5] SET domain-containing proteins G9a and its highly related homologue G9a-like protein (GLP) (also known as EHMT2 and EHMT1, respectively) catalyze mono-, di- and trimethylation of lysine 9 on histone 3 (H3K9me1/2/3, Fig. 1a) and several other proteins.[6] The highest methylation mark (H3K9me3) results in formation of heterochromatin, i.e. the transcriptionally inactive form of chromatin, and has been linked to the development and maintenance of various types of cancer.[7] For instance, recent work has shown that increased expression of G9a in aggressive lung cancer cells is associated with greater mortality in patients.[8] Therefore G9a and GLP have been recognized as validated targets for development of small molecule inhibitors for therapies against a variety of diseases, including cancer.[9]

Recent medicinal chemistry studies have demonstrated that inhibition of G9a and GLP (and other KMTs) can be achieved by small molecules that act as histone-competitive or/and SAM-competitive inhibitors.[10–12] BIX-01294, the first known selective inhibitor of G9a, was reported in 2007.[13] This small molecule inhibitor, which was identified by high throughput screening, targets the histone binding site. Since its discovery, the structure of BIX-01294 has been used in various structure-activity relationship (SAR) explorations and structure-based design studies, which led to the development of inhibitors with an improved potency and selectivity, and reduced toxicity to cells, such as UNC0638,[14] A-366,[15] E72,[16] and DCG066.[17] Few SAM-competitive inhibitors of G9a and GLP have also been recently reported, including BIX-01338 and BRD4770.[13,18] However, SAM-competitive inhibitors are often unselective due to high homology in SAM-binding sites between different methyltransferases. Most known inhibitors have similar inhibitory activity against G9a and GLP, and developing selective inhibitors for either one is considered challenging due to their high protein homology and similarity of the histone and SAM binding sites (~80%).[19] Nonetheless, recent work has shown that a high degree of selective inhibition can be achieved; MS012 and related structures have up to 140-fold selectivity for GLP over G9a.[20]

In order to establish proper folding and enzymatic activity, G9a and GLP methyltransferases each contain four structural zinc ions. Both enzymes contain two distinguishable types of zinc fingers; three Zn(II) ions are chelated in a triangular cluster by 9 cysteines (Fig. 1b, top left), whereas one Zn(II) ion is chelated by 4 cysteines in a Cys_4-type zinc finger (Fig. 1b, top right).[21] The latter zinc finger is adjacent to the SAM-binding site. Recent studies have highlighted that significant efforts have been made in developing

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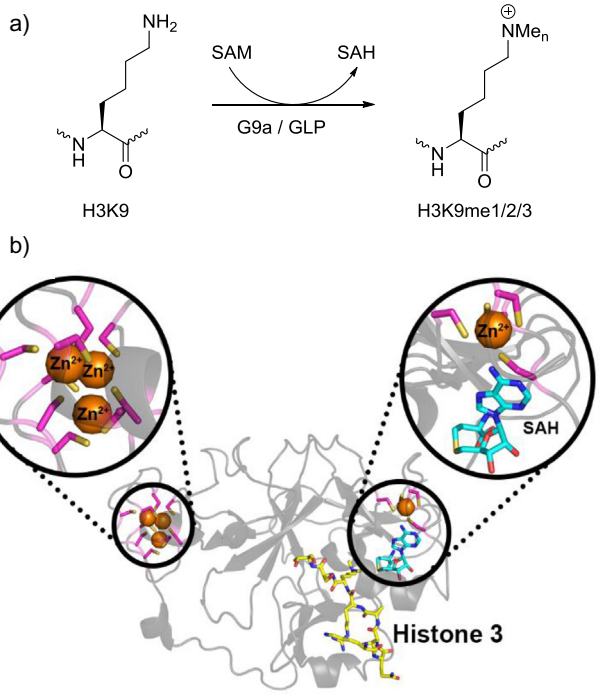


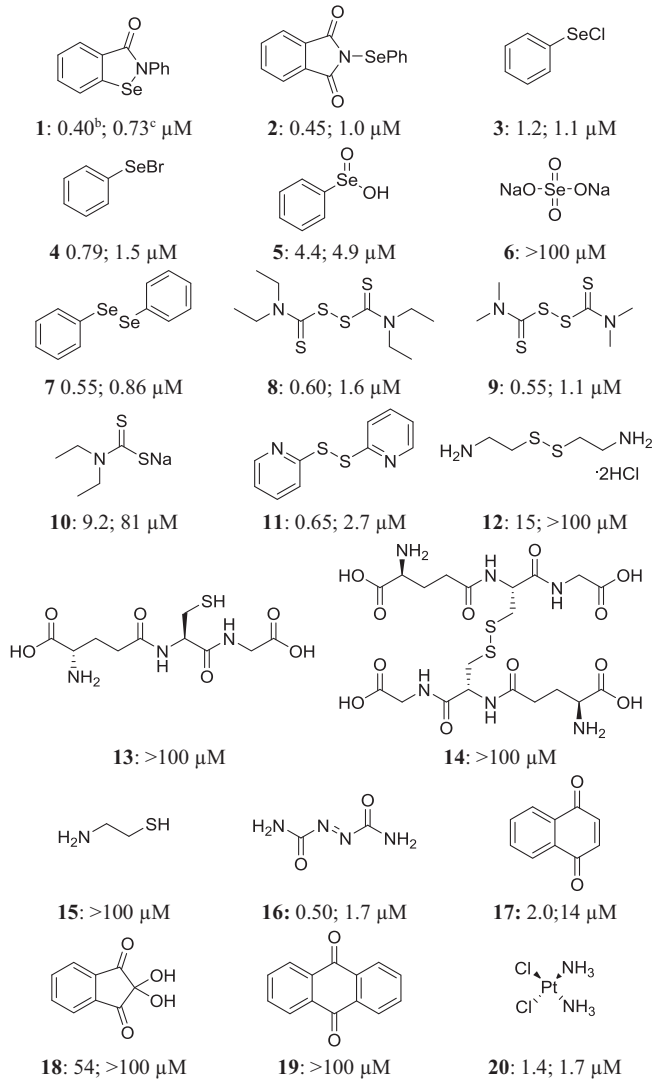
Fig. 1. a) G9a- and GLP-catalyzed methylation of H3K9 (n = 1, 2, 3); b) Crystal structure of GLP in complex with histone 3 peptide (yellow) and S-adenosylhomocysteine (SAH, light cyan); a zoomed view on the Cys residues (magenta) involved in the chelation of structural Zn(II) (orange) (PDB ID: 2RFI).

strategies for targeting labile Zn-fingers with electrophilic small molecules, most notably by ebselen and disulfiram.[22,23] Inhibition of important biological processes by the release of structural zinc ions has been shown for a variety of proteins, including nucleocapsid 7,[24] p300,[25] γ-butyrobetaine,[26] and histone lysine demethylase JMJD2A.[27] We hypothesized that it would be possible to inhibit G9a and GLP methyltransferases by small molecule-mediated ejection of structural zinc ions. It was envisioned that the ejection of Zn(II) from the Cys_4-Zn finger, which is located adjacent to the SAM-binding site (Fig. 1b, top right), would lead to a loss of the methyltransferase activity of G9a and GLP.

We initiated our investigations by testing whether 20 known and potential zinc ejectors, including clinically used ebselen, disulfiram and cisplatin, have an ability to inhibit the G9a and GLP methyltransferase activity (Table 1). The chosen examples include: selenium-based compounds **1**–**7**, sulfur-based compounds **8**–**15**, and various other potential Zn(II) ejectors **16**–**20**. Initially, all compounds were tested at a concentration of 10 μM against both G9a and GLP. Therefore, methylation of a synthetic 15-mer peptide (residues 1–15) mimic of the *N*-terminal histone 3 tail containing a lysine at position 9 (H3K9) was monitored using matrix assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry.[28] Representative inhibition data for GLP-catalyzed methylation of H3K9 can be found in Fig. 2. Molecules that did not show significant inhibition at 10 μM, were also tested at 100 μM. Inhibition data at 10 and 100 μM for all other compounds can be found in Figs. S1–S4. For those compounds that showed >50% inhibition at a concentration of 100 μM, half maximum inhibitory concentrations (IC_{50}) were obtained using a MALDI-TOF based assay using 200 nM enzyme concentrations (Table 1 and Figs. S1–S32).

In the absence of inhibitor, 15-mer H3K9 histone mimic underwent near quantitative trimethylation (m/z = 1603.1 Da, Fig. 2a); this result is consistent with our recent studies on KMT-catalyzed methylation of lysine.[29] For ebselen **1**, which is known to inhibit

Table 1
Inhibition IC_{50} data.[a]



[a]Half maximum inhibitory concentration (IC_{50}) obtained at 200 nM enzyme concentration.
[b]IC_{50} for G9a.
[c]IC_{50} for GLP.

various zinc finger containing proteins, such as metallothionein,[30] histone lysine demethylase JMJD2A,[27] and γ-butyrobetaine hydroxylase,[26] submicromolar IC_{50} values were obtained (0.40 μM for G9a and 0.73 μM for GLP), demonstrating very effective inhibition. Notably, at 10 μM concentration of ebselen, only unmethylated peptide was observed in MALDI-TOF spectrum (Fig. 2b, m/z = 1560.9). Related seleno compounds **2**–**5** were also observed to be excellent inhibitors of G9a and GLP; IC_{50} values were found to be 0.45–4.4 μM for G9a, and 1.0–4.9 μM for GLP. Sodium selenate **6** only showed ~10% inhibition for G9a and GLP at 100 μM concentration (Figs. S2 and S4). Diphenyl diselenide **7** inhibited G9a and GLP with IC_{50} = 0.55 μM and 0.86 μM, respectively.

Having shown that selenium-based compounds act as good inhibitors of G9a and GLP, we examined related sulfur-based small molecules as potential inhibitors for these two methyltransferases. Dithiocarbamates disulfiram **8** (Fig. 2c) and thiram **9** inhibited the activity of G9a with submicromolar IC_{50} values of 0.60 and

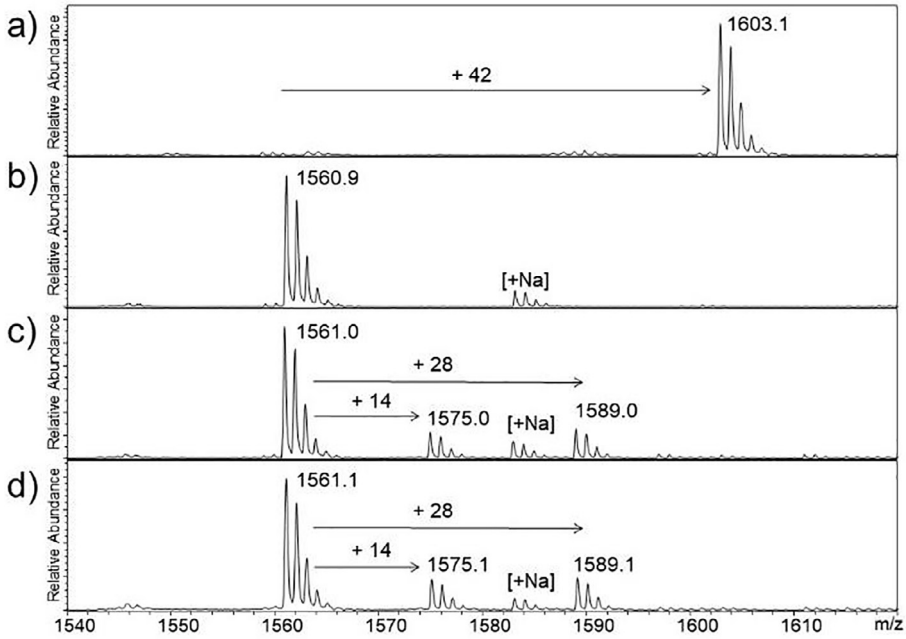


Fig. 2. Representative MALDI-TOF MS data of a) GLP-catalyzed methylation of H3K9 peptide (m/z = 1561.0); b) with 10 μM ebselen; c) with 10 μM disulfiram; d) with 10 μM cisplatin.

0.55 μM, whereas for GLP the observed IC_{50} values are 1.6 and 1.1 μM, respectively. Inhibition by clinically safe disulfiram is particularly important, as it has been used for decades for treatment alcoholism by targeting acetaldehyde dehydrogenase (ADH), thereby causing instantaneous nausea upon consumption of alcohol.[31] Besides ADH, disulfiram is also known to inhibit hepatitis C viral replication by targeting a labile zinc finger,[32] and to induce apoptosis in a number of human cancer cell lines or inhibit cancer cell growth.[33–35] Sodium diethyldithiocarbamate **10**, the reduced form of disulfiram, was found to only poorly inhibit GLP. This result is consistent with observations on inhibition of HIV-1 nucleocapsid protein.[36]

2,2′-Dithiodipyridine **11**, also known as aldrithiol, is a known zinc ejector, targeting for instance the zinc finger in nucleocapsid protein of human immunodeficiency virus type 1.[37] **11** inhibited G9a activity with IC_{50} = 0.65 μM and GLP with IC_{50} = 2.6 μM. We also tested disulfide cysteamine dihydrochloride salt **12**, but its IC_{50} value was found to be above 100 μM for GLP, whereas for G9a an IC_{50} value of 15 μM was obtained. Glutathione, in both the reduced (**13**) and oxidized (**14**) form, did not inhibit G9a and GLP. Also, no inhibition was observed for cystamine **15**, the reduced form of cysteamine dihydrochloride. Azidocarbonamide **16** showed very high inhibition activity against both G9a and GLP (IC_{50} = 0.50 and 1.7 μM, respectively). Naphthoquinone **17** and ninhydrin **18**, both known to eject zinc from p300,[25] were also tested against G9a and GLP. Naphthoquinone **17** was observed to be a potent inhibitor of G9a with an IC_{50} value of 2.0 μM, whereas it inhibited GLP with IC_{50} = 14 μM. Ninhydrin **18** only poorly inhibited GLP (~40% inhibition at 100 μM, Fig. S4) and an IC_{50} value of 54 μM was observed for G9a. In addition, anthraquinone **19**, a structurally related analogue of naphthoquinone, exhibited very poor inhibition activity (~20% for both G9a and GLP) at 100 μM. Finally, inhibitory activity of cisplatin **20** was evaluated. Cisplatin is a potent chemotherapeutic drug and is known to be highly reactive towards Cys_4 or Cys_3His type Zn-fingers.[38] Importantly, we found that cisplatin inhibits G9a and GLP with similar IC_{50} values of 1.4 and 1.7 μM respectively.

The observations that several Se- and S-based electrophiles, including clinically used ebselen and disulfiram, possess excellent inhibition activity against G9a and GLP prompted us to examine their mode of action. We envisioned that they inhibit G9a and GLP by the release of structural zinc ions, as a result of covalent modification of cysteine residues. The release of zinc from G9a and GLP was therefore monitored using the Zn^{2+}-selective indicator FluoZin™-3. In line with observed MALDI-TOF data, we found that zinc was released from both methyltransferases in the presence of those compounds that showed inhibitory activity (Figs. S33–S39). Because of their current use in clinics, we were particularly interested in inhibition of G9a/GLP by ebselen, disulfiram and cisplatin. Therefore, zinc release from G9a and GLP in the presence of various concentrations of ebselen, disulfiram and cisplatin was monitored over time in the presence of FluoZin-3 (Fig. 3a–c). We observed that ebselen ejects Zn(II) very rapidly (within minutes) for both G9a and GLP; disulfiram and cisplatin also have the ability to eject zinc ions from G9a/GLP, but require somewhat longer times to achieve it (Fig. 3b–c).

Based on a calibration curve with known Zn(II) concentrations, a dose-response curve for the amount of zinc released from each methyltransferase was plotted (GLP Fig. 3d–f, G9a Fig. S40). Each methyltransferase contains in total four structural zinc ions, one of which is very close to the active site, and three at a more distant location (Fig. 1). In the presence of ebselen (>25 μM), all 4 zinc ions were released from G9a and GLP, whereas higher concentrations of disulfiram (>50 μM) were required for complete zinc ejection. On average only ~2.5 Zn(II) ions are removed after one hour in the presence of 100 μM cisplatin, although it is possible that all four zinc ions can be released after prolonged time, and/or at even higher concentrations of cisplatin. We also tested whether UNC0638,[14] a very potent histone competitive inhibitor for G9a and GLP, was able to eject structural Zn(II); as expected, no zinc release was observed (Figs. S37 and S38).

Having shown that ebselen and disulfiram effectively inhibit G9a and GLP via zinc ejection mechanism, we next explored whether the folding of these two enzymes has been affected in

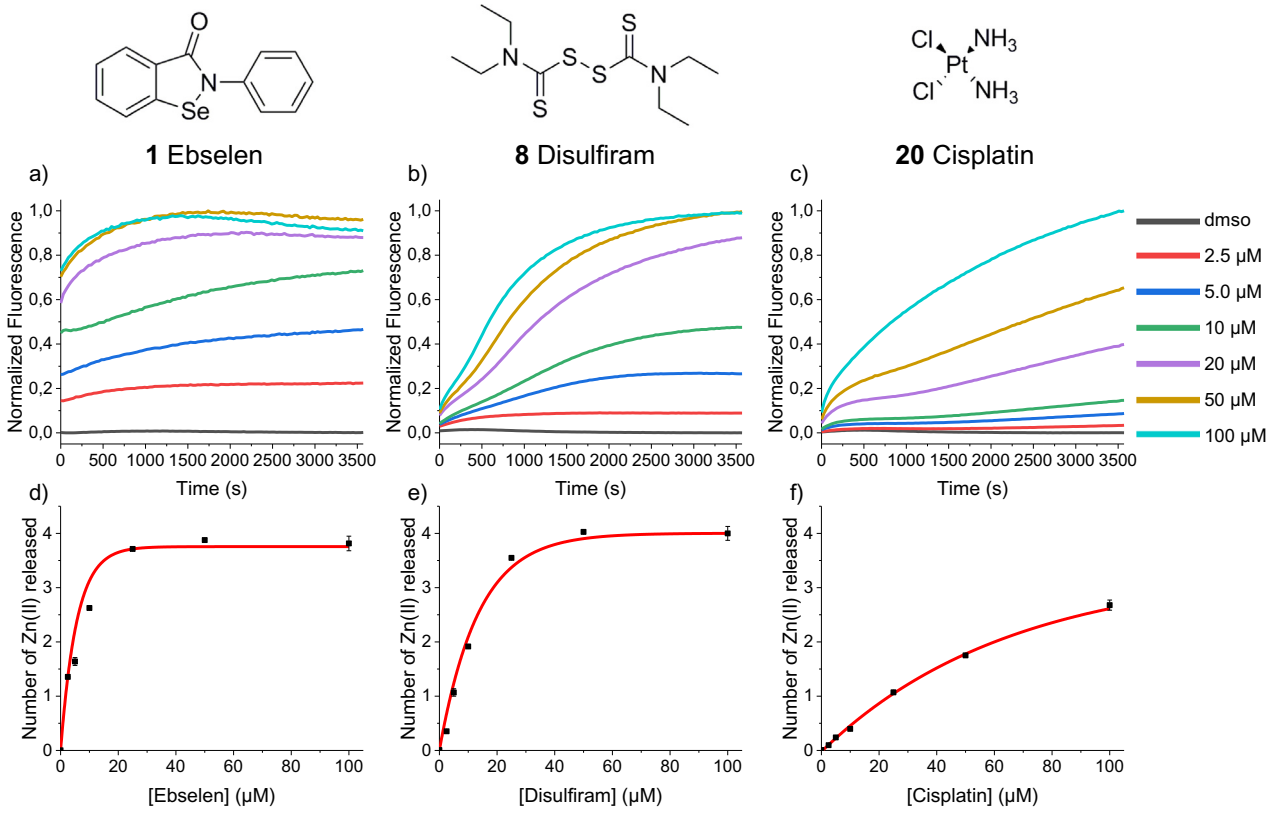


Fig. 3. a–c) Zinc ejection data for GLP (2 μM) in the presence of various concentrations of ebselen, disulfiram, and cisplatin, measured in the presence of Zn^{2+}-selective fluorophore FluoZin™-3.; d-f) Dose-response curves after 1 h incubation in the presence of ebselen, disulfiram, and cisplatin.

the presence ebselen and disulfiram. In order to investigate potential changes to the secondary and/or tertiary structure of these methyltransferases, we employed circular dichroism (CD), a well-established tool in protein/peptide research.[39] Upon the addition of 100 μM of ebselen or disulfiram to either 2 μM G9a or GLP, we observed significant distortions in the CD spectrum (Figs. S40 and S41), implying that both enzymes became partially unfolded.

In conclusion, we have demonstrated that the ejection of structural zinc ions from G9a and GLP in the presence of selenium-, and sulfur-containing electrophilic small molecules leads to inhibition of these two biomedically important epigenetic enzymes. Our work demonstrates that clinically used ebselen, disulfiram and cisplatin act as very effective inhibitors of G9a and GLP with submicromolar or low micromolar IC_{50} values. It is possible that the observed physiological effects of these molecules may in part arise as a result of their ability to affect epigenetic processes regulated by G9a and GLP methyltransferases. Inhibition of biomedically important epigenetic processes is currently a subject of intensive investigations, therefore we envision that future studies will lead to important advances in design and development of specific inhibitors of the therapeutic potential. Although it is unlikely that highly electrophilic compounds studied here, most notably ebselen,[23] act as specific inhibitors of endogeneous proteins, it might be possible that exploring a broader chemical space via substitutions on the ebselen and disulfiram scaffolds may lead to a higher degree of specificity for certain protein targets. Detailed structure-activity relationships studies on small molecules that target structural zinc fingers may direct a design of novel type of inhibitors with an improved selectivity. Towards this aim, our work highlights that targeting zinc finger sites of histone lysine methyltransferases is an alternative strategy to commonly used approaches that target histone substrate and SAM cosubstrate binding sites; this strategy leads to efficient inhibition of G9a and GLP histone lysine methyltransferases that possess structural zinc ions.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.02.043.

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