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Identification of Labile Zn Sites in Drug-Target Proteins

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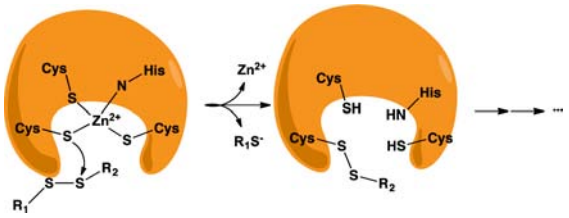
Supporting Information

ABSTRACT: Labile Zn fingers (Zfs) in proteins contain Zn-bound thiolates that can react with electrophilic agents, causing Zn^{2+} ejection and protein unfolding. Such labile Zfs have been shown to be Cys_4 or Cys_3His cores whose Zn-bound Cys have no hydrogen bonds. Our aim here is to identify labile Zfs in proteins that are promising drug targets using these features. To prove the strategy used, we showed that five proteins with predicted labile Zfs reacted with Zn-ejecting agents, whereas five proteins with no or inert Zfs did not. The comprehensive set of labile Zfs provides new drug targets and guidelines to redesign Zn-ejecting compounds with improved specificity.

Zn-bound thiolates in labile Zn-finger (Zf) cores can react with electrophilic agents, resulting in Zn^{2+} ejection and loss of protein structure and function (Scheme 1). Here, we applied the principles controlling the Zn-bound Cys reactivity that we had previously elucidated to predict labile Zfs in proteins that are promising drug targets. We then experimentally verified that Zn^{2+} could be ejected from five proteins with predicted labile Zfs but not from five proteins with no or inert Zfs. The labile Zfs found provide novel therapeutic drug targets.

Scheme 1. Proposed Reaction Mechanism for some Zn-Ejecting Compounds[1]



Zfs are small protein motifs that are abundantly (~3%) encoded in the human genome.[2a] They function as interaction modules that bind DNA,[2b] RNA,[2c] proteins,[2d] or lipids[2e] and are essential in many vital cellular processes, such as transcription, translation, DNA replication and repair, metabolism, cell proliferation, apoptosis, and signaling.[2f] They can be grouped by the type of Zn core, i.e., $Zn{\cdot}Cys_4$, $Zn{\cdot}Cys_3His$, $Zn{\cdot}Cys_2His_2$, and $Zn_2{\cdot}Cys_6$.[3] The Zn^{2+} in Zfs is needed for protein function as it induces the correct folding of Zf peptides, stabilizing the local protein structure that is needed for function.[4]

Labile Zfs are emerging drug targets. Although Zf cores typically play a structural role, some Zf cores are susceptible to electrophilic agents and can serve as promising drug targets for retroviral or cancer therapies (Figure 1).[1,5] An example is the labile Zf containing two highly conserved $Zn{\cdot}Cys_3His$ cores in the HIV-1 nucleocapsid p7 protein (Ncp7), a critical transcription factor in viral replication cycle.[6] Electrophilic agents can oxidize the Zn-bound Cys, causing Zn^{2+} ejection and loss of viral, but not human, Zf protein structure and function.[7] Labile Zfs have also been found in herpes virus[8] and arenavirus.[5e]

Protein name	Disease targeted	Year
Ncp7	HIV infection	1993
C3HC4 domain	herpes virus infection	1994
hER-DBD	breast cancer	2004
JMJD2A	prostate cancer	2009
BCA2	breast cancer	2010
Glycoprotein	arenavirus infection	2011

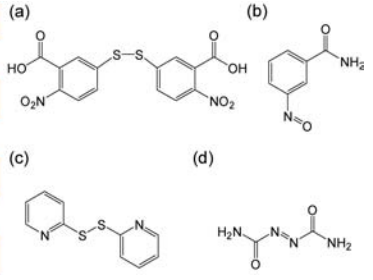


Figure 1. Current drug targets containing labile Zfs (left) and some Zn-ejecting agents: (a) 5,5′-dithiobis(2-nitrobenzoic acid), (b) 3-nitrosobenzamide, (c) aldrithiol-2, and (d) azodicarbonamide.

Labile Zfs serve not only as antiretroviral drug targets but also as cancer therapeutic drug targets. One example is the labile $Zn{\cdot}Cys_4$ core in the human estrogen receptor (hER) DNA-binding domain, which is essential to breast cancer growth.[9] Zn-ejecting agents can selectively block hER binding to DNA and subsequent transcription.[5c] Another example is the labile $Zn{\cdot}Cys_3His$ core in JMJD2A, which is a target for prostate, breast, ovarian, and pancreatic cancers.[1] Electrophilic agents can eject the structural Zn^{2+} in the $Zn{\cdot}Cys_3His$ core, thus inhibiting JMJD2A. Since labile Zfs are being continuously discovered and azodicarbonamide (Figure 1d) has undergone phase I and II trials,[10] identifying novel labile Zfs would be useful in antiviral and cancer therapies.

What factors determine the reactivity of a Zn-bound thiolate and thus the Zf core? An early analysis of 207 Zf cores from 92 protein structures showed that the degree of screening of the Zn-bound thiolate via backbone NH···S hydrogen bonds determines its reactivity.[11] Both experimental[12] and theoretical[13] studies on model Zn complexes confirmed that a single

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hydrogen bond formed by a Zn-bound thiolate with the backbone NH group can dramatically reduce its reactivity toward electrophiles.[14] However, we found several well-known inert Zf cores containing unsheltered Cys ligands with no hydrogen bonds.[15] By evaluating the free energy barriers for methylation and dissociation of a Zn-bound thiolate in $Zn{\cdot}Cys_4$, $Zn{\cdot}Cys_3His$, $Zn{\cdot}Cys_2His_2$, and $Zn_2{\cdot}Cys_6$ cores and their dependence on NH···S hydrogen bond(s), we showed that hydrogen bonds from the peptide backbone or bonds from a second Zn^{2+} to Zn-bound S atoms suppress the reactivity not only of these S atoms but also of Zn-bound S atoms with no interactions (see ref 15, Figures 4−6).

The physical principles controlling the Zn-bound thiolate reactivity in a Zf core that we had elucidated[15] provide guidelines in searching for labile Zf cores in the Protein Data Bank[16] (PDB): Labile Zn sites are Cys_4 or Cys_3His cores containing Zn-bound cysteines that are likely to have no hydrogen-bonding interactions. These guidelines have been used to screen the entire PDB for putative labile Zfs: (1) PDB structures were searched for $Zn{\cdot}Cys_3His$ or $Zn{\cdot}Cys_4$ sites. (2) Hydrogen bonds to the Zn-bound cysteines were computed using HBPLUS.[17] (3) Zfs containing no hydrogen bonds to any Zn-bound cysteines in $Zn{\cdot}Cys_4$ or $Zn{\cdot}Cys_3His$ cores were found. (4) As many structures correspond to the same protein or mutant proteins, these Zfs were grouped using BLAST-CLUST, which finds pairs of sequences with statistically significant matches and clusters them using single-linkage clustering; in each group, the wild-type Zf with the highest resolution structure was chosen.

The predicted labile Zfs included those listed in Figure 1 and the known labile Zfs in *Escherichia coli* ADA enzyme (1EYF) and *Drosophila melanogaster* U-shaped transcription factor (1FU9). To identify new potential Zf drug targets in humans, the literature was searched to see if the human protein containing the predicted labile Zf was a promising drug target. This search yielded 22 human proteins that are promising drug targets, but their Zfs are not known to be labile and are not considered to be drug targets (see Table S1).

To verify our predictions, we tested five promising cancer target proteins that were predicted to contain labile Zfs: (1) Pirh2 (p53-induced RING-H2), which is involved in many signaling pathways related to the genesis and evolution of cancer;[18] (2) BHC80,[19] an essential component of the lysine-specific demethylase 1 that is implicated in prostate, breast, lung, and bladder cancers;[20] (3) TRAF6 (tumor necrosis factor receptor associated factor 6), which is involved in oncogenic protein kinase B activation;[21] (4) UHRF1 (ubiquitin-like with PHD and ring-finger domain 1),[22] a marker of lung cancer;[23] and (5) DNMT3L (DNA methyltransferase), which is required for DNA methylation of imprinted genes and for the growth of human embryonal carcinoma.[24] The cDNA of these five cancer targets were cloned and expressed in *E. coli* (see Supporting Information). Upon addition of various Zn-ejecting compounds to these five proteins, Zn^{2+} was released, as monitored by the fluorescence change of the zinc-specific fluorophore, FluoZin-3 (Figure 2). As controls, we tested two non-Zf enzymes and three proteins with predicted inert Zn-sites; viz., (1) ribonuclease T, a Mg^{2+}-binding enzyme;[25] (2) carbonic anhydrase I, a Zn^{2+} enzyme with a $Zn{\cdot}His_3H_2O$ site;[26] (3) CRN4 (cell death-related nuclease 4) with an inert $Zn{\cdot}Cys_4$ core,[27] (4) TZD (the three-Cys_2His_2 Zf domain) of mouse testis Zf,[28] and (5) Gal4 with an inert $Zn_2{\cdot}Cys_6$ core.[29] All five control proteins were not affected by the Zn-ejecting agents.

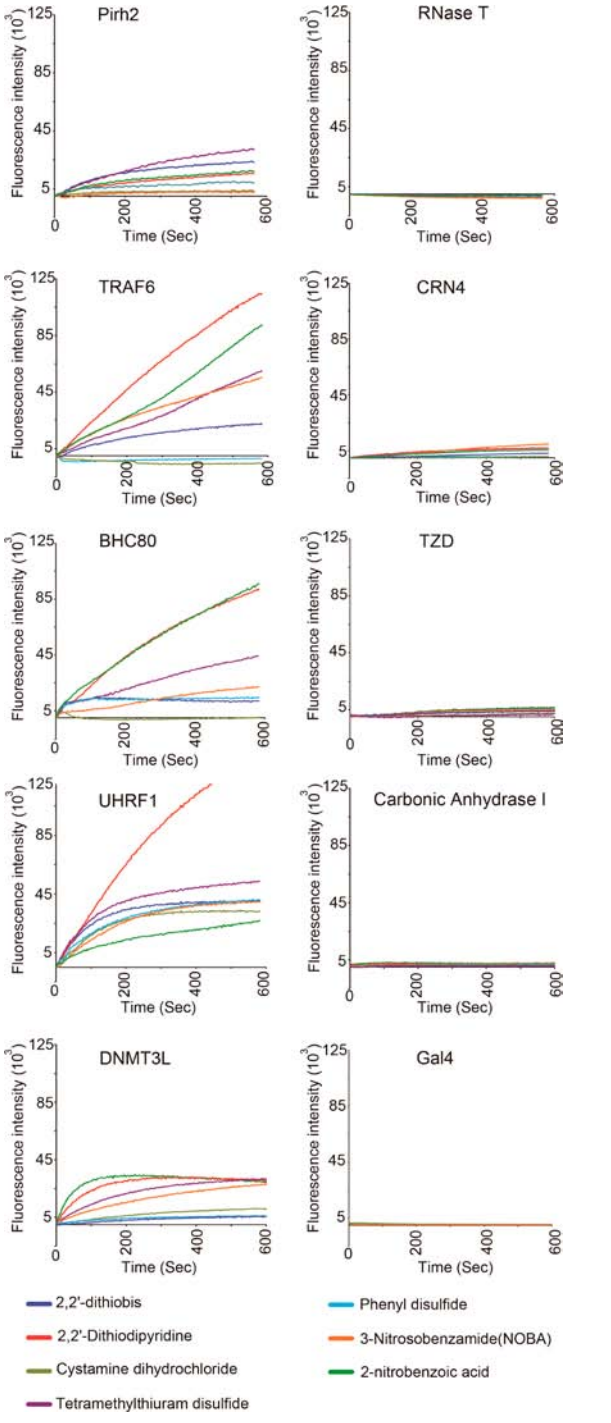

Figure 2. Zn^{2+} ejection test on Pirh2, TRAF6, BHC80, UHRF1, and DNMT3L cancer targets predicted to contain labile Zfs (left column). The Zn^{2+} in these proteins was released by adding seven Zn-ejecting agents, as monitored by the increased fluorescence signal of the Zn-specific fluorophore, FluoZin-3, using an excitation wavelength at 494 nm and emission wavelength at 516 nm for detection. Ribonuclease T, CRN4, TZD, carbonic anhydrase I, and Gal4 are negative controls (right column).

The results in Figure 2 thus verified all our predictions and lend support to the physical principles used to screen the PDB for labile Zfs.

To measure the efficiency of the Zn-ejecting compounds, the most effective Zn-ejector for each target protein was selected, and the Zn^{2+} ions released by the different doses of the Zn-

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ejector were monitored during the experiments by FluoZin-3 (Figure 3A). For a given concentration i of the Zn-ejector of a

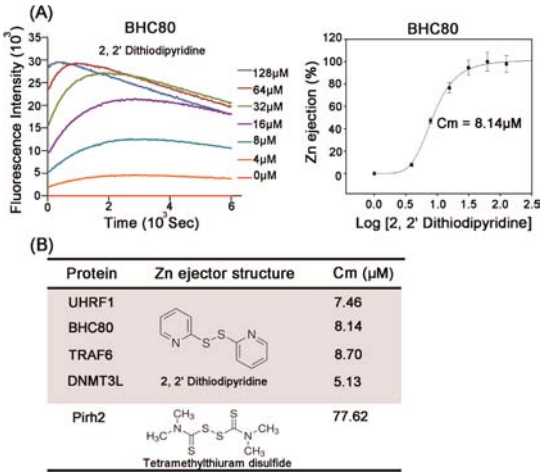

Figure 3. Estimating the efficiency of the Zn-ejecting compounds for UHRF1, BHC80, TRAF6, DNMT3L, and Pirh2. (A) The concentration of the Zn-ejecting compound corresponding to 50% Zn^{2+}ejection (Cm) from BHC80 was estimated by dose-dependent time-course experiments. (B) The Cm for the five cancer targets.

given protein, the highest fluorescence intensity of the curve (I_i) is proportional to the released Zn^{2+} concentration. At the maximum fluorescence intensity of all the curves (I_{max}), all Zn ions were assumed to be ejected from the target protein. Thus, the percentage of Zn^{2+} ejected from each target protein by a given Zn-ejector concentration i was estimated by I_i/I_{max}. For a 50% Zn^{2+} ejection, the concentration Cm of 2,2′-dithiodipyridine for UHRF1, BHC80, TRAF6, and DNMT3L ranges from 5.1−8.7 μM, while that of tetramethylthiuram disulfide for Pirh2 is 77.6 μM (see Figures 3B and S1).

To further confirm that the Zn-ejecting agent was bound to Cys in the target Zfs, the molecular weights of the Zfs before and after the addition of the Zn-ejecting agent, 2,2′-dithiodipyridine, were measured by mass spectroscopy (see Figure 4). The measured MW of BHC80 (8890 Da) was close to the calculated MW (8891.30 Da). The 2,2′-dithiodipyridine-treated BHC80 showed 7 peaks of increasing MW, each incremented by ∼110 Da, suggesting that the seven cysteines in the $Zn{\cdot}Cys_3His$ and $Zn{\cdot}Cys_4$ sites were all bound covalently to half of the dithiodipyridine (MW = 220 Da). Although only the $Zn{\cdot}Cys_3His$ site was predicted to be labile in BHC80, the Zn-ejecting agent reacted with all the Zn-bound cysteines in the target protein. Once the Zn-ejecting agent disrupted the predicted labile $Zn{\cdot}Cys_3His$ core, the protein likely underwent unfolding, resulting in a loss of protective hydrogen bonds to the cysteines in the $Zn{\cdot}Cys_4$ site.

This is the first PDB screening for labile Zfs since 2001 when Maynard and Covell screened 92 proteins using protein packing and electrostatics based on a molecular mechanics forcefield.[11] They identified two known labile Zfs in HIV-1 Ncp7 and Ada protein. They also successfully predicted labile Zfs in the hER DNA-binding domain, which was subsequently shown to be susceptible to electrophilic agents and became a promising drug target for breast cancer therapy. As the number of entries in the PDB has increased dramatically from 6270 in 2001 to over 90 000 in 2013, it is timely to search the entire PDB for new labile Zfs. A key novelty in this work lies in our

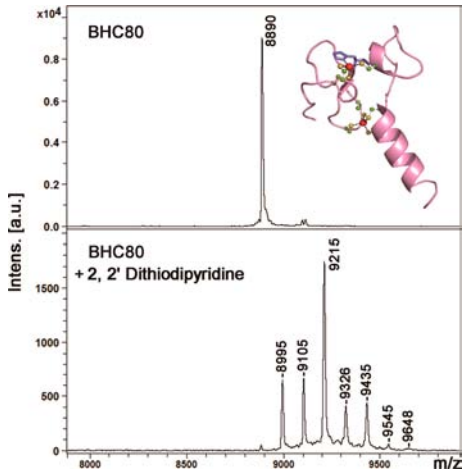

Figure 4. Zn-ejecting agent 2,2′-dithiodipyridine (MW = 220 Da) was bound covalently to the Zn-bound cysteines of BHC80, as monitored by mass spectroscopy. MW of BHC80 with two Zn sites and seven Cys, in the absence (top panel) and presence (bottom panel) of 2,2′-dithiodipyridine. All the cysteines were modified by 2,2′-dithiodipyridine, as evidenced by the increased MW.

search algorithm, which is based on physical principles derived from our previous work.[15] That Zn^{2+} ions were ejected from all five predicted labile Zfs but not from the five control proteins supports the physical principles used in our search algorithm. Another novelty in this work lies in showing that Pirh2, TRAF6, BHC80, UHRF1, and DNMT3L, which are promising cancer targets, possess labile Zfs whose structures and thus functions can be destroyed by Zn-ejecting agents. Our search algorithm allows identification of all labile Zfs with known 3D structures in a given organism, which in turn would aid in the design of Zn-ejecting agents with improved specificity. Notably, it yielded 22 putative labile Zfs in promising drug target proteins whose Zfs have not been considered to be targets for intervention. Future studies will focus on selecting the most important drug target proteins and redesigning the Zn-ejecting compounds to selectively target the labile Zfs in these proteins *in vitro* and *in vivo*.

■ ASSOCIATED CONTENT

Supporting Information

Table S1 and Figure S1 and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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