

# Using an Old Drug to Target a New Drug Site: Application of Disulfiram to Target the Zn-Site in HCV NS5A Protein

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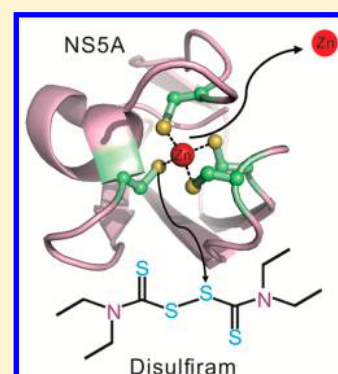
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## S Supporting Information

**ABSTRACT:** In viral proteins, labile Zn-sites, where  $\text{Zn}^{2+}$  is crucial for maintaining the native protein structure but the Zn-bound cysteines are reactive, are promising drug targets. Here, we aim to (i) identify labile Zn-sites in viral proteins using guidelines established from our previous work and (ii) assess if clinically safe Zn-ejecting agents could eject  $\text{Zn}^{2+}$  from the predicted target site and thus inhibit viral replication. As proof-of-concept, we identified a labile Zn-site in the hepatitis C virus (HCV) NS5A protein and showed that the antialcoholism drug, disulfiram, could inhibit HCV replication to a similar extent as the clinically used antiviral agent, ribavirin. The discovery of a novel viral target and a new role for disulfiram in inhibiting HCV replication will enhance the therapeutic armamentarium against HCV. The strategy presented can also be applied to identify labile sites in other bacterial or viral proteins that can be targeted by disulfiram or other clinically safe Zn-ejectors.

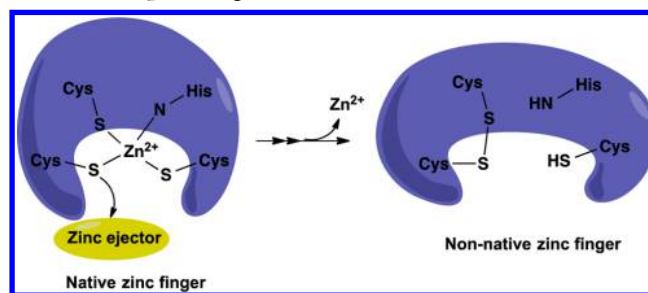


## INTRODUCTION

Many proteins have “catalytic” and/or “structural” Zn-binding sites where  $\text{Zn}^{2+}$  plays a crucial role in the enzyme-catalyzed reaction or in stabilizing the protein structure, respectively.<sup>1–3</sup> Both catalytic and structural Zn-binding sites can serve as therapeutic drug targets,<sup>4</sup> but their drug design targets differ due to differences in ligand preference and Zn–ligand distances in these two types of sites.<sup>5</sup> The presence of water ligands, lack of Zn-bound Cys, and longer mean Zn–ligand distances distinguish catalytic Zn sites from structural ones.<sup>5</sup> In catalytic Zn sites, the small size and poor charge-donating ability of water as well as the longer Zn–ligand distances enable  $\text{Zn}^{2+}$  to serve as a Lewis acid and bind another ligand, making  $\text{Zn}^{2+}$  a good target for drug design. In structural Zn sites, however,  $\text{Zn}^{2+}$  is a poor drug target due to the strong charge-donating ability and bulky size of  $\text{Cys}^-$ , which reduce Zn’s electron-accepting ability and prevent it from binding another ligand tightly.

However, in some structural Zn-sites, certain Zn-bound cysteines can react with electrophilic agents, causing  $\text{Zn}^{2+}$  to lose its ligands, leading to its ejection and thus disruption of the native protein structure and function (Scheme 1). Such labile Zn-sites containing potentially reactive Zn-bound cysteines in viral proteins are promising drug targets for retroviral therapy.<sup>6–11</sup> As an example, the human immunodeficiency virus type 1 (HIV-1) nucleocapsid p7 protein, a critical transcription factor in the viral replication cycle, contains two highly conserved Zn-Cys<sub>3</sub>His core, where the Zn-bound thiolates have been found to react with electrophilic agents

**Scheme 1. Labile Zn-Sites where Zn-Bound Cysteines React with Electrophilic Agents**

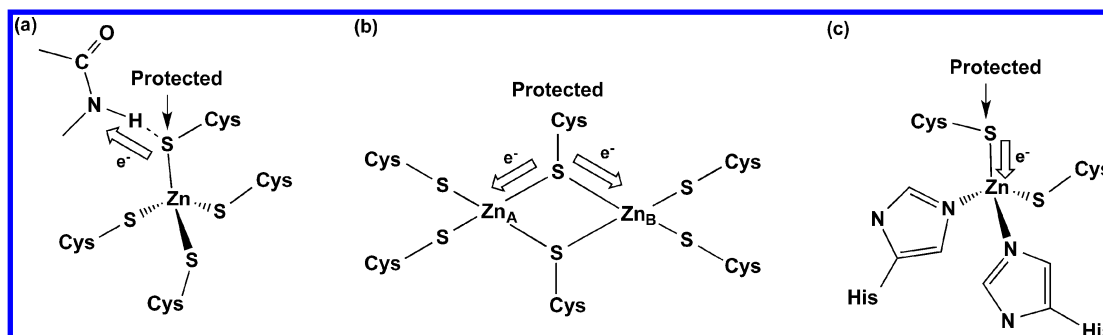


leading to  $\text{Zn}^{2+}$  ejection and loss of viral protein structure and function.<sup>12</sup> Apart from HIV-1, labile Zn fingers have also been found in herpes virus and arenavirus.<sup>11,13</sup>

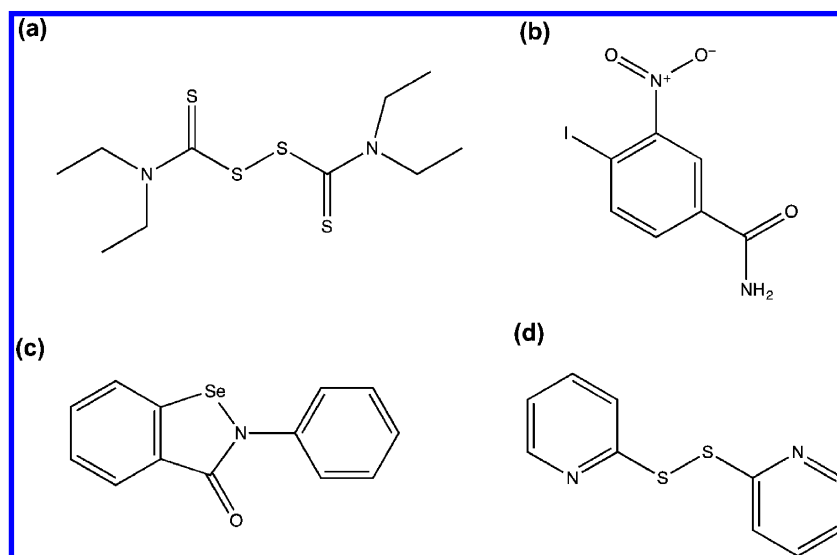
The experimental finding that certain Zn-bound cysteines in structural Zn-sites are reactive, while those in many Zn fingers are inert raises the intriguing question as to what factors determine the Zn-bound cysteine’s reactivity in structural Zn-sites. Clearly, a deciding factor is whether the Zn-site is accessible to a Zn-ejector. Assuming that the Zn-site can accommodate a Zn-ejector, its reactivity toward an electrophilic agent depends on the S(Cys) charge: decreasing the S(Cys) negative charge would suppress its reactivity. We have shown that hydrogen bonds or bonds from a second  $\text{Zn}^{2+}$  to a Zn-

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**Figure 1.** Factors suppressing the Cys reactivity: (a) N—H...S hydrogen bonds, (b) bonds to a second  $\text{Zn}^{2+}$ , and (c) bonds to His as opposed to Cys ligands.



**Figure 2.** Selected compounds with zinc-ejecting activity: (a) tetraethyl thiuram disulfide (disulfiram), (b) 4-iodo-3-nitrobenzamide (iniparib or BSI-201), (c) 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen), and (d) 2,2'-dithiodipyridine (aldriethiol-2).

bound S(Cys) atom reduce not only the latter's negative charge, but also the negative charge of the other Zn-bound S(Cys) atoms with no hydrogen bonds, making the Zn-bound cysteines less prone to react with electrophilic agents (Figure 1).<sup>14</sup> Changes in the hydrogen-bonding interactions and/or solvent exposure of the structural Zn-site upon substrate binding would thus affect the Zn-bound thiolate reactivity. Another factor determining the reactivity of a Zn-bound Cys toward electrophilic agents is the Zn's positive charge: increasing the latter enables Zn to effectively compete with electrophilic agents for the Zn-bound thiolates. The Zn's positive charge in  $\text{Zn}\cdot\text{Cys}_2\text{His}_2$  sites is greater than that in  $\text{Zn}\cdot\text{Cys}_3\text{His}$  or  $\text{Zn}\cdot\text{Cys}_4$  cores, as neutral ligands such as His transfer much less charge to Zn than negatively charged Cys.<sup>15,16</sup> Hence, the Zn-bound cysteines in  $\text{Zn}\cdot\text{Cys}_2\text{His}_2$  sites tend to be inert.

The above factors controlling the Zn-bound cysteine reactivity in structural Zn-sites have helped to establish guidelines to search for labile (druggable) structural Zn-sites given the protein structure.<sup>16,17</sup> Since neutral Zn-bound ligands or a second  $\text{Zn}^{2+}$  or hydrogen bonds to Zn-bound S(Cys) atoms would suppress the reactivity of the Zn-bound cysteines (see above), structural  $\text{Zn}\cdot\text{Cys}_4$  or  $\text{Zn}\cdot\text{Cys}_3\text{His}$  sites containing no hydrogen bond to any of the Zn-bound cysteines were predicted to be labile. These guidelines can be used to identify putative labile structural Zn-sites in viral proteins, which can be

experimentally verified using known Zn-ejecting agents in vitro. However, for the Zn-ejecting agent to serve as a potential drug candidate, it should eject  $\text{Zn}^{2+}$  from only the viral drug target protein, but not from essential human Zn-containing proteins. One way to overcome this problem is to employ Zn-ejecting agents that have been tested for safety in clinical trials or have been approved by the Food and Drug Administration. Examples of such Zn-ejecting agents are (i) disulfiram (trade names Antabuse and Antabus, Figure 2a), an old drug used to treat alcoholism because of its ability to irreversibly inhibit the enzyme, aldehyde dehydrogenase, thus producing an acute sensitivity to alcohol,<sup>18,19</sup> (ii) iniparib (Figure 2b), which has undergone ~20 clinical trials including Phase III trials for patients with various cancer types,<sup>20</sup> and (iii) ebselen (Figure 2c), a clinically safe organoselenium compound that has undergone placebo-controlled double-blind clinical trials for treating acute ischemic stroke<sup>21</sup> and is currently undergoing clinical trials for treating cardiovascular disease, arthritis, atherosclerosis, and cancer.<sup>22,23</sup>

Our aim is to determine if the aforementioned guidelines can be used to identify labile structural Zn-sites in viral proteins and if clinically safe drugs such as disulfiram could be used to eject  $\text{Zn}^{2+}$  from the predicted target site and thus inhibit viral replication. As proof-of-concept, we have used our guidelines to identify a labile structural Zn-site in the hepatitis C virus (HCV). This virus was chosen for study because it contains a

zinc-binding multifunctional nonstructural protein (NSSA) that is an attractive target for anti-HCV therapy.<sup>24–26</sup> NSSA is essential for HCV replication and assembly, modulation of signaling pathways in the host cell, and interferon response.<sup>27</sup> Its fold is stabilized by  $\text{Zn}^{2+}$ , which plays a key structural role, as mutations that disrupt binding of  $\text{Zn}^{2+}$  to NSSA are lethal for RNA replication.<sup>28</sup> Furthermore, many HCV protein structures, in particular the NSSA Zn-binding domain structure, have been solved. We predicted that the Zn-site in NSSA is labile and verified our prediction by showing that several Zn-ejecting agents including disulfiram could eject  $\text{Zn}^{2+}$  from NSSA. We further showed that disulfiram could inhibit HCV RNA replication of HCV subgenomic replicons, and that its inhibitory effects are similar to ribavirin, a general inhibitor of virus replication. We conclude that disulfiram is a promising candidate for combination therapy with other direct-acting antiviral drugs to treat HCV patients, especially, those with concurrent alcohol use disorder.

## METHODS

**Cells.** HCVrep-HA cells, HCV subgenomic replicons, were derived from the original HCV N strain 1bneo/delS (genotype 1b) and generated by insertion of a hemagglutinin (HA) tag in the C-terminal region of NSSA as previously described. These cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum, nonessential amino acids, antibiotics (100 U/mL penicillin G and 100  $\mu\text{g}/\text{mL}$  streptomycin), and 0.5 mg/mL G418 at 37 °C in a 5%  $\text{CO}_2$  incubator.

**Reagents.** Disulfiram was purchased from Sigma and solubilized in 100% DMSO (Sigma). Ribavirin was purchased from Sigma and solubilized in water. IFN $\alpha$  (HumanKine IFN $\alpha$  2A) was purchased from Millipore and solubilized in water. All the experimental concentrations were achieved by diluting drugs with culture medium.

**NSSA Expression and Purification.** The cDNA of NSSA (domain I, residues 33–207) was subcloned into the NheI/SalI sites of expression vector pET28a (Novagen) to generate recombinant NSSA with a N-terminal His-tag, MGSSHHHHHHSSGLVPRGSHM (see Figure S1 for the full recombinant protein sequence). The calculated MW of this recombinant protein is 21 471 Da. The pET28a-NSSA plasmid was transformed into *E. coli* BL21-CodonPlus(DE3)-RIPL strain (Stratagene) cultured in LB medium supplemented with 50 mg/mL kanamycin. The cultures were grown to an OD600 of 0.5 and then induced with 1 mM IPTG at 18 °C for 20 h. The harvested cells were disrupted by a microfluidizer and the crude cell extracts were first loaded onto a Ni-NTA resin affinity column (Qiagen) followed by a Superdex 200 gel filtration column (GE Healthcare) in 20 mM MOPS (pH 7.0), 300 mM NaCl and 10% glycerol.

**Zinc Ejection Assays.** Release of zinc ions from NSSA was monitored by the fluorescence emission from the zinc-specific fluorophore FluoZinTM-3 (Invitrogen/Life Technologies). The Zn-ejecting agents, tetraethyl thiuram disulfide (disulfiram), 4-iodo-3-nitrobenzamide (iniparib or BSI-201), 2-phenyl-1,2-benziselenazol-3(2H)-one (ebselen), and 2,2'-dithiodipyridine (aldrithiol-2), were dissolved in DMSO to a stock solution of 100 mM and then diluted in 50 mM phosphate buffer at pH 7.5. NSSA (5  $\mu\text{M}$ ) was mixed with the Zn-ejecting agent (5  $\mu\text{M}$ ) and 5  $\mu\text{M}$  FluoZin-3 in a total reaction volume of 100  $\mu\text{L}$  at room temperature. Fluorescence emission was monitored by EnSpire Multilabel Plate Reader (PerkinElmer, U.S.A.) at an excitation wavelength of 494 nm and emission wavelength of 516 nm for 10 min. For the molecular weight (MW) measurement by mass spectrometry, NSSA (1 mg/mL) was mixed with tetraethyl thiuram disulfide (disulfiram, 50 mg/mL) and incubated at 37 °C for 30 min. The NSSA proteins with or without disulfiram were then denatured by DTT and passed through a ZipTipC18 desalting column (Millipore) before analyzed by Bruker Daltonics-Autoflex III TOF/TOF mass spectrometer (Bremen).

**MTS Assay.** A CellTiter96 AQ<sub>ueous</sub> One solution Cell Proliferation Assay kit (Promega) was used to evaluate cell viability. Cells were seeded onto a 96-well plate, and the media was replaced with phenol red-free DMEM containing 10% CellTiter96 AQ<sub>ueous</sub> One solution reagent. The plates were incubated for an additional 30 min at 37 °C in a 5%  $\text{CO}_2$  incubator. Light absorbance was measured at 490 nm with a 96-well plate reader. Each experiment was performed in three replicated wells.

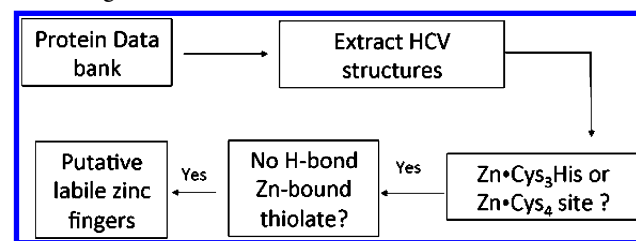
**Quantitative RT-PCR.** Total cellular RNA was extracted by using High Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer's protocol. cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen). The primers for reverse transcription are oligo (dT)20 and HCV-specific RT primer (5'-CACTCGCAAGCACCTATCA-3'). For real-time PCR analysis, we followed the standard TaqMan method with the Universal Probe Library System and LightCycler 480 (Roche Diagnostics). GAPDH was used as an internal control for the normalization of cellular RNA and intracellular viral RNA. The primers and probes were as follows: for HCV, the sense primer was 5'-CATGGCGTTAGTATGAG-TGTGCG-3' and the antisense primer was 5'-GGTTCGCGACACCA-CTATG-3' (with Universal Probe 75; Roche), and for GAPDH, the sense primer was 5'-AGCCACATCGCTCAGACAC-3' and the antisense primer was 5'-GCCCAATACGACCAAATCC-3' (with Universal Probe 60; Roche).

**Statistical Analysis.** The statistical analysis for MTS assay and qRT-PCR data were performed by using Microsoft Excel software. The graphs represent the means  $\pm$  standard deviations. *p* value were determined using Student's *t* distribution test with one tail. \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001 were considered statistically significant.

## RESULTS

**Predicting Labile Zinc Sites in HCV Proteins.** The search algorithm summarized in Scheme 2 was used to screen

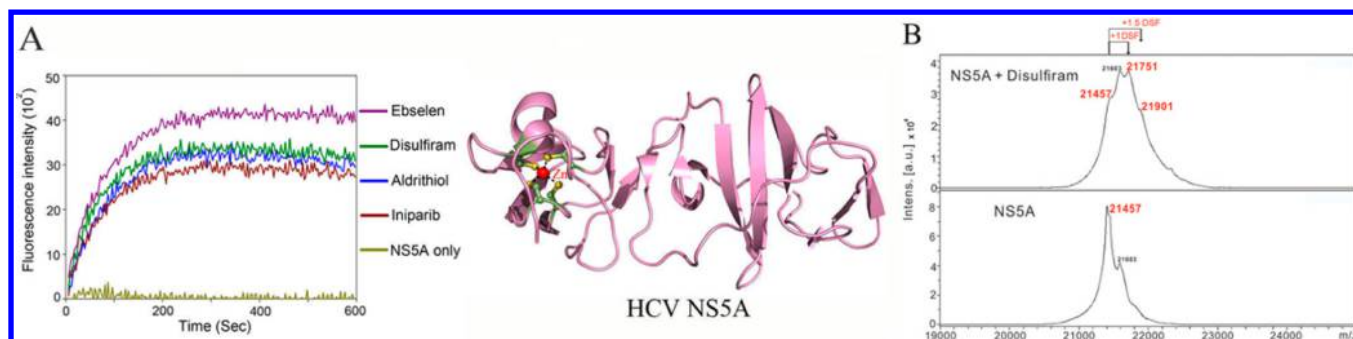
**Scheme 2.** Flowchart for Screening the PDB for Druggable Zinc Fingers



HCV protein structures in the Protein Data Bank.<sup>29</sup> HCV proteins whose PDB structures contain Zn-Cys<sub>4</sub> or Zn-Cys<sub>3</sub>His sites were identified. Hydrogen bonds to the Zn-bound cysteines were computed using HBPLUS.<sup>30</sup> No hydrogen bonds to any of the Zn-bound cysteines were found in two HCV proteins; viz., the NS3/4A serine protease containing the Zn-Cys<sub>3</sub>His site (PDB entry 3m5l) and the NSSA protein containing the Zn-Cys<sub>4</sub> site (PDB entry 3fqj). However, the Zn-bound His in the NS3/4A serine protease can be substituted with a water molecule (PDB entry 3m5o) to yield a Zn-Cys<sub>3</sub>H<sub>2</sub>O complex. Thus, according to Scheme 2, the Zn-Cys<sub>4</sub> site of the NSSA protein was predicted to be labile.

**Zn<sup>2+</sup> Can Be Ejected from HCV NSSA in Vitro.** To verify that the Zn-Cys<sub>4</sub> site in the NSSA protein is indeed labile, the cDNA of NSSA was cloned and expressed in *E. coli* (see Methods). Four Zn-ejecting compounds; viz., disulfiram, iniparib, ebselen, and aldrithiol-2, as illustrated in Figure 2, were used: Aldrithiol-2 (Figure 2d) was chosen, as it is known to inactivate the essential zinc fingers in the HIV-1 nucleocapsid protein and simian immunodeficiency virus





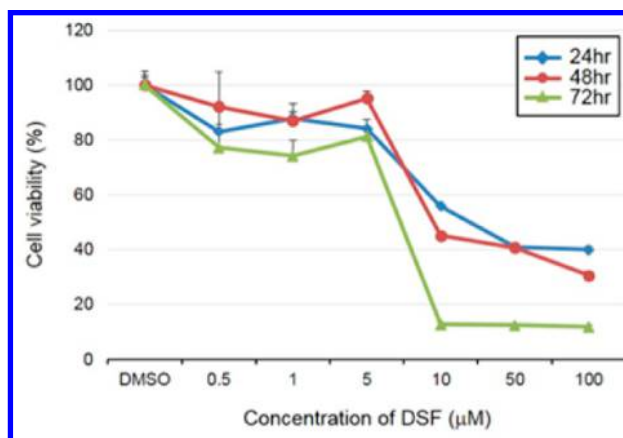
**Figure 3.** Effects of various Zn-ejecting agents on HCV NSSA: (a) NSSA protein was incubated with the four Zn-ejecting agents, ebselen, disulfiram, aldrithiol-2, and iniparib. Zinc ions were released from NSSA as shown by the increase of the fluorescence signal of the Zn-specific fluorophore, FluoZin-3, using an excitation wavelength at 494 nm and emission wavelength at 516 nm for detection. The control line was the buffer solution containing only NSSA and FluoZin-3. The ribbon model of monomeric NSSA (PDB entry: 3fqj) is displayed on the right with the Zn<sup>2+</sup> depicted as a red sphere and the four Cys side-chains in ball-and-stick. (b) The MW of NSSA before (bottom panel) and after (top panel) the addition of disulfiram. The mass spectra were measured by MALDI-TOF. Three independent experiments gave similar results, hence only spectra from one of the experiments are shown.

virions,<sup>31</sup> whereas disulfiram, iniparib, and ebselen are known to be clinically safe (see above). Upon addition of these Zn-ejecting compounds to NSSA, Zn<sup>2+</sup> ions were released, as evidenced by the fluorescence change of the zinc-specific fluorophore, FluoZin-3 (Figure 3A). Thus, all four compounds could eject zinc ions from NSSA.

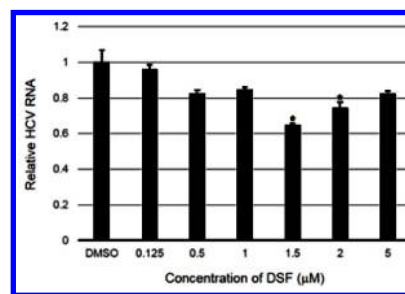
To further confirm that the Zn-ejecting agent was bound to Cys in the targeted Zn-site of NSSA, mass spectroscopy was used to detect the MW of NSSA before and after the addition of disulfiram. The MALDI-TOF mass spectrum of NSSA revealed a major peak with a measured MW of 21 457 Da (Figure 3B, bottom panel), close to the calculated MW of 21 471 Da and within the expected accuracy for MALDI-TOF (0.05–0.2%);<sup>32</sup> the shoulder at 21 603 Da likely reflects impurity in the NSSA sample. The DSF-treated NSSA had additional peaks at 21 751 and 21 901 Da (Figure 3B, top panel). As disulfiram has a MW of 296 Da, the mass difference between the DSF-treated and untreated samples of 294 and 444 Da suggest that two and three half-disulfiram were bound to two and three cysteines, respectively.

**Disulfiram Could Inhibit HCV RNA Replication.** Among the four Zn-ejecting agents, we chose disulfiram for in vivo tests, as it is already in clinical use. To examine the effects of disulfiram on HCV RNA replication in cells, we used a cell line harboring the HCV subgenomic replicon, RepHA, which expresses all the nonstructural HCV proteins, but not structural proteins, and can undergo HCV RNA replication. However, it does not produce virus particles. First, the cytotoxicity of disulfiram on RepHA cells was tested by treating the cells with different concentrations of disulfiram and determining cell viability by MTS cell proliferation assay at 24-h intervals. The results in Figure 4 showed only minor cytotoxicity up to 5  $\mu$ M disulfiram, but significant cytotoxicity at  $\geq 10$   $\mu$ M disulfiram. Thus,  $\leq 5$   $\mu$ M disulfiram was used for subsequent experiments.

To evaluate the effects of disulfiram on HCV RNA replication, RepHA cells were treated with 0.125–5  $\mu$ M disulfiram and the amounts of HCV RNA were measured after 24 h by quantitative RT-PCR. The results in Figure 5 show that disulfiram inhibited HCV replication in a concentration-dependent manner and reached 20–30% reduction at a concentration of 1.5  $\mu$ M. Paradoxically, increasing the concentration of disulfiram did not further reduce HCV RNA replication level, but increased it instead. Why higher disulfiram concentrations resulted in lower antiviral activities is unclear,



**Figure 4.** Cell viability, as determined by MTS assay, of HCV subgenomic replicons, RepHA, after treatment with various concentrations of disulfiram for 24, 48, and 72 h.

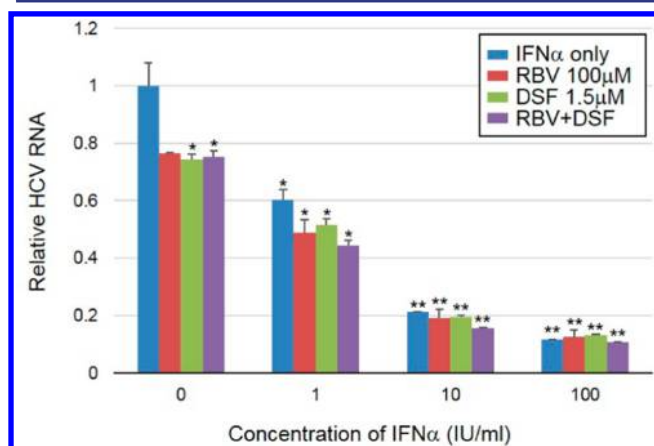


**Figure 5.** Effects of disulfiram on HCV RNA replication. HCV RNA expression levels measured by quantitative RT-PCR after 24 h of disulfiram treatment.

but it might be related to the effects of disulfiram on other cellular zinc-finger-containing proteins that may be present in the cell.

**Disulfiram Exhibit Properties That Are Similar to Ribavirin with and without Interferon.** A standard treatment for HCV infection is to combine pegylated interferon alpha (IFN $\alpha$ ) and ribavirin, a guanosine analog that can stop viral RNA synthesis. However, many adverse effects are associated with ribavirin, notably, hemolytic anemia.<sup>33</sup> Hence, we examined whether disulfiram could replace ribavirin in HCV therapy. RepHA cells were first treated with 1.5  $\mu$ M disulfiram

or ribavirin for 24 h, and then with different dosages of IFN $\alpha$  for another 24 h. The results in Figure 6 show that 1.5  $\mu$ M disulfiram inhibited HCV replication by about 26%, comparable to 100  $\mu$ M ribavirin, which also inhibited HCV replication by about 24%.



**Figure 6.** Effects of disulfiram combined with IFN $\alpha$  or ribavirin on HCV RNA replication. RepHA cells were treated with 1.5  $\mu$ M disulfiram (DSF), 100  $\mu$ M ribavirin (RBV), or both disulfiram and ribavirin for 24 h, then treated with 0–100 IU/mL IFN $\alpha$  for another 24 h. The amounts of HCV RNA after these treatments were measured by quantitative RT-PCR.

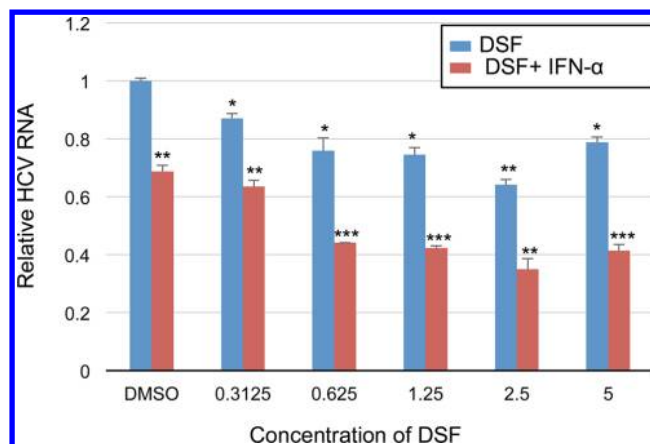
Interestingly, in different treatment combinations, we found that both disulfiram and ribavirin achieved better inhibition when combined with 1.33 IU/mL of IFN $\alpha$  (~60% inhibition) than the latter alone (~40% inhibition) (Figure 6). However, such a synergistic effect was not observed at higher IFN $\alpha$  concentrations ( $\geq 10$  IU/mL) where addition of disulfiram or ribavirin did not further inhibit HCV replication significantly (Figure 6).

We therefore further examined this phenomenon using RepHA cells treated with serially diluted disulfiram without or with IFN $\alpha$  (1.33 IU/mL). The results showed that disulfiram inhibited HCV replication by about 20–30% at every concentration of disulfiram used, but when combined with 1.33 IU/mL IFN $\alpha$ , the inhibition rate reached ~60% (Figure 7). Thus, disulfiram exhibit a synergistic effect when combined with 1.33 IU/mL IFN $\alpha$ .

## DISCUSSION

We have provided proof-of-concept for targeting predicted labile structural Zn-sites in viral proteins with clinically safe Zn-ejecting agents: We show that disulfiram, an old drug used for alcohol aversion therapy with known pharmacology and toxicology, could eject Zn<sup>2+</sup> from the predicted labile Zn-site in the viral NSSA protein and thus inhibit HCV replication. While the NSSA protein is known to be an attractive target for anti-HCV therapy because of its involvement in many critical aspects of the HCV life cycle,<sup>27</sup> this is the first report that its structural Zn-site is labile and can serve as a target for a clinically used drug. This is also the first report that disulfiram could inhibit HCV replication to a similar extent as ribavirin.

Disulfiram is an old drug long used for alcohol aversion therapy since 1949. Previous studies have reported the potential repurposing of disulfiram for treating *Giardia lamblia* infections,<sup>34</sup> multidrug-resistant and extensively drug-resistant



**Figure 7.** Effects of disulfiram combined with IFN $\alpha$  on HCV RNA replication. RepHA cells were treated with serially diluted DSF from 5–0.3125  $\mu$ M with or without 1.33 IU/mL IFN $\alpha$  for 24 h, after which expression levels of HCV RNA were measured by quantitative RT-PCR.

tuberculosis,<sup>35</sup> cocaine addiction,<sup>36</sup> and various cancers.<sup>18,19,37–40</sup> This study suggests that disulfiram in combination with interferon and/or direct-acting antiviral agents may be effective in treating patients with HCV infection and alcohol use disorders concomitantly. Compared with the general population, alcohol-dependent individuals are more likely to be infected with HCV;<sup>41</sup> conversely, alcohol use disorders are more prevalent in HCV-infected patients,<sup>42</sup> hence HCV infection and alcohol dependency often co-occur. Although direct-acting antiviral agents that target HCV NS3/4A protease (e.g., simeprevir), NSSB polymerase (e.g., sofosbuvir), or NSSA (e.g., ledipasvir) are highly effective drugs,<sup>43</sup> they are currently very expensive: Sofosbuvir costs US\$1000/pill,<sup>44</sup> whereas sofosbuvir combined with ledipasvir (trade name, Harvoni) in a single pill costs US\$1125/pill,<sup>45</sup> ~1000 times more expensive than disulfiram, which costs as little as US\$1.21/pill in Taiwan. The current high cost of these drugs prohibits their universal use. Furthermore, the heavy alcohol consumption often reported in HCV-infected patients is a large barrier to treatment even with the new drugs.<sup>46</sup> Interestingly, two early studies assessing the safety of disulfiram in HCV(+) individuals concluded that disulfiram could be a safe therapeutic option, as two indicators of hepatic injury, aspartate and alanine transaminase, did not exhibit significant elevations in HCV(+) patients taking disulfiram over 12 months.<sup>47,48</sup> These studies assumed disulfiram eliminated alcohol use and thus the purported alcohol-HCV hepatotoxic synergy, since drinking is known to accelerate HCV-related liver damage.<sup>49</sup> Our results suggest that disulfiram could also help to suppress HCV replication.

Ribavirin is often combined with direct-acting antiviral agents in current treatment of HCV-infected patients. Ribavirin is crucial in maintaining an antiviral response: Monotherapy with sofosbuvir alone achieved suboptimal efficacy: The sustained virological response, defined as undetectable HCV RNA in the blood for 24 weeks after the end of treatment, in a group of ten HCV (genotype 2 or 3) patients is only 60% with sofosbuvir alone but is 100% when combined with ribavirin.<sup>43</sup> However, ribavirin could result in hemolytic anemia, which could lead to worsening of cardiac disease and myocardial infarctions.<sup>33</sup> Since disulfiram and ribavirin show similar inhibitory effects on HCV replication, using disulfiram instead of ribavirin, might be an

alternative therapy for patients with hemoglobinopathies; e.g., thalassemia major or sickle-cell anemia.

Although pegylated IFN $\alpha$ -free and ribavirin-free therapy combining two or three direct-acting antiviral agents seem to be effective even in patients with compensated cirrhosis, viruses resistant to these new drugs have already emerged. For example, resistance to NSSA inhibitors such as dacatasvir<sup>26</sup> and samatasvir<sup>50</sup> is associated with mutations in the first 100 amino acids of NSSA, which contains the conserved Zn-binding site required for HCV replication.<sup>26,45</sup> However, disulfiram might exhibit a high barrier to viral resistance, as its target, the conserved cysteines, are involved in coordinating to Zn<sup>2+</sup>, which is critical for NSSA structural integrity, and their mutations would affect Zn binding and thus HCV replication.<sup>28</sup>

The current proof-of-concept study is limited to proteins that contain labile Zn-Cys<sub>3</sub>His or Zn-Cys<sub>4</sub> sites. Clinically safe Zn-ejectors could in principle be applied to enzymes with a catalytic Zn-site along with a structural but labile Zn-site: the Zn-ejector would be expected to eject the Zn<sup>2+</sup> from the labile Zn site, thereby disrupting the native protein structure and thus function. However, they cannot be applied to enzymes containing only catalytic Zn-sites that lack reactive cysteines; in such cases, inhibitors can be designed to bind the metal cation to turn off its catalytic role (see [Introduction](#)). Future studies could entail optimizing disulfiram and testing its derivatives using full-length replicon and infectious virus system.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/jacs.6b00299](https://doi.org/10.1021/jacs.6b00299).

Figure S1, Sequence of the recombinant NSSA (domain I, residues 33–207) ([PDF](#))

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### Author Contributions

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### Notes

The authors declare no competing financial interest.

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