Inhibition of Peroxidase Activity of Cytochrome *c*: De Novo Compound Discovery and Validation[§]

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ABSTRACT

Cytochrome *c* (cyt *c*) release from mitochondria is accepted to be the point of no return for eliciting a cascade of interactions that lead to apoptosis. A strategy for containing sustained apoptosis is to reduce the mitochondrial permeability pore opening. Pore opening is enhanced by peroxidase activity of cyt *c* gained upon its complexation with cardiolipin in the presence of reactive oxygen species. Blocking access to the heme group has been

proposed as an effective intervention method for reducing, if not eliminating, the peroxidase activity of cyt *c*. In the present study, using a combination of druggability simulations, pharmacophore modeling, virtual screening, and in vitro fluorescence measurements to probe peroxidase activity, we identified three repurposable drugs and seven compounds that are validated to effectively inhibit the peroxidase activity of cyt *c*.

Introduction

The pathophysiological consequences of excessive or sustained apoptosis, including acute tissue injuries or chronic sustained injuries, have motivated, in recent years, the development of new drugs and therapeutic strategies for modulating apoptotic pathways and events. The release of cytochrome *c* (cyt *c*) from the mitochondria into the cytosol is usually viewed as “the point of no return” in mitochondria-mediated apoptotic response. Blocking the interactions of cyt *c* at the inner mitochondrial membrane or intermitochondrial membrane space prior to its release emerged as a viable strategy for discovering antiapoptotic drugs.

Under normal physiologic conditions, cyt *c* serves as an electron carrier between the respiratory complexes III and IV. During the execution of intrinsic apoptosis, it interacts with the phospholipid cardiolipin (CL) and forms a cyt *c*–CL complex which confers a CL-specific peroxidase activity to cyt *c* in the presence of reactive oxygen species that fuels the oxidation of CL. Subsequent CL oxidation induces the opening of permeability pores at the outer mitochondrial membrane, which permits the release of cyt *c* and other proapoptotic factors from the mitochondria into the cytosol. Cytochrome *c* release, in turn, triggers a cascade of caspase interactions that lead to apoptosis

(Kagan et al., 2005). Mitochondria-targeted inhibitors of the peroxidase activity of cyt *c* can mitigate, if not prevent, apoptosis (Kagan et al., 2009a).

We recently designed and synthesized a series of imidazole-substituted analogs of stearic acid [triphenylphosphonium-conjugated imidazole-substituted stearic acid (TPP-*n*-ISA)], with the imidazole groups attached at the positions $6 \leq n \leq 14$. These compounds were shown to specifically bind the heme iron on cyt *c* and block the peroxidase activity of the cyt *c*–CL complex (Atkinson et al., 2011). Using a combination of absorption spectroscopy and electron paramagnetic resonance measurements, along with molecular dynamics simulations, we showed that TPP-*n*-ISAs were able to suppress the CL-induced structural rearrangements in cyt *c*, which would otherwise give way to peroxidase activity (Jiang et al., 2014). Among these compounds, TPP-6-ISA proved to be particularly effective in inhibiting the peroxidase function in mouse embryonic cells exposed to ionizing radiation (Jiang et al., 2014).

In the present study, we report significant progress in discovering de novo inhibitors of cyt *c*. As a first step, we performed a series of druggability simulations in the presence of small organic (probe) molecules representative of drug-like fragments to assess the druggability of cyt *c*, using a recently introduced method (Bakan et al., 2012). These simulations permitted us to evaluate the binding pauses and maximal binding affinities of probes that potentially bind the heme-binding pocket in cyt *c*, and to construct a pharmacophore model (PM) based on the identities and spatial distribution of probe molecules in this pocket. Screening of the PM against libraries

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ABBREVIATIONS: 002-126-168, 1-[4-(1-Adamantyl)-2-methoxybenzoyl]-1*H*-imidazole; CL, cardiolipin; cyt *c*, cytochrome *c*; IOA, imidazole-substituted oleic acid; L254614, 1-(4,9-dihydrothieno(2,3-*c*)(2)benzothiepin-4-yl)-1*H*-imidazole; PAINs, pan assay interference compounds; PDB, Protein Data Bank; PM, pharmacophore model; TOCL, 1,1',2,2'-tetraoleoyl cardiolipin; TPP-*n*-ISA, triphenylphosphonium-conjugated imidazole-substituted stearic acid.

of small molecules as well as a database of known drug-target pairs led to seven hits (drug-like compounds), in addition to the identification of three repurposable drugs. Biochemical experiments to inhibit cyt *c* peroxidase activity confirmed that seven of these 10 newly discovered compounds/drugs exhibit efficiencies comparable to or better than those observed (Jiang et al., 2014) for TPP-*n*-ISA molecules, opening the way to new molecular intervention strategies for modulating mitochondrial-mediated apoptosis.

Materials and Methods

Structure Analysis. From the Protein Data Bank (PDB), we retrieved and structurally aligned homologous cyt *c* structures using ProDy (Bakan et al., 2014). The code used for analysis is given as an example in the ensemble analysis tutorial of the online ProDy documentation. In brief, the procedure involves searching PDB for structures sharing 40% or more sequence identity with human cyt *c* protein (UniProt accession P99999) and automated retrieval and alignment of structures onto a template structure. The ensemble of structures retrieved from the PDB is shown Fig. 1A.

Druggability Simulations. We performed two groups of runs (Table 1): runs P1–P6 in the presence of a set of probe molecules containing drug-like fragments, and runs I1 and I2 in the presence of imidazole-containing oleic acids (IOAs), which were previously identified to mitigate radiation-induced cell death (Atkinson et al., 2011). All simulations were performed in duplicate to verify reproducibility and improve statistical accuracy.

Runs P1–P6 were prepared and analyzed using the VMD (Humphrey et al., 1996) plugin DruGUI (Bakan et al., 2012), Solvate, Autoionize, and Psfgen (Humphrey et al., 1996). Simulations were performed using NAMD (Nelson et al., 1996) software with CHARMM (MacKerell et al., 2002) and CHARMM general force fields (Vanommeslaeghe et al., 2010). System setup, equilibration, and productive simulation protocols described by Bakan et al. (2012) were used. IOA topology files were prepared using the VMD plugin Molefacture, and parameters of chemically analogous atoms in the CHARMM force field were used for simulations. IOA initial configuration was set manually at the binding site.

Fig. 1. Superposition of cytochrome *c* structures crystallographically resolved under different conditions, and comparison with open conformer. (A) Overlay of 97 cyt *c* structures available in the PDB. The backbone is colored according to a previous definition of foldons (the white foldon is colored gray), and the heme group is displayed in ball-and-stick representation. The ensemble is visualized using Chimera (<http://www.cgl.ucsf.edu/chimera/>) (Pettersen et al., 2004). (B) Comparison of crystallographic structure (PDB ID 1HRC, gray and violet) with a model of open conformation, generated using VMD. We display the disordered loop (red) of the open conformer to illustrate its departure from the closed (gray) state. His18 and Met80 are shown in stick representation, above and below the heme group, respectively. Note the significant displacement in Met80. MD, molecular dynamics simulations.

Peroxidase Activity Assays. Compounds were tested for their ability to inhibit the peroxidase activity of cyt *c* complexed with 1,1',2,2'-tetraoleoyl cardiolipin (TOCL; designated cyt *c*/TOCL) by H₂O₂-induced oxidation experiments, using Amplex Red (Life Technologies, Grand Island, NY) as a probe. The peroxidase activity of cyt *c*/TOCL complexes with Amplex Red reagent was determined by measuring the fluorescence of resorufin (oxidation product of Amplex Red) in 20 mM HEPES buffer (pH 7.4) containing 100 μ M diethylenetriaminepentaacetic acid. Cyt *c* (1 μ M) was incubated with TOCL/1,2-dioleoyl-*sn*-glycero-3-phosphocholine liposomes (TOCL/cyt *c* ratio 25:1) for 10 minutes, and peroxidase reaction was started by the addition of 50 μ M Amplex Red and 50 μ M H₂O₂. The incubation proceeded for an additional 20 minutes (reaction rate was linear in the entire time interval). Fluorescence was detected by using a Fusion α microplate analyzer (Packard BioScience, Meriden, CT) and by using an excitation wavelength of 535 nm and an emission wavelength of 585 nm. Small unilamellar liposomes were prepared from DOPC and TOCL (1:1 ratio) by sonication in 20 mM HEPES buffer containing 100 μ M DTPA (pH 7.4) (See Supplemental Tables S1 and S2).

Pharmacophore Modeling. Pharmacophore models were developed using vROCS (Rush et al., 2005). The initial model was based on probe molecules and IOA conformation that were observed to bind heme pocket tightly. Namely, we selected the probe or IOA conformations that were observed to be stabilized for 4 nanoseconds or longer. A set of conformations for a selected molecule was parsed from the trajectory using ProDy (Bakan et al., 2014), and the conformation that is closest to others was used as input to vROCS. We note that there were multiple potential combinations of probe and IOA conformations. While developing the initial model, we selected probes, water molecules, and IOA conformations that will satisfy the most exposed features on the protein surface and that will capture the pocket shape completely. These included several hydrophobic features (yellow) in the center of the pocket capturing hydrophobic interactions of isopropanol, isobutene, and IOA with hydrophobic side chains. The base of the pocket had two hydrogen bond donors (blue meshed spheres) to satisfy the exposed backbone carbonyl oxygens. On the sides of the pocket, we included several polar features, contributed by the IOA head group, imidazole, and water. In particular, we incorporated a negatively charged feature (solid red sphere) between Lys13 and Arg91 due to stable salt bridge interactions of IOA and the presence of acetate binding spots in two druggability simulations. In addition to these specific chemical features, the shape component of the ROCS model was the union of volumes of selected probes.

Virtual Screening. Initially, we screened 7520 small-molecule drugs compiled in DrugBank (Knox et al., 2011) and 150 molecules observed to be bound to the heme group in various PDB (Berman et al., 2000) structures. Nonstandard ligands that are within 3 Å of heme iron were considered for virtual screening. The Python code for screening structures is provided online in the ProDy structure analysis tutorial. In the second round, we screened lead-like (3.2 M) and clean drug-like (11 M) subsets of purchasable compounds from ZINC (version 12) (Irwin and Shoichet, 2005). Three-dimensional conformers of compounds were generated using the OpenEye application OMEGA (version 2.4.6) (Hawkins et al., 2010) with default options. Compounds were screened using OpenEye application ROCS (version 3.1.2) (Rush et al., 2005). Compounds were ranked based on Tanimoto combo scores that combine shape and color (chemical) similarity scores. A compound receives a score of 1 if its shape perfectly matches that of the model. Likewise, a compound that satisfies all chemical features of the model receives an additional score of 1. Hence, Tanimoto combo scores range from 0 to 2. Since compounds were not penalized for overlapping with the protein or the heme group, we filtered such compounds by visual inspection using VIDA (<http://www.eyesopen.com/>).

Pan Assay Interference Compound Filtering. Tested compounds were screened against PAINs (pan assay interference compounds) (Baell and Holloway, 2010) using the online webserver PAINS-Remover (<http://cbligand.org/PAINS/>).

TABLE 1
Composition of the systems simulated for assessing cyt *c* druggability

Runs	PDB ID	Time	Water	Composition of Probe Molecules ^a	Ions
P1, P2	1hrc	40 × 2	2400	84 isopropanol, 12 acetamide, 12 acetate, 12 isopropylamine	8 Cl [−]
P3, P4	1hrc	40 × 2	2400	60 isopropanol, 12 acetamide, 12 acetate, 12 isopropylamine, 24 isobutane	8 Cl [−]
P5, P6	1hrc	40 × 2	2400	48 imidazole, 24 neutral methyl phosphate, 24 isobutane, 12 isopropanol, 12 acetate	4 Na ⁺
I1, I2	1hrc	40 × 2	2421	1 imidazole-substituted oleic acid	14 Cl [−]

^aFor details, see Bakan et al. (2012).

Results

Native Cyt *c* Has a Compact, Closed Structure Resistant to Small-Molecule Binding. Cytochrome *c* is a 105-residue protein with a compact structure (Bushnell et al., 1990). Its heme group is protected from the environment, and the heme iron is coordinated by His18 and Met80. Cyt *c* folding involves stepwise assembly of five folding units (foldons) shown in different colors in Fig. 1A (Maity et al., 2005). Upon complexation with CL, cyt *c* assumes a partially unfolded conformation whereby the Fe-Met80 bond is ruptured (Kagan et al., 2005), although the details of the conformational changes involved in this process are not available from spectroscopic methods (Sinibaldi et al., 2010; Hanske et al., 2012).

As a first step, we analyzed the structural variations among the 97 PDB structures (from different organisms and/or under different crystallization conditions) resolved for cyt *c*. Despite

fluctuations in atomic coordinates, evidenced by the superposition of the PDB structures, all resolved structures were in the closed form (Fig. 1A). Repeated 200-nanosecond-long simulations of cyt *c* dynamics under native state conditions confirmed the high stability of the closed form. No partial unfolding was observed, and the heme-binding pocket remained closed to the environment (unpublished data). The high stability of the closed conformation of cyt *c* under native state conditions in the absence of interactions with CL is consistent with our in vitro experiments showing negligible peroxidase activity (Kagan et al., 2009b).

An Open Conformer of Cyt *c* Is Stabilized upon Small-Molecule Binding. To visualize the potential interaction of cyt *c* with small molecules, we adopted as an initial conformer an open form of cyt *c* (Atkinson et al., 2011) where the red foldon is open and Met80 is displaced by 12.5 Å away from

Fig. 2. Heme site is the only druggable site with a nanomolar achievable affinity. Snapshots from the simulations P1, P2, P3, and P4 are displayed in the respective panels (A), (B), (C), and (D). Probe binding spots are shown as spheres and colored based on their binding free energies. Acetate and isobutane binding spots are indicated by orange and green arrows, respectively. Lowest and highest binding free energies (kcal/mol) are specified in each panel. Total binding free energies are shown in Table 2. Twenty cyt *c* conformations evenly spaced in each trajectory are shown as ribbons and colored as in Fig. 1A. Protein side chains and heme atoms are colored yellow. Sim, simulation.

Fig. 3. Cyt *c* simulations in the presence of diverse fragments and IOA inhibitors. Snapshots from simulations P5 and I1 are shown in (A) and (B), respectively. Sim, simulation.

the heme (Fig. 1B), and we performed a series of simulations in the presence of small molecules with diverse physicochemical properties. This conformer (cytc_open.pdb) is available in the Supplemental Material. In this conformer, the pocket enclosing the heme group is solvent-exposed and large enough to accommodate the binding of a drug-like molecule. As previously noted (Jiang et al., 2014), this conformation naturally tends to close down (into the native compact conformer) unless stabilized by a bound ligand. The ligand-binding properties of this site and the conformational space accessible upon ligand binding were characterized by two sets of runs (Table 1). Runs P1–P6 used the protocol that we optimized previously for identifying druggable sites (Bakan et al., 2012). Probe molecules in these simulations flooded the heme-binding pocket and stabilized the open conformation of the red foldon loop (Figs. 2 and 3A). Among probe molecules, isopropanol and isobutene were observed to frequently bind the center of the pocket, indicating the strong potential of the heme and the side chains lining the pocket to undergo hydrophobic interactions (hence their resistance to solvent exposure in the native state). Residues that lined the pocket and interacted with bound probes included Tyr67, Leu68, Pro71, Ile75, Met80, Phe82, and Ile85. The binding free energies of these two probes ranged from -2.0 to -2.8 kcal/mol.

On the peripheries of the pocket, acetate, imidazole, and methyl phosphate were the probes observed to interact with Lys13, Asn52, and Glu90. Acetamides were found to bind weakly. Isopropylamine, on the other hand, did not bind the heme pocket at all, presumably due to the excess positively charged residues on the surface of cyt *c*. In runs P1–P4, the iron was continually coordinated by the same water molecule throughout the entire duration of simulations. In P5 and P6, we placed an imidazole molecule adjacent to the heme iron so that it would coordinate the iron and provide input for developing a pharmacophore model (Fig. 3A).

Finally, we simulated cyt *c* complexed with IOA (runs I1 and I2; Table 1). An IOA molecule was initially placed in the

binding pocket such that its imidazole group would coordinate the heme iron. During the simulations, the carboxy-head group of IOA explored the pocket and optimized binding interactions. In particular, we observed formation of salt bridges with Lys13 and Arg91 side chains (Fig. 3B; Supplemental Fig. 1).

Heme Binding Site Is a Nanomolar Druggable Site.

Runs P1–P4 were performed for assessing the druggability of the heme-binding pocket. The maximal achievable ligand-binding affinity calculation was based on the probe binding spots displayed in Fig. 2. The maximal affinity is calculated from the sum of binding free energies of individual probes. Previously, we showed for a diverse set of targets that maximal affinities derived from these simulations are generally close to or better than the affinities measured for best known drug-like inhibitors of the particular targets (Bakan et al., 2012). The maximal binding affinity for the heme pocket was found here to vary from 0.11 to 1.12 nM (Table 2). This suggests that it is possible to identify drug-like compounds that will bind the heme pocket, and with further design, their affinity can reach the nanomolar region.

Druggability Data Permit Us to Build a First PM. We used binding data from simulations to develop a PM for virtual screening of compound libraries (Fig. 4A; Supplemental Fig. 2). Tightly bound probe and water molecules (Supplemental Fig. 2A) and favorably bound IOA conformations (Supplemental Fig. 2B) were selected as the basis for PM development using OpenEye software vROCS (Rush et al., 2005). The initial model that combines selected molecules is shown in Fig. 4A. The gray shaded region corresponds to the union of the volumes of the individual probe molecules. Within this region, colored spheres correspond to moieties that bear specific features or satisfy specific interactions, such as hydrogen bonds acceptor, as labeled.

Experiments Confirmed the Peroxidase Inhibitory Activity of Three Repurposable Drugs Deduced from Initial PM. We screened for compounds that fit this shape and satisfy some of the feature requirements. To ensure prioritization of compounds that coordinate heme iron, we increased the weight of features that capture iron coordination (green ring and adjacent red acceptor) by a factor of 8.

In the first round of virtual screening, we evaluated two sets of compounds: 1) 7520 approved or investigational drugs from DrugBank (Knox et al., 2011), and 2) 150 molecules that are cocrystallized bound to heme in PDB structures. Compounds were ranked based on their Tanimoto similarity to the PM, which takes shape and feature similarities into account (Supplemental Fig. 3). We eliminated compounds that overlapped with cyt *c* or heme and those that were not available for purchase. We selected 12 dissimilar compounds (Supplemental Fig. 4; Table 3) with the aim of

TABLE 2
Druggability of cyt *c* binding pocket

Simulation	$\Delta G_{\text{probe binding}}$	Affinity	Fractional Contribution of Probes	Charge
	kcal/mol	nM		
P1	−12.3	1.12	5.5 isopropanol, 1 acetate, 0.5 acetamide	−1e
P2	−13.3	0.19	6.6 isopropanol, 0.3 acetamide	0e
P3	−13.7	0.11	3.7 isopropanol, 3.3 isobutane	0e
P4	−13.1	0.28	3 isopropanol, 2.9 isobutane, 1 acetate, 0.1 acetamide	−1e

$\Delta G_{\text{probe binding}}$, free energy change upon binding.

Fig. 4. Initial PM and three hits experimentally verified to inhibit cyt *c*. (A) Initial PM based on probe molecules and IOA conformations. Supplemental Fig. 2 provides details of the model. (B–D) Overlay of top-three hits obtained from screening the PM against Food and Drug Administration–approved drugs compiled in DrugBank. (E) Dose-dependent activity of compounds tested in the first round. Bars indicate cyt *c* peroxidase activity in arbitrary units (au). Amount of compound is measured by the compound to cyt *c* molecular ratio. Standard deviations are calculated from three repeats. HQL-79, 4-(benzhydryloxy)-1-[3-(1*H*-tetraazol-5-yl)propyl]piperidine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

identifying features that are required for inhibitory activity. For iron coordination, we considered compounds bearing 5- and 6-membered aromatic heterocycles, and paroxetine that contains the methylenedioxy group. Thiamine pyrophosphate was selected to test whether a negatively charged moiety is essential for binding.

These 12 compounds were tested for their ability to inhibit the peroxidase activity of the complex cyt *c*/TOCL by H₂O₂-induced oxidation assays probing the transition of Amplex Red to resorufin (see *Materials and Methods* section). Experiments were performed at three different doses for each compound measured as molar ratio of compound to cyt *c*. Results are presented in Fig. 4E. Three of the 12 compounds, bifonazole, econazole, and abiraterone, demonstrated very strong inhibition of peroxidase activity at low doses. Fifty percent inhibition of peroxidase activity was achieved at a ratio of 2:1, and more than 80% inhibition at a ratio of 5:1. Four of the tested compounds showed moderate inhibitory activity, and five were found inactive.

Among the three highly active compounds, two, bifonazole and econazole, are imidazole antifungal drugs. Both are found in PDB structures bound to members of the cytochrome P450 family. The third, abiraterone, on the other hand, is a Food and Drug Administration–approved drug used in castration-resistant prostate cancer. It inhibits 17 α -hydroxylase/C17,20 lyase (CYP17A1), an enzyme which is expressed in testicular, adrenal, and prostatic tumor tissues.

Refined PM Using Data from First Round Further Led to Seven Novel Cyt *c* Inhibitors. We performed a second round of studies for lead identification using the three

previously identified and experimentally confirmed compounds (together with the data obtained from druggability simulations) to further improve our PM and screen larger libraries of purchasable compounds (Fig. 5A). The incorporation of the three active compounds into the model contributed several ring features in the center of the pocket (Supplemental Fig. 5). We also removed features from the initial model which did not appear to be required for activity. For example, anionic features based on the carboxyl head of IOA and donor/acceptor features based on isopropanol and water molecules were removed

TABLE 3

Scores, molecular properties, and inhibitory concentrations of tested drugs (from DrugBank and the PDB)

Compound	Tanimoto	Log P ^a	Percent activity ^b	IC ₅₀
Bifonazole	0.994	4.966	16	0.83
Econazole	0.941	5.117	51	2.39
Abiraterone	0.903	4.423	20	0.44
Paroxetine	0.884	4.053	91	N/D
Zimelidine	0.884	0.206	76	N/D
Etomidate	0.936	2.399	79	N/D
Thioperamide	0.955	2.665	91	N/D
Metyrapone	0.968	1.763	100	N/D
Ancymidol	0.861	1.122	95	N/D
NNK	1.005	0.594	96	N/D
Thiamine pyrophosphate	0.983	−5.716	100	N/D
HQL-79	0.874	2.654	96	N/D

HQL-79, 4-(benzhydryloxy)-1-[3-(1*H*-tetraazol-5-yl)propyl]piperidine; N/D, not determined; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

^aLog P is calculated using Molinspiration online cheminformatics tools (<http://www.molinspiration.com/>).

^bPercent activity at 2:1 compound to cyt *c* ratio is displayed.

Fig. 5. Pharmacophore modeling and experimental verification of a second round of compounds identified for cyt *c* inhibition. (A) Optimized PM refined upon incorporation of the features of validated hits shown in Fig. 4. See Supplemental Fig. 5 for details of the model. (B–D) Overlay of top-three hits on the refined PM. (E) Dose-dependent activity of 14 compounds identified in this round (same format as in Fig. 4E). 004-212-014, 1-(4-fluorophenyl)-4-[3-(4-fluorophenyl)imidazole-4-carbonyl]piperazine; 004-301-433, 2-[4-(1*H*-imidazol-1-ylmethyl)phenyl]benzoic acid; 004-430-932, 1-(3-phenylimidazole-4-carbonyl)-4-[3-(trifluoromethyl)phenyl]piperazine; 016-629-555, 1-[4'-(1*H*-imidazol-1-ylmethyl)biphenyl-3-yl]ethanone; 020-139-730, 5-(imidazol-1-ylmethyl)-4-methyl-2-phenyl-1,3-thiazole; 9009967, 1-[2-[(1-chloro-2-naphthyl)oxy]ethyl]-1*H*-imidazole hydrochloride; 9291732, 1-[4-(1*H*-imidazol-1-ylmethyl)benzoyl]indoline; ADM19808287, (3*S*)-3-imidazol-1-yl-1-[4-[3-methyl-4-(trifluoromethyl)isoxazole[4,5-*e*]pyridin-6-yl]-1-piperidyl]but; Z1006910000, (2*S*)-2-imidazol-1-yl-*N*-methyl-*N*-[2-(1-naphthyl)ethyl]propanamide; Z131771408, *N*-[(1-benzylpyrazol-4-yl)methyl]-*N*-methyl-3-phenyl-imidazole-4-carboxamide; Z219193164, [4-(imidazol-1-ylmethyl)phenyl]-pyrrolidin-1-yl-methanone; Z66113547, 3-(4-fluorophenyl)-*N*-methyl-*N*-[4-(trifluoromethyl)phenyl]methylimidazole-4-carboxamide.

(Supplemental Fig. 5, A and B). Since the two most active compounds bore imidazole rings that coordinate the heme iron, we added a cation feature with weight 5 and increased the weights of ring/acceptor features to 10.

Using the improved model, we screened drug-like (11 million) and lead-like (3.1 million) subsets of the ZINC (Irwin and Shoichet, 2005) purchasable compound library. Supplemental Figure 7 displays the Tanimoto score distribution of the top-ranking 4615 compounds. After eliminating the compounds that gave rise to steric overlap with the protein, we selected 14 compounds (Supplemental Fig. 6; Table 4) for experimental testing. In this case, all compounds bore an imidazole group for iron coordination. Performing the previously described assays (see also *Materials and Methods*) showed that seven of these 14 compounds had peroxidase inhibitory activities in a concentration-dependent manner. These were able to inhibit half of cyt *c* peroxidase activity at a 2:1 compound:cyt *c* ratio (Figs. 5E and 6). Figure 5, B–D illustrates the close match between each of the three most potent compounds and the refined PM.

Discussion

Using computational methods based on first principles, we developed a pharmacophore model that captures the shape of the heme-binding pocket in the open (druggable) form of cyt *c* along with chemical features that are required for ligand binding. This model helped us identify three Food and Drug Administration–approved (repurposable) drugs and seven novel compounds that inhibit the peroxidase activity of cyt *c* in a dosage-dependent manner. Four of the compounds were at

least as effective as TPP-6-ISA, which we recently optimized for the inhibition of the peroxidase activity of cyt *c* using structure-based modeling (Jiang et al., 2014). Interestingly, compound

TABLE 4

Scores, molecular properties, and inhibitory concentrations of tested purchasable compounds (from the ZINC library)

Compound	Tanimoto	Log P ^a	Percent activity ^b	IC ₅₀
L254614	1.441	3.264	3	0.15
9009967	1.436	3.465	25	0.71
002-126-168	1.344	4.359	8	0.67
016-629-555	1.484	3.259	18	0.61
020-139-730	1.423	2.899	56	2.38
Z1006910000	1.389	2.672	61	3.1
9291732	1.360	2.717	53	1.74
ADM19808287	1.389	2.371	68	N/D
004-430-932	1.423	3.406	67	N/D
Z66113547	1.353	3.403	77	N/D
004-212-014	1.420	2.862	83	N/D
Z219193164	1.400	0.959	93	N/D
Z131771408	1.353	2.316	87	N/D
004-301-433	1.352	−0.162	95	N/D

004-212-014, 1-(4-fluorophenyl)-4-[3-(4-fluorophenyl)imidazole-4-carbonyl]piperazine; 004-301-433, 2-[4-(1*H*-imidazol-1-ylmethyl)phenyl]benzoic acid; 004-430-932, 1-(3-phenylimidazole-4-carbonyl)-4-[3-(trifluoromethyl)phenyl]piperazine; 016-629-555, 1-[4'-(1*H*-imidazol-1-ylmethyl)biphenyl-3-yl]ethanone; 020-139-730, 5-(imidazol-1-ylmethyl)-4-methyl-2-phenyl-1,3-thiazole; 9009967, 1-[2-[(1-chloro-2-naphthyl)oxy]ethyl]-1*H*-imidazole hydrochloride; 9291732, 1-[4-(1*H*-imidazol-1-ylmethyl)benzoyl]indoline; ADM19808287, (3*S*)-3-imidazol-1-yl-1-[4-[3-methyl-4-(trifluoromethyl)isoxazole[4,5-*e*]pyridin-6-yl]-1-piperidyl]but; Z1006910000, (2*S*)-2-imidazol-1-yl-*N*-methyl-*N*-[2-(1-naphthyl)ethyl]propanamide; Z131771408, *N*-[(1-benzylpyrazol-4-yl)methyl]-*N*-methyl-3-phenyl-imidazole-4-carboxamide; Z219193164, [4-(imidazol-1-ylmethyl)phenyl]-pyrrolidin-1-yl-methanone; Z66113547, 3-(4-fluorophenyl)-*N*-methyl-*N*-[4-(trifluoromethyl)phenyl]methylimidazole-4-carboxamide.

^aLog P is calculated using Molinspiration online cheminformatics tools.

^bPercent activity at 2:1 compound to cyt *c* ratio is displayed.

Fig. 6. Activity profiles of hits at lower concentrations. Percent residual cyt c peroxidase activity is shown for varying ratios of compound to cyt c. 020-139-730, 5-(imidazol-1-ylmethyl)-4-methyl-2-phenyl-1,3-thiazole; 9009967, 1-[2-[(1-chloro-2-naphthyl)oxy]ethyl]-1H-imidazole hydrochloride; 9291732, 1-[4-(1H-imidazol-1-ylmethyl)benzoyl]indoline; Z1006910000, (2S)-2-imidazol-1-yl-N-methyl-N-[2-(1-naphthyl)ethyl]propanamide.

L254614 [1-(4,9-dihydrothieno(2,3-c)(2)benzothiepin-4-yl)-1H-imidazole] exhibited remarkable potency, practically inhibiting complete cyt c peroxidase activity. This compound differs from others by its compact, relatively rigid structure and the central sulfur-containing heptameric ring.

Postanalysis of compounds showed that high lipophilicity, in addition to ideal coordination of heme iron, is a required property for binding. Calculated partition coefficients (log P) of the best seven hits are higher than 3.3 (Tables 3 and 4). These values are, however, more favorable than the log P of IOA and imidazole stearic acid, which are 6.2 and 6.7, respectively. Whereas such high lipophilicity indicates nonspecific interactions, econazole (first round, from DrugBank) and 002-126-168 [1-[4-(1-adamantyl)-2-methoxybenzoyl]-1H-imidazole] (second round, ZINC library) make a specific hydrogen bond with a backbone carbonyl. Additionally, we screened these compounds against the pan assay interference compounds (PAINS) filter (Baell and Holloway, 2010). All passed the filter, showing that they are free of reactive intermediates and functional groups that were considered promiscuous, poorly soluble, or unstable.

The compounds presented in this study provide a unique basis for identifying even more potent compounds. Four of the newly discovered compounds showed remarkable peroxidase inhibitory activities, and the inhibitory activities of the other six tested compounds were comparable to those of triphenylphosphonium-imidazole stearic acids, which were very effective in inhibiting proapoptotic oxidative events in cells, suppressing cyt c release, preventing cell death, and protecting mice against lethal doses of irradiation (Atkinson et al., 2011). These de novo compounds can thus serve as promising candidates for developing next-generation antiapoptotic agents and radioprotectors.

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Authorship Contributions

Participated in research design: Bahar, Bakan, Kagan.

Conducted experiments: Kapralov, Bakan, Hu.

Performed data analysis: Bahar, Bakan, Bayir, Kagan, Kapralov.

Wrote or contributed to the writing of the manuscript: Bahar, Bakan, Bayir, Kagan.

References

- Atkinson J, Kapralov AA, Yanamala N, Tyurina YY, Amoscato AA, Pearce L, Peterson J, Huang Z, Jiang J, and Samhan-Arias AK et al. (2011) A mitochondria-targeted inhibitor of cytochrome c peroxidase mitigates radiation-induced death. *Nat Commun* **2**:497.
- Baell JB and Holloway GA (2010) New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. *J Med Chem* **53**:2719–2740.
- Bakan A, Dutta A, Mao W, Liu Y, Chennubhotla C, Lezon TR, and Bahar I (2014) Evol and ProDy for bridging protein sequence evolution and structural dynamics. *Bioinformatics* **30**:2681–2683.
- Bakan A, Nevins N, Lakdawala AS, and Bahar I (2012) Druggability assessment of allosteric proteins by dynamics simulations in the presence of probe molecules. *J Chem Theory Comput* **8**:2435–2447.
- Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, and Bourne PE (2000) The protein data bank. *Nucleic Acids Res* **28**:235–242.
- Bushnell GW, Louie GV, and Brayer GD (1990) High-resolution three-dimensional structure of horse heart cytochrome c. *J Mol Biol* **214**:585–595.
- Hanske J, Toffey JR, Morenz AM, Bonilla AJ, Schiavoni KH, and Pletneva EV (2012) Conformational properties of cardiolipin-bound cytochrome c. *Proc Natl Acad Sci USA* **109**:125–130.
- Hawkins PCD, Skillman AG, Warren GL, Ellingson BA, and Stahl MT (2010) Conformer generation with OMEGA: algorithm and validation using high quality structures from the Protein Databank and Cambridge Structural Database. *J Chem Inf Model* **50**:572–584.
- Humphrey W, Dalke A, and Schulten K (1996) VMD: visual molecular dynamics. *J Mol Graph* **14**:33–38, 27–28.
- Irwin JJ and Shoichet BK (2005) ZINC—a free database of commercially available compounds for virtual screening. *J Chem Inf Model* **45**:177–182.
- Jiang J, Bakan A, Kapralov AA, Silva KI, Huang Z, Amoscato AA, Peterson J, Garapati VK, Saxena S, and Bayir H et al. (2014) Designing inhibitors of cytochrome c/cardiolipin peroxidase complexes: mitochondria-targeted imidazole-substituted fatty acids. *Free Radic Biol Med* **71**:221–230.
- Kagan VE, Bayir A, Bayir H, Stoyanovsky D, Borisenko GG, Tyurina YY, Wipf P, Atkinson J, Greenberger JS, and Chapkin RS et al. (2009a) Mitochondria-targeted disruptors and inhibitors of cytochrome c/cardiolipin peroxidase complexes: a new strategy in anti-apoptotic drug discovery. *Mol Nutr Food Res* **53**:104–114.
- Kagan VE, Bayir HA, Belikova NA, Kapralov O, Tyurina YY, Tyurin VA, Jiang J, Stoyanovsky DA, Wipf P, and Kochanek PM et al. (2009b) Cytochrome c/cardiolipin relations in mitochondria: a kiss of death. *Free Radic Biol Med* **46**:1439–1453.
- Kagan VE, Tyurin VA, Jiang J, Tyurina YY, Ritov VB, Amoscato AA, Osipov AN, Belikova NA, Kapralov AA, and Kini V et al. (2005) Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol* **1**:223–232.
- Knox C, Law V, Jewison T, Liu P, Ly S, Frolkis A, Pon A, Banco K, Mak C, and Neveu V et al. (2011) DrugBank 3.0: a comprehensive resource for 'omics' research on drugs. *Nucleic Acids Res* **39**:D1035–D1041.
- MacKerell AD, Brooks B, Brooks C, Nilsson L, Roux B, Won Y, and Karplus M (2002) CHARMM: the energy function and its parameterization. *Encyclopedia of Computational Chemistry* John Wiley & Sons, Chichester, UK.
- Maity H, Maity M, Krishna MMG, Mayne L, and Englander SW (2005) Protein folding: the stepwise assembly of foldon units. *Proc Natl Acad Sci USA* **102**:4741–4746.
- Nelson MT, Humphrey W, Gursoy A, Dalke A, Kale LV, Skeel RD, and Schulten K (1996) NAMD: a parallel, object-oriented molecular dynamics program. *Int J High Perform Comput Appl* **10**:251–268.
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, and Ferrin TE (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* **25**:1605–1612.
- Rush TS, 3rd, Grant JA, Mosyak L, and Nicholls A (2005) A shape-based 3-D scaffold hopping method and its application to a bacterial protein-protein interaction. *J Med Chem* **48**:1489–1495.
- Sinibaldi F, Howes BD, Piro MC, Polticelli F, Bombelli C, Ferri T, Coletta M, Smulevich G, and Santucci R (2010) Extended cardiolipin anchorage to cytochrome c: a model for protein-mitochondrial membrane binding. *J Biol Inorg Chem* **15**:689–700.
- Vanommeslaeghe K, Hatcher E, Acharya C, Kundu S, Zhong S, Shim J, Darian E, Guvench O, Lopes P, and Vorobyov I et al. (2010) CHARMM general force field: A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. *J Comput Chem* **31**:671–690.

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