Inhibition of Peroxidase Activity of Cytochrome c: De Novo Compound Discovery and Validation


Affiliations

Departments of Computational and System Biology, School of Medicine (A.B., F.H., I.B.) and Department of Environmental and Occupational Health (A.A.K., H.B., V.E.K.), University of Pittsburgh, Pittsburgh, PA 15219
Running Title:

Inhibition of Cytochrome c Peroxidase Activity

Corresponding Author:

Ivet Bahar, Distinguished Professor and John K. Vries Chair
Department of Computational & Systems Biology
School of Medicine, University of Pittsburgh
3064 BST3, 3501 Fifth Ave, Pittsburgh, PA 15213
Phone: 412 648 3332 - Fax: 412 648 3163
bahar@pitt.edu  http://www.ccbb.pitt.edu/Faculty/bahar/

Number of text pages: 15
Number of tables: 4
Number of figures: 6
Number of references: 23
Number of words in the Abstract: 132
Number of words in the Introduction: 518
Number of words in the Discussion: 342

Abbreviations

cyt c: cytochrome c
PM: pharmacophore model
TOCL: 1,1’2,2’-tetraoleoyl cardiolipin
CL: cardiolipin
IOA: imidazole-substituted oleic acid
PDB: Protein Data Bank
Abstract

Cytochrome c (cyt c) release from the 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. The latter 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 (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 inter-mitochondrial-membrane space prior to its release emerged as a viable strategy for discovering antiapoptotic drugs.

Under normal physiological 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 cyt c a CL-specific peroxidase activity in the presence of reactive oxygen species that fuel 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 pro-apoptotic 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 analogues of stearic acid (triphenylphosphonium-conjugated imidazole-substituted oleic and stearic acids, 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 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 ten 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. We retrieved from the PDB and structurally aligned homologous cyt c structures using ProDy (Bakan et al., 2014). The code used for analysis is given as an example in Ensemble Analysis Tutorial of the online ProDy documentation. Briefly, 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 Figure 1A.

Druggability simulations. We performed two groups of runs (Table I): runs P1 to 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 (shortly designated as IOA), 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 to P6 were prepared and analyzed using VMD plugin DruGUI (Bakan et al., 2012), Solvate, Autoionize, and Psfgen. 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. (Bakan et al., 2012) were used. IOA topology files were prepared using VMD plugin Molefacture, and parameters of chemically analogous atoms in CHARMM force field were used for simulations. IOA initial configuration was set manually at the binding site.
**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) (shortly designated cyt c/TOCL) by H$_2$O$_2$-induced oxidation experiments, using Amplex Red 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 $\mu$M DTPA. Cyt c (1 $\mu$M) was incubated with TOCL/DOPC liposomes (TOCL/cyt c ratio 25:1) for 10 min and peroxidase reaction was started by addition of 50 $\mu$M Amplex Red and 50 $\mu$M H$_2$O$_2$. The incubation proceeded for an additional 20 min (reaction rate was linear in the entire time interval). Fluorescence was detected by employing a “Fusion R” microplate analyzer 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 $\mu$M DTPA (pH 7.4).

**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 four nanoseconds or longer. A set of conformations for 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 to 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 7,520 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. Non-standard ligands that are within 3Å of heme iron were considered for virtual screening. Python code for screening structures is provided online in 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 (Irwin and Shoichet, 2005) (version 12). 3D conformers of compounds were generated using OpenEye application OMEGA (Hawkins et al., 2010) (v2.4.6) with default options. Compounds were screened using OpenEye application ROCS (Rush et al., 2005) (v3.1.2). 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.
PAINS filtering. Tested compounds were screened against PAINs (for Removal of Pan Assay Interference Compounds) (Baell and Holloway, 2010) using the online webserver PAINS-Remover (http://cbligand.org/PAINS/).

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 Figure 1A (Maity et al., 2005). Upon complexation with CL, cyt c assumes a partially unfolded conformation whereby 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 (Figure 1A). Repeated 200 ns 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 (data not shown). 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 a 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 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 the heme (Figure 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 (Figures 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 (Figure 3A).

Finally, we simulated cyt c complexed with imidazole-substituted oleic acid (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 (Figure 3B and Suppl Figure S1).

**Heme binding site is a nanomolar druggable site**

Runs P1 to 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 Figure 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 nM 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 nanomolar region.

**Druggability data permit us to build a first pharmacophore model (PM)**

We used binding data from simulations to develop a PM for virtual screening of compound libraries (**Figures 4A** and **S2**). Tightly bound probe and water molecules (**Figure S2A**) and favorably bound IOA conformations (**Figure S2B**) 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 **Figure 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 (i) 7,520 approved or investigational drugs from DrugBank (Knox et al., 2011), and (ii) 150 molecules that are co-crystallized 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 (Figure S3). We eliminated compounds that overlapped with cyt c or heme and those that were not available for purchase. We selected 12 dissimilar compounds (Figure S4 and Table 3) with the aim of 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 methylenedioxy-group. Thiamine pyrophosphate was selected to test whether a negatively charged moiety is essential for binding.

These twelve compounds were tested for their ability to inhibit the peroxidase activity of the complex cyt c/TOCL by H$_2$O$_2$-induced oxidation assays probing the transition of Amplex Red to resorufin (see Experimental Section). Experiments were performed at three different doses for each compound measured as molar ratio of compound to cyt c. Results are presented in Figure 4E. Three of the 12 compounds, bifonazole, econazole, and abiraterone demonstrated very strong inhibition of peroxidase activity at low doses. 50% 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 cytochrome P450 family. The third, abiraterone, on the other hand, is an FDA-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 above identified and experimentally confirmed three compounds (together with the data obtained from druggability simulations) to further improve our PM and screen larger libraries of purchasable compounds (Figure 5A). The incorporation of the three active compounds into the model contributed several ring features in the center of the pocket (Figure S5). We also removed features from the initial model which did not appear to be required for activity. For example, anionic feature based on carboxyl head of IOA and donor/acceptor features based on isopropanol and water molecules were removed (Figure S5 A-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 ZINC (Irwin and Shoichet, 2005) purchasable compound library. Figure S7 displays the Tanimoto score distribution of top-ranking 4,615 compounds. After eliminating the compounds that gave rise to steric overlap with the protein, we selected 14 compounds (Figure S6 and Table 4) for experimental testing. In this case, all compounds bore an imidazole group for iron coordination. Performing the above described assays (see also Methods) showed that seven out 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 2:1 compound:cyt c ratio (Figs 5E and 6). Figure 5B-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 FDA-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 that we recently optimized for the inhibition of the peroxidase activity of cyt c using structure-based modeling (Jiang et al., 2014). Interestingly, compound L254614 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.

Post-analysis of compounds showed that high lipophilicity, in addition to ideal coordination of heme iron, are required properties for binding. Calculated partition coefficients (logP) of the best seven hits are higher than 3.3 (Tables 3 and 4). These values are, however, more favorable than logP of IOA and ISA, which are 6.2 and 6.7, respectively. While such high lipophilicity indicates non-specific interactions, econazole (first round, from DrugBank) and 002-126-168 (2nd round, Zinc library) make a specific hydrogen bond with a backbone carbonyl. Additionally, we screened these compounds against 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 set 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 TPP-imidazole stearic acids, which were very effective in inhibiting pro-apoptotic 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 anti-apoptotic agents and radioprotectors.

Acknowledgments

Fellowship by Tsinghua University is acknowledged by FH. Authors thank OpenEye Scientific Software for free access to their software.

Authorship Contributions:

Participated in research design: Bahar, Bakan, and Kagan
Conducted experiments: Kapralov
Conducted computations/simulations: Bakan, and Hu
Performed data analysis: Bahar, Bakan, Bayir, Kagan and Kapralov
Wrote or contributed to the writing of the manuscript: Bahar, Bakan, Bayir, and Kagan
References


Footnotes:

This work was supported by the National Institutes of Health, Institute of General Medical Sciences [5R01 GM099738-03] to I.B. and Institute of Allergy and Infectious Diseases [5U1 9AI068021] to V.E.K., H.B., and I.B.
Figure legends

Figure 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 previous definition of foldons (white foldon is colored gray), and the heme group is displayed in ball-and-stick CPK representation. The ensemble is visualized using Chimera. 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.

Figure 2. Heme site is the only druggable site with a nanomolar achievable affinity. 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 Figure 1A. Protein side-chains and heme atoms are colored yellow.

Figure 3. Cyt c simulations in presence of diverse fragments and IOA inhibitors. Snapshots from simulations P5 and I1 are shown in panels A and B, respectively.

Figure 4. Initial pharmacophore model (PM) and three hits experimentally verified to inhibit cyt c. A. Initial PM based on probe molecules and IOA conformations. Figure S2 provides details of the model. B-D. Overlay of top three hits obtained from screening the PM against FDA-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. Amount of compound is measured by compound to cyt c molecular ratio. Standard deviations are calculated from 3 repeats.
Figure 5. Pharmacophore modeling and experimental verification of second round of compounds identified for cyt c inhibition. A. Optimized PM refined upon incorporation of the features of validated hits shown in Figure 4. See Figure S5 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 Figure 4E). Figure 6. Activity profiles of hits at lower concentrations. Percent residual cyt c peroxidase activity of is shown for varying compound to cyt c ratio.

cytc_open.pdb Cyt c open conformation used as initial configuration in molecular dynamics and druggability simulations.
Tables

Table 1: Composition of the systems simulated for assessing cyt c druggability

<table>
<thead>
<tr>
<th>Runs</th>
<th>PDB ID</th>
<th>Time (ns)</th>
<th>Water</th>
<th>Composition of Probe molecules*</th>
<th>Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>P 1, P2</td>
<td>1hrc</td>
<td>40 x 2</td>
<td>2400</td>
<td>84 isopropanol, 12 acetamide, 12 acetate, 12 isopropylamine</td>
<td>8 Cl⁻</td>
</tr>
<tr>
<td>P 3, P4</td>
<td>1hrc</td>
<td>40 x 2</td>
<td>2400</td>
<td>60 isopropanol, 12 acetamide, 12 acetate, 12 isopropylamine, 24 isobutane</td>
<td>8 Cl⁻</td>
</tr>
<tr>
<td>P5, P6</td>
<td>1hrc</td>
<td>40 x 2</td>
<td>2400</td>
<td>48 imidazole, 24 neutral methyl phosphate, 24 isobutane, 12 isopropanol, 12 acetate</td>
<td>4 Na⁺</td>
</tr>
<tr>
<td>I1, I2</td>
<td>1hrc</td>
<td>40 x 2</td>
<td>2421</td>
<td>1 imidazole-substituted oleic acid</td>
<td>14 Cl⁻</td>
</tr>
</tbody>
</table>

(* for details, see ref 5.)
Table 2: Druggability of cyt c binding pocket

<table>
<thead>
<tr>
<th>Sim</th>
<th>ΔG&lt;sub&gt;probe binding&lt;/sub&gt; kcal/mol</th>
<th>Affinity</th>
<th>Fractional contribution of probes</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>-12.3</td>
<td>1.12 nM</td>
<td>5.5 isopropanol, 1 acetate, 0.5 acetamide</td>
<td>-1e</td>
</tr>
<tr>
<td>P2</td>
<td>-13.3</td>
<td>0.19 nM</td>
<td>6.6 isopropanol, 0.3 acetamide</td>
<td>0e</td>
</tr>
<tr>
<td>P3</td>
<td>-13.7</td>
<td>0.11 nM</td>
<td>3.7 isopropanol, 3.3 isobutane</td>
<td>0e</td>
</tr>
<tr>
<td>P4</td>
<td>-13.1</td>
<td>0.28 nM</td>
<td>3 isopropanol, 2.9 isobutane, 1 acetate, 0.1 acetamide</td>
<td>-1e</td>
</tr>
</tbody>
</table>
Table 3: Scores, molecular properties, and inhibitory concentrations of tested drugs (from DrugBank\textsuperscript{13} and the PDB)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tanimoto</th>
<th>logP*</th>
<th>% activity**</th>
<th>IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifonazole</td>
<td>0.994</td>
<td>4.966</td>
<td>16</td>
<td>0.83</td>
</tr>
<tr>
<td>Econazole</td>
<td>0.941</td>
<td>5.117</td>
<td>51</td>
<td>2.39</td>
</tr>
<tr>
<td>Abiraterone</td>
<td>0.903</td>
<td>4.423</td>
<td>20</td>
<td>0.44</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>0.884</td>
<td>4.053</td>
<td>91</td>
<td>N/D</td>
</tr>
<tr>
<td>Zimelidine</td>
<td>0.884</td>
<td>0.206</td>
<td>76</td>
<td>N/D</td>
</tr>
<tr>
<td>Etomidate</td>
<td>0.936</td>
<td>2.399</td>
<td>79</td>
<td>N/D</td>
</tr>
<tr>
<td>Thioperamide</td>
<td>0.955</td>
<td>2.665</td>
<td>91</td>
<td>N/D</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>0.968</td>
<td>1.763</td>
<td>100</td>
<td>N/D</td>
</tr>
<tr>
<td>Ancymidol</td>
<td>0.861</td>
<td>1.122</td>
<td>95</td>
<td>N/D</td>
</tr>
<tr>
<td>NNK</td>
<td>1.005</td>
<td>0.594</td>
<td>96</td>
<td>N/D</td>
</tr>
<tr>
<td>Thiamine pyrophosphate</td>
<td>0.983</td>
<td>-5.716</td>
<td>100</td>
<td>N/D</td>
</tr>
<tr>
<td>HQL-79</td>
<td>0.874</td>
<td>2.654</td>
<td>96</td>
<td>N/D</td>
</tr>
</tbody>
</table>

* logP is calculated using Molinspiration online cheminformatics tools (http://www.molinspiration.com/).

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

N/D: Not determined.
Table 4: Scores, molecular properties, and inhibitory concentrations of tested purchasable compounds (from Zinc library)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tanimoto</th>
<th>logP*</th>
<th>% activity**</th>
<th>IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>L254614</td>
<td>1.441</td>
<td>3.264</td>
<td>3%</td>
<td>0.15</td>
</tr>
<tr>
<td>9009967</td>
<td>1.436</td>
<td>3.465</td>
<td>25%</td>
<td>0.71</td>
</tr>
<tr>
<td>002-126-168</td>
<td>1.344</td>
<td>4.359</td>
<td>8%</td>
<td>0.67</td>
</tr>
<tr>
<td>016-629-555</td>
<td>1.484</td>
<td>3.259</td>
<td>18%</td>
<td>0.61</td>
</tr>
<tr>
<td>020-139-730</td>
<td>1.423</td>
<td>2.899</td>
<td>56%</td>
<td>2.38</td>
</tr>
<tr>
<td>Z1006910000</td>
<td>1.389</td>
<td>2.672</td>
<td>61%</td>
<td>3.1</td>
</tr>
<tr>
<td>9291732</td>
<td>1.360</td>
<td>2.717</td>
<td>53%</td>
<td>1.74</td>
</tr>
<tr>
<td>ADM19808287</td>
<td>1.389</td>
<td>2.371</td>
<td>68%</td>
<td>N/D</td>
</tr>
<tr>
<td>004-430-932</td>
<td>1.423</td>
<td>3.406</td>
<td>67%</td>
<td>N/D</td>
</tr>
<tr>
<td>Z66113547</td>
<td>1.353</td>
<td>3.403</td>
<td>77%</td>
<td>N/D</td>
</tr>
<tr>
<td>004-212-014</td>
<td>1.420</td>
<td>2.862</td>
<td>83%</td>
<td>N/D</td>
</tr>
<tr>
<td>Z219193164</td>
<td>1.400</td>
<td>0.959</td>
<td>93%</td>
<td>N/D</td>
</tr>
<tr>
<td>Z131771408</td>
<td>1.353</td>
<td>2.316</td>
<td>87%</td>
<td>N/D</td>
</tr>
<tr>
<td>004-301-433</td>
<td>1.352</td>
<td>-0.162</td>
<td>95%</td>
<td>N/D</td>
</tr>
</tbody>
</table>

* LogP is calculated using Molinspiration online cheminformatics tools.

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

N/D: Not determined.
Figure 2
Figure 4
Figure 5
Figure 6

The graph shows the compound/cyt c ratio for various compounds across different concentrations: 0.5, 1, 2, and 5. The horizontal axis lists the compounds, and the vertical axis represents the percentage of activity. The error bars indicate the variability in the data.
Figure S1. Distance between imidazole-substituted oleic acids (IOA) and cyt c residues Lys13 and Arg91. The time evolutions of the distances between side-chains of Lys13 and Arg91 and the carboxylic acid group of IOA from simulation I1 are shown in green and blue, respectively. In the second half of the simulation, IOA forms two stable salt bridges with the aforementioned residues. Heme iron is stably coordinated by the nitrogen of imidazole group in IOA throughout the entire duration of simulation, as shown by the flat orange curve.
Figure S2. Details of the initial pharmacophore model (PM). A. Select probes and water molecules that are used in the design of the model (top), their selected features (middle), and the resulting PM (bottom). Note that some probe and water molecules were selected to contribute only to the shape of the PM. B. Select IOA conformations that are used in design the model (top), selected features that correspond to the inhibitor head and tail (middle), and union of volumes of individual molecules and selected features to obtain the PM (bottom). Color code in PM: yellow, hydrophobic; red, acceptor; blue, donor; green, aromatic; dark red, negatively charged.
**Figure S3. Distribution of Tanimoto combo scores of compounds.** A. Scores of 150 compounds observed in PDB structures as heme iron coordinators. B. Scores of top 2,700 out of 7,520 compounds from DrugBank\(^\text{13}\). Compounds that scored below the default cutoff are omitted.
Figure S4. Structures of the 12 compounds tested in the first round.
Figure S5. Optimized pharmacophore model after incorporating features from initial hits. A. Initial model after refinements. The volume contributions by all probes were preserved. Color features, except iron coordinating features and two hydrophobic features, were removed. Weights of iron coordinating features (acceptor and ring) were increased from 8 to 10. Cation (blue solid sphere) feature was added onto the ring feature to capture imidazole groups. B. Features based on IOA conformations, after removal of anion feature. C-E. Features based on the three hits identified in the first round of screening. Final optimized model that is the union of displayed color features and volumes is shown in Figure 5A.
Figure S6. Structures of compounds tested in the second round.
Figure S7. Distribution of Tanimoto combo scores for top 4,615 compounds from ZINC library.