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More than just GPCR Ligand: Structure-based Discovery of Thioridazine Derivatives as Pim-1 Kinase Inhibitors †‡

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Pim-1 kinase is a serine/threonine kinase which plays an important role in cell proliferation and differentiation. The Pim-1 kinase expression is elevated in leukemia and prostate cancer. Accordingly, we employed structure-based hierarchical virtual screening approach to identify potential unknown Pim-1 kinase activity for existing drugs. Among the LOPAC library of pharmacologically active compounds, one top-ranked drug molecule thioridazine, a well-known antipsychotic agent which exerted its biological function as a dopamine receptor antagonist, showed low micromolar activity in the Pim-1 enzymatic assay. We determined the co-crystal structure of thioridazine bound with Pim-1 kinase, and defined the key elements of the pharmacophore by analyzing the structure-activity relationship of thioridazine analogues. In addition, we also assessed our pharmacophore by successfully predicting the Pim-1 activity for the selective Akt inhibitor 10-DEBC. Our discovery of the unknown Pim-1 inhibitory activity of thioridazine and 10-DEBC might provide novel insights in understanding their molecular mechanism of action, and inspire the computation-driven multiple-target drug discovery.

The target binding profile of a drug molecule is essential for understanding its mechanism of action (MOA), as its off-target binding propensities may lead to better clinical efficacy in some circumstances, while causing side effects in other cases. Many marketed drugs today were discovered serendipitously, and the elucidation of their MOA has been usually retrospective. Notably, their therapeutic efficacies were often attributed to their multiple target actions. One example is clozapine. One of the most successful atypical antipsychotic drugs, it was discovered half a century ago, and its clinical efficacy was reported to be associated with its promiscuous binding to dozens of G protein-coupled receptors (GPCRs).1 Many compounds even designated as single-target drugs are in fact not as specific as originally expected, and have been discovered to bind to other targets. For example, Gleevec was initially designed to inhibit the abnormal tyrosine kinase BCR-ABL. Subsequently, it was shown to target several other kinases, including e-KIT and PDGFR kinases, which led to its expanded clinical applications in advanced or metastatic gastrointestinal stromal tumor (GIST).2,3 Experimentally testing all drugs on all potential biomolecular targets is practically impossible; therefore, computationally predicting the novel polypharmacology of drug molecules has recently attracted great interest in the field of drug discovery.4,6 One notable method was the development of a statistics-based chemoinformatics approach, similarity ensemble approach (SEA), which had been applied in systematically relating the ligand chemical similarity to biological targets.5,9 Complementary to such ligand-based approaches, protein structure-based methods require the structures of known targets to predict protein-ligand binding complexes, and to estimate the binding affinities, like the inverse docking protocol INVDOCK.10,11

GPCRs and kinases are two most important drug target families, and many of their ligands are promiscuous within their own protein families. However, the ligand cross-reactivity between kinase inhibitors and GPCR ligands has rarely been reported. Interestingly, a well-known kinase drug, sorafenib, was identified to bind to 5-hydroxytryptamine receptors (5-HTRs) by computationally screening the FDA approved drug molecules against the modelled structure of the 5-HT2A receptor.12 Therefore, we were wondering whether we could discover known...
GPCR ligands with unexpected kinase activities by virtual screening GPCR ligands against therapeutically relevant kinases. The proviral integration site in Moloney murine leukemia virus (Pim) kinases are a family of proto-oncogenic serine/threonine kinases regulating several signalling pathways that are fundamental to cancer development and progression. As the most studied member of Pim kinases, Pim-1 kinase is highly expressed in a wide range of hematopoietic malignancies and solid cancers, especially in leukemia and prostate cancer. Pim-1 kinase adopts a typical kinase fold consisting of N-terminal and C-terminal domains linked by a hinge region. However, the presence of residue P123 at the hinge region disrupts the canonical hydrogen bonding pattern with the adenine moiety of ATP or bound inhibitors due to the lack of the hydrogen bond donor. This unique hinge region architecture suggests that Pim-1 kinase inhibitors might be structurally distinguishable from many classical kinase inhibitors. Therefore, we further asked whether we could discover the unexpected Pim-1 kinase activity of GPCR ligands.

In our continuous endeavor to computationally find novel ligand cross-reactivity, in combination with experimental evaluation to explore the unknown Pim-1 kinase activity of existing drugs and chemical probes with known biological activity (Fig. 1). Briefly, we docked 1,280 LOPAC library compounds (Sigma-Aldrich Corp.) against the ATP binding site of Pim-1 kinase (PDB ID: 2J2I) using the fast-compute docking program DOCK 3.5.54. The automated docking pipeline as described previously includes the sphere generation, scoring grid and docking calculations. All generated docking poses were subjected to the MM-GB/SA refinement and rescoring procedure. The energy minimizations were performed using Protein Local Optimization Program (PLOP) with all-atom molecular-mechanics (MM) force field and a generalized born surface area (GB/SA) implicit solvent model with variable internal dielectric constant. The ligand binding energy was computed by subtracting the energies of the optimized free ligand in solution and the free protein in solution from the optimized ligand-protein complex’s energy in solution, accounting for protein-ligand interaction energy, desolvation energies of ligand and protein, and ligand strain energy. Finally, we visually examined the top 1% ranked poses, and chose the compounds of interest for experimental testing.

1,280 compounds
Structure-Based Virtual Screening via Molecular Docking
Second (per compound)
11,044 docking poses
MM-GB/SA Refinement and Rescoring
Minute (per pose)
top 1% binding poses
Visual Examination
2 compounds
Experimental Testing
Fig. 1. Flowchart of our structure-based virtual screening strategy.

Fig. 2 (a) Dose response inhibition of Pim-1 by thioridazine. The IC₅₀ value was determined from three independent tests. (b) Fitting dextrorotatory form (+) of thioridazine (gray) into an electron density map in the ATP binding site of Pim-1. The sigmaA weighted 2Fo-Fc electron density map contoured at a level of 1.0σ. Coordinates and detailed methods for the solved crystal structure were deposited to the PDB with the accession ID 4IAA. Molecular images were generated with PyMOL.

Thioridazine represents a novel class of Pim-1 inhibitors. We submitted thioridazine to the SEA server (http://sea.bkslab.org), but none of kinases were predicted to be thioridazine’s potential target based on ligand structural similarity. We also performed pair-wise similarity comparison of thioridazine against the crystal inhibitors of Pim-1 using ChemMine Web Tools. None of the calculated Tanimoto Coefficients are greater than 0.35 (ESI, Table S1†). In addition, the structural similarity analysis against all reported 669 Pim-1 inhibitors in ChEMBL database didn’t identify any structural analogues of thioridazine. Therefore, thioridazine could not be predicted as a Pim-1 inhibitor using these ligand-based approaches.

Next, we determined the X-ray crystal complex structure of Pim-1 kinase bound with thioridazine at 2.85 Å resolution (Fig. 2b). Note that we used the racemic mixture in the crystallization study due to the inadequate availability of pure enantiomers, and we fitted the dextrorotary form (+) of thioridazine into the electron density map in the ATP-site. The detailed experimental procedure is included in Supporting Information (ESI, Table S2†). The overall structure of the Pim-1 kinase resembles published crystal structures. The crystal binding pose of thioridazine is consistent with our docking prediction with an RMSD value of 3 Å using dextrorotary form for comparison. The electron density of phenothiazine group in thioridazine is very clear, it interacts favorably with hydrophobic residues V52, L174 and
I185 in the ATP binding site, while no hydrogen bond is formed between thioridazine and the kinase hinge region. Nevertheless, the week electron density map features at aliphatic chain and piperidine ring of thioridazine suggest the conformational diversity and/or the ambiguous configurations of the piperidine ring in the racemic mixture.

To explore the key elements of the binding pharmacophore of thioridazine, we obtained three thioridazine analogues from the National Cancer Institute (NCI) compound library. Compounds NCI1186055 and NCI1186058, with a chloro or bromo group replacing the methylsulfanyl group on the phenothiazine ring of thioridazine, exhibited similar inhibitory activity to thioridazine (Fig. 3a, Table 1). This result was consistent with the crystallographic position of methylsulfanyl group, which did not form direct contacts with binding-site residues by extending into the solvent (Fig. 2b). On the contrary, compound NCI64076 was nearly inactive (Fig. 3a, Table 1). The major difference between NCI64076 and thioridazine was the length of the aliphatic chain bridging the piperidine ring and the phenothiazine scaffold, which may largely reduce the favorable electrostatic interaction between the positively charged nitrogen atom on the piperidine group and negatively charged residue D128. To further assess the contribution of the aliphatic amines, we synthesized and tested compound b4 along with three intermediates (Fig. 3b, Table 1). All three intermediate compounds were inactive while only compound b4 was as active as thioridazine, which could support the basic components of the pharmacophore: a tricyclic ring scaffold occupying the hydrophobic ATP binding site, a bridging aliphatic chain with proper length and an aliphatic amine group interacting with negatively charged residue D128.

Additionally, we noticed that one widely used selective Akt inhibitor, 10-DEBC (Fig. 3c), satisfied our designated Pim-1 pharmacophore. As expected, 10-DEBC showed strong inhibitory activity with an IC_{50} value of 1.28 µM. We also determined the X-ray crystal complex structure of 10-DEBC bound with Pim-1 (Fig. 4, Table S2†). Comparing two co-crystal structures, the tricyclic scaffolds in both compounds overlap to a great extent while the methylsulfanyl group in thioridazine and the chloro group in 10-DEBC point in opposite directions. At the same crystallographic resolution of 2.85 Å, the electron density map in the 10-DEBC bound structure is sufficiently resolved to place the aliphatic chain and diethylamino group. The charged tertiary amine group in 10-DEBC forms a more favorable electrostatic interaction with residue D128 than the thioridazine (3.5 Å in 10-DEBC vs. 4.5 Å in thioridazine), which may indicate the structural basis of strong inhibitory activity of 10-DEBC.

### Table 1. Inhibition of Pim-1 by thioridazine derivatives.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>IC_{50} (µM)</th>
</tr>
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<tbody>
<tr>
<td>Thioridazine (-/+)</td>
<td>6.89 ± 1.51</td>
</tr>
<tr>
<td>Thioridazine (-)</td>
<td>7.39 ± 2.59</td>
</tr>
<tr>
<td>Thioridazine (+)</td>
<td>7.03 ± 2.52</td>
</tr>
<tr>
<td>NCI1186055</td>
<td>8.16 ± 2.47</td>
</tr>
<tr>
<td>NCI1186058</td>
<td>6.73 ± 0.45</td>
</tr>
<tr>
<td>NCI164076</td>
<td>34.0 ± 7.2% (100 µM)</td>
</tr>
<tr>
<td>b1</td>
<td>9.7 ± 2.1% (100 µM)</td>
</tr>
<tr>
<td>b2</td>
<td>22.6 ± 2.9% (100 µM)</td>
</tr>
<tr>
<td>b3</td>
<td>17.6 ± 8.7% (100 µM)</td>
</tr>
<tr>
<td>b4</td>
<td>8.70 ± 3.39</td>
</tr>
<tr>
<td>10-DEBC</td>
<td>1.28 ± 0.28</td>
</tr>
</tbody>
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* Data are shown as (mean values ± SD) from three independent determinations.

Fig. 3. (a) The chemical structures of three thioridazine derivatives obtained from the National Cancer Institute. (b) Synthesis of phenothiazine derivatives. General synthetic approaches. Reagents: 1. sulfur, I_{2}, 170 °C, 2 h, 60%; 2. 1,4-dibromobutane, NaH, 25°C, DMF, overnight, 78%; 3. NaNO_{3}, DMSO/H_{2}O (5:1), 25°C, 48h, 55%; 4. Na, H_{2}, Pd/C, MeOH, 50°C overnight, 50%; 4. Na, NH_{4}H_{2}O, CsCO_{3}, DMF, 80°C, 2h, 80%. (c) Chemical structure of 10-DEBC.

Fig. 4. The detail view of Pim-1 kinase domain with the 10-DEBC (PDB code: 4MED) compared with the thioridazine (PDB code: 4IAA). The protein was shown in a green cartoon representation. The compound 10-DEBC was represented in orange stick and thioridazine was in cyan stick. Several important binding site residues were highlighted. The sigma-A weighted 2Fo-Fc electron density map contoured at a level of 1.0σ.
Thioridazine is an antipsychotic drug known to exert its biological function as a dopamine receptor antagonist. Interestingly, thioridazine has recently been found to selectively target the neoplastic cells, and impair human somatic cancer stem cells capable of in vivo leukemic disease initiation without showing effects on normal blood stem cells. The authors proposed that dopamine receptors might serve as a biomarker involved in diverse malignancies. However, it is not clear whether thioridazine’s Pim-1 kinase inhibition activity might also contribute to its effect of eradicating cancer stem cells. Several pieces of experimental evidence are available to support our hypothesis. Firstly, Pim-1 kinase has been shown to express in haematopoietic stem cells and embryonic stem cells. Pim-1 mediates the homing and migration of haematopoietic cells through phosphorylation of chemokine receptor 4 (CXCR4). Pim-1 kinase is also involved in mouse embryonic stem cell self-renewal. Secondly, Pim-1 plays an important physiological role in c-Myc driven tumorigenesis via effective oncosphere collaboration, where elevated expression level of Pim-1 is often observed in the context of increased c-Myc levels. Pim-1 phosphorylates c-Myc to stabilize c-Myc in vivo and to enhance c-Myc transforming activity, where c-Myc exerts critical function in both self-renewal and differentiation of stem cells and early progenitor cells. Thirdly, elevated levels of Pim-1 kinase have been observed in leukemia cancer cells. Nevertheless, the mechanism of Pim-1 inhibition by thioridazine in cancer stem cell differentiation requires further elucidation.

In addition, thioridazine was reported to induce apoptosis in ovarian, cervical and endometrial cancer cells by targeting Akt/mTOR signalling pathway, where the phosphorylation levels of Akt and 4E-BP1 were decreased by thioridazine treatment. Notably, both Akt and Pim-1 kinases share several common substrates that are involved in apoptosis. Pim-1 inhibitor has recently been reported to reduce the phosphorylation of Akt. 4E-BP1, one of the best characterized targets of the mTOR complex, was identified as a substrate of Pim-1 kinase. Therefore, it is likely that thioridazine induces apoptosis by modulating Akt/mTOR signalling pathway through Pim-1 inhibition.

In the present study, we also predicted the Pim-1 activity of a selective Akt inhibitor 10-DEBC based on our derived pharmacophore from thioridazine derivatives. Our biochemical and structural analysis validated its Pim-1 inhibitory activity. 10-DEBC was reported to be a micromolar inhibitor of Akt (5 µM). However, it was also reported to induce autophagy through an Akt-independent mechanism in neurons. Our result suggests that the cellular functions of 10-DEBC might relate to its Pim-1 inhibitory activity.

In summary, a selective dopamine receptor antagonist, thioridazine, was identified to be a low micromolar inhibitor of Pim-1 kinase by a structure-based hierarchical virtual screening approach. The co-crystal structure, SAR study and pharmacophore evaluation indicated that a tricyclic scaffold with a proper aliphatic side chain bridged tertiary amine might present a novel class of Pim-1 inhibitor. Moreover, a selective Akt inhibitor 10-DEBC possessing the key pharmacophore elements was shown to exhibit strong in vitro Pim-1 activity. Therefore, it is intriguing whether the Pim-1 inhibitory activity of thioridazine and 10-DEBC contributes to their mode of action. We hope that our present results will not only provide a novel scaffold for Pim-1 inhibitor design, but also inspire the computation-driven multiple-target drug discovery.

Acknowledgements

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References


A structure-based hierarchical virtual screening method was employed to identify a GPCR ligand, thioridazine as a Pim-1 kinase inhibitor.