Privileged Structures and Polypharmacology within and between Protein Families


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Privileged Structures and Polypharmacology within and between Protein Families


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KEYWORDS Polypharmacology, Privileged Structure, Binding Site Comparison, Pirin, B-Raf.

ABSTRACT: Polypharmacology is often a key contributor to the efficacy of a drug, but is also a potential risk. We investigated two hits discovered via a cell-based phenotypic screen, the CDK9 inhibitor CCT250006 and the pirin ligand CCT245232, to establish methodology to elucidate their secondary protein targets. Using computational pocket-based analysis, we discovered intra-family polypharmacology for our kinase inhibitor, despite little overall sequence identity. The inter-family polypharmacology of CCT245232 with B-Raf was used to discover a novel pirin ligand from a very small but privileged compound library, despite no apparent ligand or binding site similarity. Our data demonstrates that in areas of drug discovery where intra-family polypharmacology is often an issue, ligand dissimilarity cannot necessarily be used to assume different off-target profiles and that understanding inter-family polypharmacology will be important in the future to reduce the risk of idiopathic toxicity and in the design of screening libraries.

Polypharmacology, where small molecules bind to more than one protein target at concentrations relevant to their therapeutic free exposure, is a key element in drug discovery and development. It can be beneficial as an essential part of the efficacy and phenotype of a drug, particularly in complex diseases such as cancer, but can also be detrimental, leading to toxicity by hitting undesirable protein targets. This balance in therapeutic index can represent a significant challenge in lead optimization.

Privileged structures are defined as small molecule scaffolds that are able to bind more than one receptor, a concept which has been useful in compound library design and in understanding polypharmacology.

We recognized that there are four types of polypharmacology that are directly relevant to drug discovery. i) The first is often encountered in small molecule probes used in chemical biology, for example PROTACs, and in the rational design of dual inhibitors, such as the combination of histone deacetylase (HDAC) inhibitors with janus kinase and IDO1-targeting compounds. In this approach, two distinct compounds, usually with different functions and selectivities, are attached to each other via a flexible linker, with the aim that despite being attached, each motif of the chemical probe will retain its activity.

ii) The second is described by weak and often non-specific interactions with proteins outside of the target protein’s family. These anti-targets are broadly characterized in vitro safety pharmacology screens, where binding to promiscuous targets can cause adverse drug reactions at high dose; for example, adenosine A2a receptor and hERG.

iii) Owing to high sequence similarity in the active sites within a protein family, small molecule ligands often bind with high affinity to multiple members of that family. This type of polypharmacology is particularly well established in kinase drug discovery, where privileged structures of certain hinge-binding motifs display low selectivity within the family, an effect that can lead to a poor therapeutic index (staurosorpin) or be crucial for efficacy (aurora/FLT3).

iv) The final type of polypharmacology that can impact drug discovery has only recently been recognized, largely due to broad proteome screening platforms becoming readily available. High affinity specific interactions can occur for ligands binding to different protein families, despite no apparent binding site or sequence similarity. This concept was recently highlighted by the discovery that the well-characterized potent kinase inhibitor, BI-2536 (PLK1, IC50=0.83 nM), binds with high affinity to the bromodomain family protein, BRD4 (IC50=25-37 nM). However, it is unclear whether the inter-family activity encountered represents an inherent conservation in protein structure, expressed through an underlying similarity between protein binding sites, or is simply an anomaly.

We recently carried out a cell-based high-throughput phenotypic screen to discover inhibitors of the heat shock transcription factor 1 (HSF1) stress pathway. From the screen, we identified two novel series and exploited several molecular target identification strategies to discover direct...
Figure 1. Rational Polypharmacology within the Kinase Family. A: A close analogue of aminopyrimidine 1 bound to CDK2 (PDB: 4BZD, green) and overlaid with CDK9 (PDB: 4EC8, turquoise) and homology models of TAOK1 (brown) and HIPK2 (yellow); despite little overall sequence identity, the proteins show clear pocket shape similarity, which was detected by the pocket analysis tool. B: Key interactions of the close analogue of 1 with CDK2 (PDB: 4BZD, green), compared to the equivalent residues in CDK9 (blue), TAOK1 (brown) and HIPK2 (yellow), showing that these key residues are retained. C: Chemical structure of aminopyrimidine 1 and the well validated CDK9 inhibitor dinaciclib 3.

The SiteHopper pocket analysis tool was then applied to our 21 kinase dataset, comparing detected pockets of each kinase with reference pockets derived from structures of CDK9. Each kinase was analyzed with respect to the maximum PatchScore observed with any of the reference CDK9 pockets. This analysis identified 11 kinases with a maximum PatchScore of greater than 1.3 (Tables 1 and S2), a cut-off proposed to describe significant structural similarity between binding sites.25 As expected, CDK9 itself was the top-ranked hit, as the score simply represents conformation differences between different protein structures. The second ranked hit, CDK2 and CDK14, had already been confirmed as a direct protein target of 1 from our previous work.24 From the remaining hits, TAOK1 and HIPK2 (discovered from analysis of homology models) were selected for further study as they were evolutionarily more distant from CDK9, with sequence identities of 24% and 8%, and sequence homology of 48% and 40%, respectively, and for which functional assays were available. The activity of aminopyrimidine 1 was assessed using radio labeled filter binding assays, to give IC₅₀ values of 490 nM and 30 nM for TAOK1 and HIPK2, respectively (Tables S4 and S5).35 Therefore, despite little total sequence identity between TAOK1, HIPK2 and CDK9, each protein still shares a common kinase fold and possesses similar binding sites, resulting in intra-family polypharmacology (Figure 1A, 1B, S3 and S4).

Although from these data we cannot conclude that either kinase will significantly contribute to the efficacy or in vivo toxicology of aminopyrimidine 1, they do suggest that binding site pocket analysis can be a useful tool for prioritizing off-target proteins for further evaluation as part of a lead optimization selectivity assessment. Particularly as neither protein kinase selected using pocket analysis displays high sequence similarity to CDK9,36 so may not have been chosen.

Table 1. SiteHopper Analysis comparing the Kinase Binding Site Similarity with CDK9

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<tr>
<th>Rank</th>
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Only the top 11 hits are shown using the maximum score obtained against all available conformations, see the SI for details.
### Table 2. Inter-Family Polypharmacology Screen of Pirin and B-Raf

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ND=Not determined aAll compounds were purchased from Selleckchem (www.selleckchem.com accessed June 2016). bCompared to the dual pirin/B-Raf ligand bisamide 2, calculated using ECFC, 4 molecular fingerprint Tanimoto coefficients as implemented in Pipeline Pilot v9.5. cFor references of the described B-Raf activities, see the SI. dMeasured using the pirin FP-assay and represents the geometric mean of at least n=2 repeats.

Using in vitro kinase recombinant protein profiling, we had previously shown that bisamide 2 possessed moderate affinity for the protein kinase, B-Raf (IC$_{50}$=420 nM), concurrent with its high affinity for pirin (K$_{D}$=38 nM).23 The inter-family protein activity of bisamide 2 for both B-Raf and pirin is surprising, as the two proteins possess no significant binding site sequence similarity. To rationalize this result, we solved the crystal structure of bisamide 2 bound to pirin (Figure 2A, S8, S14 and Table S6) and compared its binding mode to the docked structure of bisamide 2 in B-Raf (Figure 2B). Bisamide 2 displays a distinct binding conformation in pirin, with the two amide carboxyls forming an eclipsed conformation around the central ring. In the docked structure of bisamide 2 in B-Raf, assumed to be ATP competitive, the two amide carboxyls form a perpendicular conformation, consistent with the crystal structures of the bisamide chemotype bound to EphA3 (PDB: 3DZQ) and p38 (PDB: 3KQT) kinases (Figures S5 and S6). This is in contrast to the BRD4/PLK1 example of inter-family polypharmacology,20 where BI-2536 adopts essentially an identical conformation against both protein targets (Figure S7).

Even though pocket analysis had been unable to detect any similarity between the pirin and B-Raf binding sites, we hypothesized that if the affinity of bisamide 2 to both proteins was a more general phenomenon, then it should be possible to...
Figure 2. Structural Comparison of Pirin and B-Raf. N=blue, O=red, S=yellow, Cl=green, Metal=brown, H omitted for clarity. Only key residues have been shown and the solvent, except the metal-bound water, has been omitted for clarity. Hydrogen bonds are shown as yellow dashes.

A: Bisamide 2 bound to pirin demonstrating the key hydrogen bonds to Asp43 and the metal-bound water, and the amide-eclipsed conformation. The quinoline ring motif is solvent exposed.

B: Docked structure of bisamide 2 bound to B-Raf (PDB: 4G9C, see Figures S4 and S5 for additional pictures), 2 binds in a distinct staggered conformation and the quinoline ring motif is placed in close proximity to the hinge region.

C: Structure of PLX4720 7 bound to pirin. The azaindole-ketone motif forms an equivalent hydrogen bonding array to the amide of 2. The chloro-substituent is buried deeply within the narrow binding tunnel. Because the sulfonamide moiety is disordered it cannot be observed in the electron density but is shown here for clarity.

D: PLX4720 7 bound to B-Raf (PDB: 3C4C). The azaindole motif interacts with the hinge region and the chloro-substituent points on a solvent exposed vector.

E and F: Key interaction of PLX4720 7 and bisamide 2, respectively, bound to pirin. Hydrogen bonds are represented by an arrow, π-stacking interactions by a dotted line. Key interactions of both ligands bound to B-Raf are shown in Figure S14.

G: Chemical structure of FP-Probe 4.

discover a second ligand of a distinct chemotype that could bind to both proteins.

The bisamides are currently the only known high affinity chemotype for pirin but B-Raf has been extensively studied resulting in multiple inhibitors of distinct chemotype being discovered. To rapidly screen B-Raf inhibitors in an efficient manner, we developed a new pirin binding assay. Fluorescence polarization (FP) assays are highly versatile and have been widely used to characterize proteins that do not possess enzymatic activity. The design of the FP-probe was carried out by analysis of the crystal structure of 2 bound to recombinant pirin, and through strong precedent from previous pirin ligand design (Figures 2B, 2G and S13).

The bisamide 2 (Figure 2G) was synthesized using a 9-atom linker in 7 steps and 1.2% overall yield (see Chemistry Experimental section in SI).

Titration of recombinant pirin against a fixed concentration (2.0 nM) of FP-probe 4 revealed a very high apparent affinity for pirin \(K_D=11\) nM, within 5-fold of the FP-probe 4 concentration (Figure S1). Using the FP-assay, bisamide 2 and our pirin chemical probe CCT251236 51 gave IC_{50} values of 44 nM (pIC_{50}=7.36±0.10, n=4) and 33 nM (pIC_{50}=7.48±0.09, n=46), respectively, at the tight-binding limit of the assay (for an example see Figure S2). Our negative control regioisomer 6 failed to displace the FP-probe 4 at concentrations greater than 1 μM. These results were comparable to our previous surface plasmon resonance (SPR) data for pirin.23

A panel of seven well-validated and potent B-Raf inhibitors (Entries 4 and 6-11 in Table 2),43 representing several chemotypes distinct from bisamide 2 \(T_C=0.38-0.67\), were purchased and screened in the pirin FP-assay. No activity was observed against pirin with six of the B-Raf inhibitors. However, when we screened the ligand with the lowest \(T_C\) compared to bisamide 2, the azaindole derived inhibitor, PLX4720 7, the ligand demonstrated a high affinity for pirin \(IC_{50}=0.67\) μM, Table 2, Entry 4). Therefore, despite sharing little chemical similarity to bisamide 2, PLX4720 7 was also able bind both pirin and B-Raf, representing a 14% hit-rate from our very small privileged compound library.

To rationalize the high affinity of PLX4720 7 for pirin, we solved its crystal structure (Figures 2C, S10, S16, and Table S6) and compared it to bisamide 2 (Figure S12). PLX4720 7, like bisamide 2, forms no direct interactions with the metal-center of pirin, instead forming hydrogen bonds through the metal-bound water molecule and the central ketone carbonyl. The NH-group of the azaindole moiety acts as a hydrogen bond donor with Asp43, in a similar manner to the methyl-dial amide of bisamide 2. The remaining portion of the azaindole ring forms a π-stacking interaction with Phe53 and points deeply into the lipophilic narrow binding tunnel, leaving the propyl-sulfonamide moiety essentially solvent exposed and highly flexible as it could not be observed in the electron density. This is in contrast to the binding mode of PLX4720 7 bound to B-Raf (Figure 2D, PDB: 3C4C), where the propyl-sulfonamide moiety is now buried deeply within a lipophilic region of the protein and it is the azaindole motif which points towards the solvent channel.

The dissimilarity in binding mode demonstrates that although our two privileged ligands (2 and 7) could bind proteins across families with high affinity, their structure-activity relationships (SAR) towards each target remain essentially distinct. Against pirin, we could exploit the
solvent-exposed quinoline region of bisamide 2 to substitute
the methylene group with a larger substituent. The t-butyl-
piperazine bisamide analogue 8 retains its high affinity for
pirin (Table 2, Entry 5) but the piperazine moiety is predicted
to be in close proximity to the hinge region of B-Raf, where
bulky substituents should not be tolerated. This orthogonal
SAR was confirmed when the B-Raf polypharmacology was
lost in the case of 8 (IC\textsubscript{50}>10 \textmu M). A similar contrast in SAR
is observed for PLX4720 7. Against B-Raf, the chloro-
substituent is on an essentially solvent-exposed vector and can
be readily substituted with a phenyl ring, which gives the high
affinity B-Raf ligand, vemurafenib 9 (Table 2, Entry 6).
However, against pirin, the chloro-substituent of 7 is buried in
the lipophilic tunnel, so the phenyl substitution in 9 now
results in a clear clash with the protein and the complete loss
of the pirin polypharmacology, enhancing the selectivity for
B-Raf.\textsuperscript{44}

Understanding the role of polypharmacology in drug
discovery is essential to improve the efficiency of compound
development and decrease drug attrition. Using two
chemotypes discovered from a high-throughput phenotypic
screen, we have demonstrated that binding site pocket
similarity analysis can be a useful tool in selecting off-target
proteins from within the same family for further investigation.
This approach could offer clear benefits in lead optimization,
prioritizing the study of secondary targets for their importance
in efficacy or toxicity. However, when searching for proteins
outside of the family of the target protein, pocket analysis was
not predictive. We could demonstrate that, despite there being
no detectable sequence or shape similarity in the binding sites
of pirin and B-Raf, there is “inherent” or “underlying”
similarity between these proteins, resulting in inter-family
polypharmacology and limited SAR homology\textsuperscript{45} from two
distinct chemotypes. Consistent with the BRD4/PLK1
example, there are no computational methods we are currently
aware of that are able to detect, quantify or predict this
phenomenon. An analysis by Shoichet et al. of ligands that
bound more than one protein from different families\textsuperscript{46} found
that the majority of examples of inter-family polypharmacology were not as a result of matched residues in
the ligand binding site and there was no simple code for ligand
recognition. Nonetheless, using a bottom-up approach, we
discovered a novel pirin ligand after screening a very small
privileged compound library.

As more examples of inter-family polypharmacology
become available, we will be able to better analyze and
understand what factors control the underlying similarity in
protein binding sites and the conservative evolution that nature
has used to generate them. We will then be better able to
predict efficacy and idioptic toxicities before compounds
reach the clinic and design more efficient high-throughput
screening libraries of privileged structures possessing the
appropriate complexity so that they are likely to hit targets
across the proteome.

ASSOCIATED CONTENT

Supporting Information.
The Supporting Information is available free of charge on the
ACS Publications website.

Pocket analysis, B-Raf docking study, chemistry experimental,
pirin fluorescence polarization assay, pirin and B-Raf
crystallography analysis, kinase assay data, crystallography experimental (PDF)

PDB ID Codes
Atomic coordinates and structure factors for the crystal structures of
pirin with compound 2 and 7 can be accessed using PDB
codes: 6H1I and 6H1H respectively. The authors will release the
atomic coordinates and experimental data upon article publication.

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Matthew D. Cheeseman: 0000-0003-1121-6985

Author Contributions
All authors have given approval to the final version of the manuscript. J.M., N.Y.M., M.C., J.B. and N.B. carried out and
designed the computational studies. N.E.A.C., B.W., A.E.P.,
M.J.T., L.E.E., C.S.R., P.W., K.J. and M.D.C. designed and
synthesized the small molecules and chemical probes. S.A., Y.B.
and R.L.M.V.M. designed and carried out the crystallography
experiments. M.R., L.E.E., L.O.F and R.B. designed and carried out
the FP-assay experiments. J.M. and M.D.C. wrote the manuscript.

Notes
The Institute of Cancer Research has a potential financial interest
in inhibitors of the HSF1 pathway and operates a Rewards to
Discoverers scheme.

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Cancer Investment Trust. Paul Workman is Cancer Research UK
Life Fellow.

ABBREVIATIONS
PROTAC, proteolysis targeting chimera; HDAC, histone
decetylase; IDO1, indoleamine 2,3-dioxogenase; hERG, human
ether-\textsuperscript{a}-go-go-related gene; PLK1, polo-like kinase 1; BRD4,
bromodomain-containing protein 4; HSF1, heat shock
transcription factor 1; CDK9, cyclin-dependent kinase 9; Tc,
Tanimoto coefficient; SPR, surface plasmon resonance
spectroscopy; MOA, mechanism of action; PDB, protein data
bank; PMP, protein model portal; HIPK2, Homeodomain-
interacting protein kinase 2.

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SiteHopper Analysis comparing the Kinase Binding Site Similarity with CDK9

314x297mm (96 x 96 DPI)
### Inter-Family Polypharmacology Screen of Pirin and B-Raf

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Structural Comparison of Pirin and B-Raf. N=blue, O=red, S=yellow, Cl=green, Metal=brown, H omitted for clarity. Only key residues have been shown and the solvent, except the metal-bound water, has been omitted for clarity. Hydrogen bonds are shown as yellow dashes. A: Bisamide 2 bound to pirin demonstrating the key hydrogen bonds to Asp43 and the metal-bound water, and the amide-eclipsed conformation. The quinoline ring motif is solvent exposed. B: Docked structure of bisamide 2 bound to B-Raf (PDB: 4G9C, see Figures S4 and S5 for additional pictures). 2 binds in a distinct staggered conformation and the quinoline ring motif is placed in close proximity to the hinge region. C: Structure of PLX4720 7 bound to pirin. The azaindole-ketone motif forms an equivalent hydrogen bonding array to the amide of 2. The chloro-substituent is buried deeply within the narrow binding tunnel. Because the sulfonamide moiety is disordered it cannot be observed in the electron density but is shown here for clarity. D: PLX4720 7 bound to B-Raf (PDB: 3C4C). The azaindole motif interacts with the hinge region and the chloro-substituent points on a solvent exposed vector. E and F: Key interaction of PLX4720 7 and bisamide 2, respectively, bound to pirin. Hydrogen bonds are represented by an arrow, pi-stacking interactions by a dotted line. Key interactions of both ligands bound to B-Raf are shown in Figure S14. G: Chemical structure of FP-Probe 4.

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