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Structural Characterization of Inhibitor Complexes with Checkpoint Kinase 2 (Chk2), a Drug Target for Cancer Therapy

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Abstract

Chk2 (checkpoint kinase 2) is a serine/threonine kinase that participates in a series of signaling networks responsible for maintaining genomic integrity and responding to DNA damage. The development of selective Chk2 inhibitors has recently attracted much interest as a means of sensitizing cancer cells to current DNA-damaging agents used in the treatment of cancer. Additionally, selective Chk2 inhibitors may reduce p53-mediated apoptosis in normal tissues, thereby helping to mitigate adverse side effects from chemotherapy and radiation. Thus far, relatively few selective inhibitors of Chk2 have been described and none have yet progressed into clinical trials. Here, we report crystal structures of the catalytic domain of Chk2 in complex with a novel series of potent and selective small molecule inhibitors. These compounds exhibit nanomolar potencies and are selective for Chk2 over Chk1. The structures reported here elucidate the binding modes of these inhibitors to Chk2 and provide information that can be exploited for the structure-assisted design of novel chemotherapeutics.

Keywords

structure-based drug design; kinase inhibitor; crystal structure

1. Introduction

Checkpoint kinase 2 (Chk2) is a serine/threonine protein kinase which, when activated by DNA damage, leads to the downstream phosphorylation of various substrates that are involved in cell cycle arrest, DNA damage repair, and apoptosis (Hirao et al., 2000; Hirao et al., 2002; Matsuoka et al., 2000; Pommier et al., 2005; Stolz et al., 2010). Chk2 is activated primarily by ATM or DNA-PK (also ATR and hMPs1) via phosphorylation of Thr68 in the

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SQ/TQ cluster domain (Ahn et al., 2000), which initiates homodimerization of Chk2 monomers followed by *trans*-activating autophosphorylation of residues Thr383 and Thr387 (Ahn et al., 2002; Oliver et al., 2007; Oliver et al., 2006) and then *cis*-phosphorylation of Ser516 (Wu and Chen, 2003). Upon activation, Chk2 phosphorylates a number of downstream targets involved in regulation of the cell cycle (e.g. Cdc25a and Cdc25c) (Bartek and Lukas, 2003; Matsuoka et al., 1998), in DNA repair and chromosome stability (e.g. BRCA1 and FOXM1) (Stolz et al., 2010; Tan et al., 2007; Zhang et al., 2004) and/or proteins that have functional roles in apoptosis such as p53, PML, and E2F1 (Antoni et al., 2007; Pommier et al., 2006).

Chk2 has attracted much attention in recent years as a potential therapeutic target for anti-cancer drug design in part because studies have shown that Chk2 may play an important role in the proliferation of cancer cells (Antoni et al., 2007; Poehlmann and Roessner, 2010; Pommier et al., 2006; Stolz et al., 2010; Zhou et al., 2003). For instance, Chk2 is activated in precancerous lesions with genomic instability and in cancer cells grown in culture (Bartkova et al., 2005; Gorgoulis et al., 2005). Chk2 has also been implicated in playing important functional roles in tumor cell adaptation to changes that result from the cycling nature of hypoxia and reoxygenation found in solid tumors (Freiberg et al., 2006), the activation of BRCA1 (Stolz et al., 2010; Zhang et al., 2004), and in the release of survivin, a protein which is involved in tumor survival (Ghosh et al., 2006). Therefore, a selective inhibitor of Chk2 could potentially abrogate the proliferation of cancer cells with endogenously activated Chk2. Indeed, prior studies with the selective cell-permeable Chk2 inhibitor PV1019 demonstrated that this compound exerts anti-proliferative effects in tumor cell lines from the NCI-60 (Shoemaker, 2006) that exhibit high endogenous levels of activated Chk2 in contrast to those with low levels of Chk2 (Jobson et al., 2009).

A second and arguably more compelling reason to be interested in Chk2 inhibitors is that selective inhibition of Chk2 in p53-defective tumor cell lines may increase their sensitivity to DNA-damaging drugs and radiation by targeting the G2 checkpoint (Levesque and Eastman, 2007; Pommier et al., 2006; Zhou and Sausville, 2003; Zhou et al., 2003). In principle, such a dual-therapy approach could increase the therapeutic efficacy of radiation and current drugs used in chemotherapy. This rationale is supported by prior evidence demonstrating that down-regulation of Chk2 in p53-mutant tumor cells results in enhanced apoptotic activity in response to ionizing radiation (Yu et al., 2001). Furthermore, the inhibitor PV1019 was shown to potentiate the cytotoxicity of camptothecin and topotecan in three ovarian cancer cell lines that had high levels of endogenous activated Chk2 (Jobson et al., 2009). The compound CCT241533 was also shown to be a selective inhibitor of Chk2 and to potentiate the cytotoxicity of PARP inhibitors (Anderson et al., 2011). Finally, selective Chk2 inhibition may also afford some protection of normal cells (Hirao et al., 2000). As a result of therapies involving ionizing radiation and chemotherapeutics, the p53-mediated apoptotic pathway often leads to the initiation of cell death in normal tissues as a result of exposure to these agents (Pommier et al., 2006). Therefore, selective inhibition of Chk2 may help alleviate undesirable side effects due to radiation and chemotherapy. Indeed, such effects were demonstrated in Chk2^{-/-} transgenic mice that showed resistance to apoptosis after exposure to ionizing radiation (Takai et al., 2002), and the treatment of mouse thymocytes and human T-cells with selective Chk2 inhibitors also provided radioprotective effects (Arienti et al., 2005; Carlessi et al., 2007; Jobson et al., 2009).

Because only a small number of Chk2 inhibitors have been identified to date (Arienti et al., 2005; Caldwell et al., 2011; Carlessi et al., 2007; Curman et al., 2001; Hilton et al., 2010; Janetka and Ashwell, 2009; Jobson et al., 2007; Jobson et al., 2009; Sharma et al., 2007; Yu et al., 2002), the discovery of novel chemotypes remains of significant interest. We previously described a novel Chk2 inhibitor obtained from a high-throughput screen, NSC

109555 (Fig. 1A), and reported co-crystal structures of this inhibitor and one of its derivatives, PV1019 (Fig. 1B), with the catalytic domain of Chk2 (Jobson et al., 2009; Lountos et al., 2009). In the present study, we present crystal structures of the Chk2 catalytic domain in complex with five more inhibitors (Fig. 1C–G) derived from NSC 109555 that were designed on the basis of information obtained from previous co-crystal structures to improve their potency and selectivity for Chk2. The new structures reported here should lead to the development of even more potent and selective inhibitors by iterative structure-assisted optimization.

2. Materials and Methods

2.1 Protein expression and purification

The catalytic domain of human Chk2 (Ser210-Glu531) was expressed and purified as previously reported (Lountos et al., 2009). The purified fraction was concentrated to 35–45 mg/mL (estimated at 280 nm using a molar extinction coefficient of $32,890 \text{ M}^{-1} \text{ cm}^{-1}$) and aliquots were flash frozen in liquid nitrogen and stored at -80°C . The final product was judged to be >90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight was confirmed by LC electrospray mass spectrometry.

2.2 Biochemical characterization of inhibitors

The inhibitors used in this study were synthesized by Provid Pharmaceuticals (Monmouth Junction, NJ). The IMAP Screening Express Kit (Molecular Devices, Sunnyvale, CA) was used for conducting the inhibition assays as previously reported (Jobson et al., 2007). The compounds were dissolved in DMSO and reactions were performed using recombinant human Chk2 and RSK2 (Millipore, Billerica, MA) and Chk1 (Upstate) with compounds in reaction buffer (10 mM Tris-HCl, pH 7.2, 10 mM magnesium chloride, 0.1% bovine serum albumin, 1 mM dithiothreitol, 10 mM ATP, and 100 nM peptide substrate) in a total volume of 5 μL in 384-well plates for 60 minutes at room temperature. Substrates used in the assay were 5FAM-AMRLERQDSIFYPK-NH₂ for Chk2, 5FAM-ALKLVRYPSFVITAK-NH₂ for Chk1, and 5FAM-AKRRRLSSLRAOH for RSK2 (all from Molecular Devices). 15 μL of IMAP binding reagent were added to each well, the plates were incubated for 30 minutes at room temperature, and fluorescence polarization was measured using a Tecan Ultra plate reader at wavelengths of 485 nm for excitation and 535 nm for emission. Each screening plated contained staurosporine as a positive control.

2.3 Crystallization of the Chk2-inhibitor complexes

All crystallization reagents were obtained from Hampton Research (Aliso Viejo, CA). The inhibitors used for this study were synthesized by Provid Pharmaceuticals (Monmouth Junction, NJ) and were dissolved in DMSO. A solution of 10 mg/mL Chk2 catalytic domain in 25 mM Tris pH 7.2, 150 mM NaCl, and 2 mM TCEP) was incubated with each inhibitor (1 mM in 10% v/v DMSO). The protein-inhibitor mixtures were incubated at room temperature for 30 minutes followed by an additional incubation for 1.5 hours at 4°C . Insoluble compounds were removed by centrifugation prior to crystallization setups. All crystals were grown using the hanging-drop vapor diffusion technique. A 1:1 ratio of protein-inhibitor solution and well solution (0.1M HEPES pH 7.8, 0.1M magnesium nitrate, 14% w/v polyethylene glycol 3350, and 16% v/v ethylene glycol) were mixed and sealed over 1 mL of well solution using a 24-well crystallization tool from Qiagen (Valencia, CA). The tray was placed in a 4°C incubator overnight and then the drops were streak-seeded the following day by transferring microseeds from previously grown Chk2-PV1019 crystals (Jobson et al., 2009) to the drops with a whisker. Crystals grew to final dimensions of approximately $0.3 \text{ mm} \times 0.1 \text{ mm} \times 0.1 \text{ mm}$ within a week. The crystals were removed from

the drop with a litho-loop (Molecular Dimensions, Apopka, FL) and were flash frozen in liquid nitrogen without the need for additional cryoprotectant.

2.4 X-ray data collection, structure determination, and refinement

X-ray diffraction data were collected from crystals held at approximately 100 K at beamlines 22-ID and 22-BM of the SER-CAT facilities at the Advanced Photon Source. Data sets were collected using a 1.0 Å wavelength, oscillation angle of 1.0° and a 3 second exposure time. The data were integrated and scaled using HKL3000 (Minor et al., 2006). The Chk2-inhibitor complex structures were solved by molecular replacement using the MOLREP (Vagin and Teplyakov, 2010) program from the CCP4 suite (1994). The coordinates of the Chk2-PV1019 structure (PDB code: 2W7X) were used as a search model after stripping away the inhibitor and all solvent molecules (Jobson et al., 2009). Cross-rotation and translational searches for 1 molecule in the asymmetric unit were conducted using a maximum resolution of 3.0 Å followed by rigid-body refinement with REFMAC5 (Murshudov et al., 1997). The inhibitors were unambiguously identified using cross-validated σ_A -weighted $2mF_o-DF_c$ and mF_o-DF_c electron density maps (Read, 1997). The coordinates for the inhibitors were prepared using the Dundee PRODRG server (Schüttelkopf and van Aalten, 2004) and were placed into the σ_A -weighted mF_o-DF_c difference electron density maps contoured at 3 σ level. Iterative rounds of model building with *Coot* (Emsley and Cowtan, 2004) and refinement with REFMAC5 were carried out to extend the data up to the maximum resolution for each respective data set. The refinement was monitored by setting aside 5% of the reflections for use in the calculation of the R-free value (Brunger, 1992). Water molecules were located with *Coot*. Model validation was performed using MolProbity (Davis et al., 2007). All data collection and refinement statistics are outlined in Table 2. All figures were prepared using PyMOL (www.pymol.org) or LIGPLOT (Wallace et al., 1995).

2.5 Accession numbers

Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers 2YCQ (Chk2/PV1115), 2YCR (Chk2/PV976), 2YCS (Chk2/PV788), 2YCF (Chk2/PV1531) and 2XK9 (PV1533).

3. Results and Discussion

3.1 Biochemical characterization of inhibitors

The compounds were tested for inhibition against Chk2 using the IMAP Screening Express Kit (Molecular Devices, Sunnyvale, CA). They were also screened against Chk1 and RSK2 kinases to test for specificity. The results are presented in Table 1. In the assay, a fluorescently labeled peptide is phosphorylated in a kinase reaction. The addition of the IMAP binding reagent stops the kinase reaction and binds specifically to the phosphorylated peptides through a high affinity interaction of trivalent metal-containing nanoparticles with phosphogroups on the substrate. Phosphorylation and binding of the substrate to the beads can be detected by fluorescence polarization. The compounds exhibited sub-micromolar IC₅₀ values against Chk2 and were selective for Chk2 versus Chk1 and RSK2. The broad-based kinase inhibitor staurosporine was used as a positive control as it inhibits Chk2, Chk1, and RSK2.

3.2 Crystallization, structure solution, and overall structure

All of the Chk2-inhibitor complexes described in this study were obtained by co-crystallization and streak seeding, using previously grown Chk2-PV1019 crystals as a seed source (Jobson et al., 2009). Streak seeding was absolutely essential to obtain high quality crystals, as most of the complexes failed to crystallize without seeding or resulted in poor

quality crystals that were not suitable for data collection. The crystals utilized in this study typically diffracted to 2.35–1.77 Å resolution in space group P3₂21 with slightly varying unit cell dimensions. The structures were solved by molecular replacement, using the coordinates of the Chk2-PV1019 crystal structure as a search model (PDB code: 2W7X) (Jobson et al., 2009). The resulting σ_A -weighted $2mF_o-DF_c$ and mF_o-DF_c electron density maps allowed for the unambiguous identification of the binding site of the inhibitors. All structures exhibited more than 90% of the residues in the most favored region of the Ramachandran plots and favorable geometry after analysis with MolProbity (Davis et al., 2007). All of the inhibitors described in this study were found in the ATP-binding pocket of Chk2 (Fig. 2) with no significant global structural changes to Chk2 when compared with previous Chk2-inhibitor complex structures (typical r.m.s.d values were approximately 0.16 Å). Data collection and refinement statistics for these inhibitor complexes are summarized in Table 2.

3.3 Structure of Chk2 in complex with PV1115

NSC 109555 (Fig. 1A) was identified as a potent and selective inhibitor of Chk2 by high-throughput screening, yet this compound was found to be inactive in cellular assays (Jobson et al., 2007). Due to their double charge and high polarity, the bisguanidines in NSC 109555 were considered to be sub-optimal with respect to pharmacokinetic and ADME profiles, and there was reason to believe that they might give rise to off-target effects such as DNA binding and interference with polyamine function (Byczkowski and Porter, 1983; Denny and Cain, 1979; Marcus et al., 1987). Therefore, optimization efforts were focused on developing mono-guanidinohydrazines such as PV1019 (Fig. 1B), which was found to be active in cells against Chk2 (Jobson et al., 2009).

The design of compound PV1115 (Fig. 1C) was inspired by analysis of the crystal structure of Chk2 in complex with PV1019. The molecular scaffold of PV1019 was modified by introducing a cyclic guanidine analog at the guanidino head group that interacts with residue Glu273 (Jobson et al., 2009). The strategy behind this modification was to maintain the binding interaction with Glu273 while addressing the need to replace the highly basic guanylylhydrazone moiety (pK_a~12), which could reduce cellular penetration. The cyclic guanidine removes two hydrogen bond donors and increases the overall hydrophobicity. PV1115 exhibited an IC₅₀ value of 0.14 nM for Chk2 vs. an IC₅₀ of greater than 100 μM for Chk1 *in vitro* (Table 1).

Crystals of Chk2 in complex with PV1115 were obtained by co-crystallization and diffracted to 2.05 Å resolution (Table 2). PV1115 is situated within the ATP-binding pocket of Chk2 in a manner very similar to PV1019 (Fig. 3A and B) (Jobson et al., 2009). The 7-nitroindole group binds into the hinge region primarily by hydrogen bonding between the oxygen of the nitro group to the backbone amide NH of Met304 and also a water-mediated (Wa2161) hydrogen bond to the backbone carbonyl oxygen of Glu302. The nitrogen atom in the indole ring also is involved in a water-mediated (Wa2161) hydrogen bond link to the backbone carbonyl oxygen of Glu302. Additionally, several van der Waals interactions between the aliphatic portions of the indole and Leu226, Val234, Gly307, Leu354, and the aliphatic portions the side chains of Met304 and Glu308 contribute to important binding interactions in this region. The urea carbonyl oxygen in PV1115 is also linked to the backbone carbonyl oxygen of Glu302 through a water-mediated (Wa2161) hydrogen bond. A water-mediated hydrogen bond between the nitrogen adjacent to the carbonyl group to the Glu308 side chain is also observed. The aryl ring of PV1115 is surrounded by a cluster of aliphatic residues that include Val234, Ile299, Leu301 and Leu354. Therefore, this region provides favorable hydrophobic packing interactions with the inhibitor. Additionally, the aliphatic portion of the Lys249 side chain is positioned directly above the aryl ring, resulting in favorable van der Waals contacts. In the Chk2-ADP complex, Lys249 forms a stabilizing

salt bridge with Glu273 that couples the C- α helix with nucleotide binding (Huse and Kuriyan, 2002; Oliver et al., 2006). The binding of PV1115 to Chk2 causes the Lys249 residue to move approximately 3.9 Å away from Glu273, thereby abolishing this salt-bridge and allowing for the cyclic guanidine head group of PV1115 to fill the space occupied by Lys249 in the Chk2-ADP structure. The new orientation of the Lys249 residue is directed towards the phosphate-binding loop. This movement suggests a potential structural role for Lys249 in influencing the conformation of the phosphate-binding loop to accommodate inhibitor binding. Most importantly, the structure illustrates that replacement of the guandino head group with the cyclic guanidine analog maintains the important binding interaction with Glu273 via two hydrogen bonds.

As PV1115 is highly selective for Chk2 over Chk1, we examined the structural features that may be responsible for the preferential binding of PV1115 to Chk2. The coordinates of apo Chk1 (PDB code: 1IA8) were superimposed upon the Chk2-PV1115 structure to compare the positioning of PV1115 with respect to Chk2 and Chk1. Fig. 4 A,B provide a view of the hinge region in the Chk2-PV1115 complex and Chk1 with PV1115 modeled into its binding pocket, respectively. It is apparent that the presence of a bulky Tyr86 residue in Chk1, as opposed to the Leu303 in Chk2, would sterically hinder the binding of the 7-nitroindole group of PV1115 to the Chk1 hinge region, whereas the smaller side chain of Leu303 in Chk2 allows this moiety to fit. Fig. 4C (Chk2) and Fig. 4D (Chk1), illustrate the binding of PV1115 near Glu273 on the C- α helix (Glu55 in Chk1). Again, a bulkier Tyr20 residue on the phosphate-binding loop of Chk1 would sterically hinder the proper positioning of the imidazole group to maintain the hydrogen bonding interactions with Glu55. In Chk2, the smaller Cys231 residue, along with displacement of Lys249, provide ample space for binding of the imidazole group near Glu273. This cysteine residue is part of the highly conserved GXGX Φ G motif in the phosphate binding loop where Φ is generally a Tyr or Phe residue (Huse and Kuriyan, 2002). Due to the distribution of conserved glycine residues, the phosphate-binding loop has been observed to exhibit conformational plasticity so as to allow structural distortions that can accommodate the binding of small molecule inhibitors. The phosphate-binding loop in the Chk2-PV1115 complex is well-ordered whereas the same loop in the Chk2-NSC 109555 and PV1019 complexes is disordered (residues 229–231) (Jobson et al., 2009; Lountos et al., 2009). The presence of a cysteine residue in the Φ position, which is generally occupied by a Tyr or Phe residue, is of interest in inhibitor design because the Tyr/Phe residue has been implicated in interactions with small molecule inhibitors that can cause large structural changes within the loop (Mohammadi et al., 1997). Thus, these structural differences likely contribute to the preference of PV1115 binding to Chk2 over Chk1.

As observed in previous crystal structures of Chk2 in complex with NSC 109555 and PV1019 (Jobson et al., 2009; Lountos et al., 2009), a hydrophobic pocket is directly accessible near the methyl moiety of the PV1115 scaffold (Fig. 3C). The shape and size of the pocket along with the presence of a small Leu301 gatekeeper residue allow for direct access to the pocket by additional substituents on the methyl group. The structure indicates that this pocket in Chk2 is lined almost entirely by small hydrophobic residues. However, superposition of the coordinates of Chk2 upon Chk1 illustrates that Leu277 in Chk2 (which is at the “top” of the pocket) is replaced by a polar Asn59 residue in Chk1 (Fig. 5A). Thus, it is likely that hydrophobic chemical entities would preferentially bind into the Chk2 pocket rather than the corresponding pocket in Chk1 (Foloppe et al., 2006). Sequence alignments with various kinases also indicate the presence of polar or bulkier residues among other kinases in the equivalent position of Leu277 (Fig. 5B).

Additionally, some kinases also have bulkier residues at the gatekeeper position that would restrict access to this pocket (Scapin, 2002; Toledo et al., 1999). This structural observation

suggests that modification of Chk2 inhibitors by the addition of hydrophobic chemical entities on the methyl group that would fit into this pocket may potentially increase binding affinity while retaining selectivity. Future efforts in our laboratories will focus on designing such compounds.

3.4 Structural analysis of the binding modes of PV976 and PV788

Further structural analogs of NSC 109555 were examined by replacing the bisguanidines with cyclic guanidine analogs to create PV976 (Fig. 1D), which exhibits an IC_{50} value of 69.60 nM for Chk2 (IC_{50} for Chk1 is $>100 \mu M$) [Table 1]. Co-crystals of Chk2 in complex with PV976 diffracted X-rays to 2.2 Å resolution (Table 2). Like NSC 109555, PV976 adopts an extended conformation in the ATP-binding pocket (Fig. 6A,C) (Lountos et al., 2009). The primary binding interactions involve two water-mediated (Wa2032) hydrogen bond links between the urea carbonyl oxygen of PV976 and the backbone amide NH of Met304 and backbone carbonyl oxygen of Glu302 in the hinge region. As in the Chk2-NSC 109555 complex, the aryl moieties are stabilized by hydrophobic packing interactions with aliphatic residues and Lys249 is also observed to undergo a conformational shift that disrupts the salt-bridge with Glu273 and places the aliphatic portion of its side chain over the aryl ring. The binding interactions with Glu273 are mediated by hydrogen bonds between the nitrogen in the imidazole ring and adjacent nitrogen of PV976 with the Glu273 carboxylate side chain. The structure of the Chk2 inhibitor complex with NSC 109555 revealed that the guanyldihydrazone group near the hinge region is not involved in any direct hydrogen-bonding interactions with the ATP-binding pocket. The modification of the guanyldihydrazone to a cyclic guanidine moiety, however, results in an approximately 3.0 Å shift away from the position occupied by the guanyldihydrazone toward Leu226, which creates a new interaction involving a hydrogen bond between the nitrogen adjacent to the imidazole and the backbone carbonyl oxygen of Leu226. Furthermore, a new interaction is observed where Wa2059 is involved in a water-mediated hydrogen bonding bridge between the urea nitrogen and the backbone carbonyl oxygen of Glu351. A water molecule was not observed in this position in the Chk2-NSC 109555 complex.

PV788 (Fig. 1E) was created by replacing the 7-nitroindole group of PV1019 with a 3-indole substituent. The IC_{50} for this compound was 1.36 nM for Chk2 vs. $>100 \mu M$ for Chk1) [Table 1]. The 2.35 Å crystal structure revealed that the indole group adopts two conformations in the hinge region, each refined at 0.5 occupancy (Fig. 6B,D; Table 2). One conformation is stabilized by hydrogen bonding between the indole nitrogen and the backbone carbonyl oxygen of Met304. In the second conformation of the indole, the ring is flipped almost 180 degrees and is stabilized by hydrophobic packing against the aliphatic portion of the Met304 side chain. Wa2036 mediates hydrogen-bonding bridge between the urea carbonyl oxygen of PV788 and backbone amide NH of Met304 and carbonyl oxygen of Glu302 in the hinge. The guanidine head group is involved in a combination of hydrogen bonding and ionic interactions with the Glu273 carboxylate side chain as previously observed in the Chk2 complexes with NSC 109555 and PV1019 (Jobson et al., 2009; Lountos et al., 2009). Additionally, in this complex the phosphate-binding loop residues 229–231 are disordered.

3.5 Structures of the PV1531 and PV1533 inhibitor complexes

Further efforts at optimizing the guanyldihydrazone functionalities resulted in the design of inhibitors PV1531 and PV1533 (Fig. 1F and G, respectively). Given the observed binding interactions of the guanyldihydrazone with Glu273 in the crystal structures of prior analogs, it was desirable to maintain a similar hydrogen bonding potential. Therefore, N-hydroxy guanidines were considered as potential isosteres (Gustafsson et al., 2001; Morao et al., 2003; Tai et al., 1983). The advantage of such an isostere would be that while maintaining

the hydrogen bonding interactions with Glu273, the pKa would be reduced to approximately 7–8, which would be more favorable for cellular penetration. Compound PV1531 was created by modifying the guanidine head groups on NSC 109555 with the N-hydroxy oxime moiety. PV1533 was derived from the PV1019 scaffold by introducing an N-hydroxy oxime at the guanidine head group. Remarkably, these two compounds exhibited a significant improvement in their IC₅₀ values *in vitro*. PV1531 had an IC₅₀ of 0.03 nM (86 μM for Chk1) and PV1533 had an IC₅₀ of 0.07 nM (>100 μM for Chk1) [Table 1]. Co-crystals of the Chk2-PV1531 complex diffracted X-rays to 1.77 Å, the highest resolution reported for any Chk2-inhibitor complex to date [Table 2]. This structure revealed several new interactions that probably contribute to the improved potency of the inhibitor (Fig. 7A,B). PV1531 binds in an extended conformation like the parent NSC 109555 molecule and is capped by Cys231 of the phosphate-binding loop, which is well-defined in this complex. A direct hydrogen bond between a nitrogen atom of PV1531 and the backbone carbonyl of Leu226 near the hinge is observed and the water-mediated hydrogen bonding network between the carbonyl oxygen of PV1531 and the backbone amide of Met304 and carbonyl of Glu302 is maintained. The urea nitrogen atoms now are both involved in water-mediated hydrogen bonds. One is linked with the side chain carboxylate of Glu308 and the other with the backbone carbonyl oxygen of Glu351 and side chain carboxylate of Glu308. The hydrogen bonding network with Glu273 is maintained. However, due to the addition of the N-oxime, the oxygen atom is involved in a water-mediated hydrogen bond with the carboxylate side chain of Asp368 of the conserved DFG loop. This is the first significant interaction with the DFG motif observed for this series of inhibitors.

The 2.35 Å crystal structure of Chk2 in complex with PV1533 revealed a similar binding mode for PV1533 as observed with the PV1019 complex (Fig. 7C,D; Table 2). The oxygen of the 7-nitroindole participates in a direct hydrogen bonding interaction to the backbone amide of Met304. However, the electron density for the water that has been observed to mediate hydrogen bonding interactions with the urea carbonyl oxygen in the previous inhibitors is poorly defined. This may be attributed to the lower resolution of the maps. The head group hydrogen bonding interaction is maintained with Glu273 but the oxygen atom of the oxime does not directly interact with any residue.

Although the introduction of the N-oxime guanidine groups results in a significant gain in the potency of the compounds *in vitro*, prior reports have indicated that in N-hydroxylated compounds such as amidines, guanidines, and aminohydrazones, reversible metabolism at nitrogen can occur *in vivo* (Clement, 2002). Both oxidation at nitrogen to form N-hydroxy metabolites and reduction of N-hydroxy compounds occurs readily, which enables N-hydroxy derivatives to function as effective prodrugs for their more basic amidine counterparts (Clement et al., 2006; Gustafsson et al., 2001). Such enzymatic reducing systems have been found in kidney, liver, brain, lung, and intestine across many species. In mammals, this enzyme system is a three-component system consisting of cytochrome b5, its reductase, and a novel molybdenum sulfatase (Havemeyer et al., 2006). Given the reversible nature of hydroxylation on nitrogen in amidines and guanidines, it would be of interest to evaluate the pharmacokinetic and ADME profiles of this class of compounds.

3.6 Comparison with crystal structures of Chk2 in complex with 2-aminopyridine inhibitors

A recent report by Hilton and coauthors identified and characterized a series of 2-aminopyridine inhibitors of Chk2 and described crystal structures of the inhibitor complexes. The crystal structures of two of the complexes, (PDB codes: 2WTI and 2WTJ) were superimposed upon the coordinates of the Chk2-PV1115 complex to compare the binding modes (Hilton et al., 2010). The structure of Chk2 in complex with compound 53 (Fig. 1H) and 63 (Fig. 1I) shows that the 2-aminopyridine group is anchored into the hinge region via hydrogen bonds to Leu303 and Met304 and that the carboximide substituent is

hydrogen bonded to Lys249, which engages in an ionic interaction with Glu273 of the C- α helix (Fig. 8A). The binding to the hinge region differs with respect to the Chk2-PV1115 complex in that upon binding of PV1115, both direct hydrogen bonding and water-mediated hydrogen bonds are involved in interactions with the hinge region. On the other hand, in compound 53, the carboximide NH₂ group is hydrogen-bonded to the side chain carboxylate of Asp368 in the DFG loop while in compound 63, the dimethylamine group interacts with the side chain carboxylate of Asp368. The direct interactions with the DFG motif observed in the inhibitor complexes with compounds 53 and 63 differ with respect to the Chk2-PV1115 complex and its analogs, in that no direct hydrogen bonding interactions with the DFG motif are observed with the exception of the water-bridge to Asp368 that observed in the complex with PV1531. The main difference, however, is that the guanlylhydrazone and cyclic guanidine substituents of the NSC 109555/PV1019 analogs displace and fill the space occupied by Lys249 thereby allowing the binding interaction with Glu273. A significant advantage of this binding mode is that it enables direct access to the hydrophobic pocket via placement of novel chemical entities on the methyl group, which may result in improved binding affinities.

3.7 Implications for future inhibitor design and conclusions

The structures described in this study include a variety of structural analogs of the initial lead compounds NSC 109555 and PV1019 that were previously identified by our laboratories. As illustrated in Fig. 8B, all of the inhibitor complexes reported here bind in a similar fashion with respect to the core scaffold. The key structural determinants for binding of this class of compounds includes a combination of direct hydrogen bonds and water-mediated hydrogen bonds to the hinge region, hydrophobic packing interactions contributed by the aryl and methyl groups, and ionic and hydrogen bonding interactions with Glu273 contributed by either the guanlylhydrazone or cyclic guanidine functionalities. The availability of crystal structures of Chk2 in complex with this series of compounds suggests several areas that may be modified to improve inhibitor design. The urea carbonyl oxygen, which is conserved throughout this series of inhibitors, appears to play an important role by participation in water-mediated hydrogen bonds to the hinge region. Indeed, it has been shown in prior NSC 109555 analogs that replacement of the urea with a thiourea functionality results in a poorly active compound (Jobson et al., 2007). By retaining this urea linker and examining additional functional replacements in the hinge region, it may be possible to create new binding interactions with the hinge region. The hydrophobic pocket near the methyl group adjacent to the guanidine head group is also of significant interest because it provides the opportunity to add new chemical entities to the methyl group that may occupy this pocket and therefore gain binding affinity while retaining selectivity for Chk2. Finally, additional work needs to be done to optimize the guanidine head group since the guanlylhydrazone functionality may exhibit unfavorable pharmacokinetic properties. As this group is involved in important binding interactions with Glu273, it is desirable to find a functional replacement that retains this interaction but that also imparts more favorable drug-like properties to the inhibitors. Indeed, elimination of the guanidine head group results in significant decrease in the potency of the compounds (data not shown). With the current crystal structures in hand, it is now possible to apply a rational, structure-based approach to achieve these goals.

At the same time, it will be important to evaluate the pharmacological properties of these inhibitors in cell culture (Jobson et al., 2009), to investigate their antiproliferative effects as single agents in cancer cells with endogenous Chk2 activation (Zoppoli et al., 2011; Jobson et al., 2009), and to demonstrate their synergism with DNA damaging agents in normal and p53-dependent cells (Garrett and Collins, 2011). The Chk2 inhibitor PV1019 meets all of

these criteria (Jobson et al., 2009). Similar studies are currently underway with the inhibitors described here.

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Abbreviations

ADME	adsorption, distribution, metabolism and elimination
ADP	adenosine diphosphatase
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad3 related
BRCA1	breast cancer 1
Cdc25a	cell division cycle 25 homolog a
Cdc25c	cell division cycle 25 homolog c
Chk1	checkpoint kinase 1
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA protein kinase
E2F1	E2F transcription factor 1
FOXM1	forkhead box protein M1-like
hMPs1	mitotic checkpoint kinase
IC₅₀	half maximal inhibitory concentration
p53	p53 tumor suppressor
PARP	poly-(ADP-ribose) polymerase
PML	promyelocytic leukemia
RSK2	N-terminal kinase domain of p90 ribosomal S6 kinase 2

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Fig. 1. Chemical structures of the Chk2 inhibitors used in this study A) NSC 109555, B) PV1019, C) PV1115, D) PV976, E) PV788, F) PV1533, G) PV1531, H) compound 53, I) compound 63.

Fig. 2.
Three-dimensional structure of the Chk2 catalytic domain (blue) in complex with the small molecule inhibitor PV1115 (red spheres).

Fig. 3.

A) The binding mode of PV1115 in the Chk2 ATP-binding pocket superimposed onto the F_o-F_c OMIT map (wheat, 2.05 Å resolution, contoured at 3σ). The enzyme residues are depicted in green stick form whereas the inhibitor is in gray stick form. Nitrogen atoms are blue and oxygen atoms are red. The blue dashed lines represent hydrogen bonds. B) LIGPLOT diagram of the inhibitor interactions with Chk2. The dashed half-circles represent residues involved in hydrophobic interactions with the inhibitor and the green dashed lines represent hydrogen bonds. The residues involved in hydrogen bonding interactions are illustrated in ball-and-stick format and colored as follows: carbon (black), nitrogen (blue), oxygen (red), and sulfur (yellow). Water molecules are depicted as blue spheres. C) A view of the Chk2 surface (gray) highlighting the binding mode of the PV1115 inhibitor (yellow stick) and the location of the hydrophobic pocket.

Fig. 4.

Structural comparison of the Chk2 and Chk1 ATP binding pockets. a) The AX-ray crystal structure of the Chk2-PV1115 complex highlighting the binding mode of the inhibitor near the kinase hinge. B) The modeled position of PV1115 in the ATP binding pocket of Chk1 based on superposition of the coordinates of the Chk2-PV1115 complex onto those of apo-Chk2 (PDB code: 1IA8), highlighting a potential steric clash with the Chk1 hinge. C) A view of the interactions of PV1115 near residue Glu273 in Chk2. D) The same view of Chk1 with PV1115 modeled in the binding pocket, highlighting potential steric clashes.

Fig. 5.

A) A view of the residues lining the hydrophobic pocket in Chk2 and Chk1 and its location near the bound PV1115 inhibitor (yellow sticks). The gray stick residues correspond to the Chk2-PV1115 complex and the cyan-stick residues correspond to the Chk1 residues. The figure was prepared by superimposing the coordinates of Chk1 (PDB code: 1IA8) onto the coordinates of the Chk2-PV1115 complex. B) Sequence alignment of residues that form the hydrophobic pocket region in other kinases. The kinases compared were as follows: Chk2 (checkpoint kinase 2), Chk1 (checkpoint kinase 1), AURORA (aurora kinase), ERK (extracellular regulated MAP kinase), AKT1 (v-akt murine thymoma viral oncogene homolog 1), PIM1 (proto-oncogene PIM1 serine/threonine kinase), 1K3A (insulin-like growth factor 1 receptor kinase).

Fig. 6. Schematic illustration of PV976 (A) and PV788 (B) in the ATP-binding pocket of Chk2. The enzyme residues are illustrated in green stick format and the inhibitor residues in gray stick format. Nitrogen, oxygen and sulfur atoms are colored blue, red and yellow, respectively. Water molecules are depicted by red spheres and blue dashed lines represent hydrogen bonds. The σ_A -weighted $2mF_o - DF_c$ electron density maps (blue) for the inhibitors in the (C) Chk2-PV976 complex (2.2 Å resolution) and (D) Chk2-PV788 complex (2.35 Å resolution) are contoured at 1.0σ .

Fig. 7.

A) The binding mode of PV1531 in the ATP-binding pocket of Chk2. The enzyme and inhibitor atoms and the interactions between them are as depicted in Fig. 6A, B) LIGPLOT diagram of the hydrogen bonding interactions between PV1531 and Chk2, with atoms and interactions as depicted in Fig. 3B, C) The binding mode of PV1533 in the ATP-binding pocket of Chk2. D) LIGPLOT diagram of the hydrogen bonding and hydrophobic contacts between PV1533 and the ATP-binding pocket of Chk2.

Fig. 8.

A) A comparison of the conformations of the Chk2 inhibitors PV1115 (blue stick), compound 53 (wheat stick), and compound 63 (green stick) in the ATP-binding pocket. B) Major interactions of the inhibitors PV1115 (blue), PV976 (yellow), PV788 (cyan), PV1531 (red), and PV1533 (green) in the Chk2 ATP-binding pocket.

Table 1IC₅₀ (nM) values for inhibitors

Inhibitor	Chk2 Mean $\pm$ SD (n=3–4)	Chk1	RSK2
PV1115	0.14 $\pm$ 0.02	66000	>100000
PV976	69.60 $\pm$ 40.22	>100000	>100000
PV788	1.36 $\pm$ 0.71	>100000	>100000
PV1531	0.03 $\pm$ 0.03	86000	>100000
PV1533	0.07 $\pm$ 0.03	>100000	47000

Table 2

Data collection and refinement statistics

Chk2 inhibitor	PV1115	PV976	PV788	PV1531	PV1533
<i>Data Collection</i>					
Space group	P3 ₂ 21	P3 ₂ 21	P3 ₂ 21	P3 ₂ 21	P3 ₂ 21
Unit cell <i>a=b, c</i> (Å)	91.0, 93.3	90.8, 93.4	91.2, 92.7	90.5, 93.6	90.9, 93.4
Resolution (Å) ^a	50-2.05 (2.1-2.05)	50-2.2 (2.28-2.2)	50-2.35 (2.43-2.35)	50-1.77 (1.82-1.77)	50-2.35 (2.43-2.35)
Total/Unique Reflections	82448/26596	169677/22945	133926/19012	297679/44051	118737/18895
Completeness (%)	93.2 (95.9)	99.6 (99.5)	99.9 (100)	99.4 (99.8)	99.5 (99.9)
Redundancy	3.1 (3.0)	7.4 (7.4)	7.0 (7.0)	6.8 (5.9)	6.3 (5.4)
<i>I</i> / σ (<i>I</i>)	28.3 (2.6)	47.7 (4.6)	41.7 (4.9)	48.3 (2.9)	10.4 (2.2)
<i>R</i> _{Sym}	0.055 (0.401)	0.066 (0.510)	0.069 (0.452)	0.057 (0.660)	0.116 (0.663)
<i>Refinement</i>					
Resolution (Å)	50-2.05	50-2.20	50-2.35	50-1.77	50-2.35
No. of reflections (refinement/ <i>R</i> _{free})	24780/1330	21747/1178	18003/980	41231/2183	17840/974
<i>R</i> / <i>R</i> _{free}	0.209/0.247	0.200/0.233	0.200/0.239	0.196/0.218	0.200/0.249
No. of atoms/ Mean B factor (Å)					
Protein	2280/46.4	2258/45.9	2255/47.1	2350/36.5	2264/45.0
Inhibitor	30/42.4	34/67.2	36/44.2	32/36.3	29/47.8
Water	163/38.8	145/36.9	123/32.6	259/42.3	72/45.2
Ion	4/47.1	4/46.5	4/32.6	5/34.4	-/-
Rms from ideal					
Bond lengths (Å)	0.013	0.013	0.015	0.011	0.015
Bond angles (°)	1.3	1.4	1.5	1.2	1.4
Ramachandran plot					
Most favored (%)	90.2	92.9	90.0	93.0	90.9
Additionally allowed (%)	8.6	6.0	8.4	6.2	8.7
Generously allowed (%)	0.8	0.8	1.2	0.4	0.0
Disallowed (%)	0.4 (Ala507)	0.4 (Thr506)	0.4 (Thr432)	0.4 (Thr506)	0.4 (Glu295)

$$C_R = \Sigma |F_{Obs}(hkl) - F_{Calc}(hkl)| / \Sigma |F_{Obs}(hkl)|. R_{free} \text{ is the R value calculated for 5\% of the data set not included in the refinement.}$$