Crystal Structures of Two Aminoglycoside Kinases Bound with a Eukaryotic Protein Kinase Inhibitor

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Abstract

Antibiotic resistance is recognized as a growing healthcare problem. To address this issue, one strategy is to thwart the causal mechanism using an adjuvant in partner with the antibiotic. Aminoglycosides are a class of clinically important antibiotics used for the treatment of serious infections. Their usefulness has been compromised predominantly due to drug inactivation by aminoglycoside-modifying enzymes, such as aminoglycoside phosphotransferases or kinases. These kinases are structurally homologous to eukaryotic Ser/Thr and Tyr protein kinases and it has been shown that some can be inhibited by select protein kinase inhibitors. The aminoglycoside kinase, APH(3’)-IIIa, can be inhibited by CKI-7, an ATP-competitive inhibitor for the casein kinase 1. We have determined that CKI-7 is also a moderate inhibitor for the atypical APH(9)-la. Here we present the crystal structures of CKI-7-bound APH(3’)-IIIa and APH(9)-la, the first structures of a eukaryotic protein kinase inhibitor in complex with bacterial kinases. CKI-7 binds to the nucleotide-binding pocket of the enzymes and its binding alters the conformation of the nucleotide-binding loop, the segment homologous to the glycine-rich loop in eukaryotic protein kinases. Comparison of these structures with the CKI-7-bound casein kinase 1 reveals features in the binding pockets that are distinct in the bacterial kinases and could be exploited for the design of a bacterial kinase specific inhibitor. Our results provide evidence that an inhibitor for a subset of APHs can be developed in order to curtail resistance to aminoglycosides.

Introduction

The waning prospect of an effective treatment for bacterial infections due to the emergence and spread of resistance to antibiotics in pathogens has been exacerbated by the lack of novel antibacterials being introduced to the market [1]. An alternative and parallel approach in supporting the mitigation of the antibiotic resistance problem is to develop adjuvants that could interfere with the mechanism of resistance and hence restore the action of antibiotics [2]. Such a strategy has been effectively employed to combat resistance to β-lactams due to β-lactamase activity [3]. For aminoglycosides, a group of antibiotics used to treat serious nosocomial infections, the main mechanism of resistance is via the enzymatic inactivation of the drug by acetyltransferases, nucleotidylyltransferases, or phosphotransferases [4]. This implies that inhibitors of these enzymes could be exploited for the development of drug-adjuvant therapy [5,6]. Among the three types of aminoglycoside-modifying enzymes, aminoglycoside phosphotransferases or kinases (APHs) yield the highest levels of resistance thereby providing a rationale for focusing inhibitor development for these specific resistance factors [7].

The investigation of APH inhibitors that target the ATP-binding pocket was facilitated by the structural similarities between the aminoglycoside resistance enzyme APH(3’)-IIIa and serine/threonine and tyrosine eukaryotic protein kinases (ePKs), especially in the N-terminal lobe [8] (Figure 1A,C). It was subsequently shown that APH(3’)-IIIa can be inhibited by protein kinase inhibitors of the isoquinolinesulfonamide family and they are competitive with ATP-binding [9]. For example, the protein kinase inhibitor N-(2-aminoethyl)-3-chloro-isquinoline-8-sulfonamide (CKI-7) (Figure 1D) has an inhibition constant of 65 μM for APH(3’)-IIIa. Unfortunately, these compounds are only able to inhibit the resistance enzymes in vitro and cannot rescue the function of aminoglycosides in enterococcal strains harboring the aph(3’)-IIIa gene [9]. Nonetheless, this study identified lead compounds for adjuvant development aimed at reversing APH-mediated resistance to aminoglycosides.

X-ray structures of several members in the APH family have since been determined [8,10,11,12,13,14]. However, APH(3’)-IIIa remains the most extensively studied due to its broad substrate spectrum [9,15,16,17,18,19]. The crystal structure of APH(3’)-IIIa in the apo, ADP- or AMP-PNP-bound forms [8,20], as well as its ternary complex of three structurally dissimilar aminoglycosides...
Inhibition of APHs by CKI-7

Results and Discussion

The APH(3\textsuperscript{9})-Ia and APH(9)-Ia complexes with the nucleotide-bound APH(3\textsuperscript{9})-Ia ([20]). For APH(9)-Ia, this same loop (residues 28–34) also adopts a flexible conformation observed in the apo state of APH(3\textsuperscript{9})-Ia, i.e. the loop of APH(9)-Ia also dips into the phosphate-binding area of the nucleotide binding pocket.

Inhibitor Binding Site

As expected, for both APH(3\textsuperscript{9})-IIa and APH(9)-Ia, the ATP-competitive inhibitor CKI-7 occupies the nucleotide-binding pocket, between the N- and C-terminal lobes. The binding of the inhibitor did not alter the main or side chain conformation of any residues lining the binding pocket in either enzyme, except for the APH homolog of the glycine-rich loop mentioned above. The isoquinoline ring of the inhibitor is buried in the hydrophobic adenine-binding cleft (Figure 2A,B) and its position and orientation mimics that of the adenine ring of the nucleotide (Figure 2C,D). A principal contact between the isoquinoline ring and APH adenine ring with APH is the stacking interactions conferred by the aromatic ring side chain of Tyr42 in APH(3\textsuperscript{9})-Ia, is also affected by this protein kinase inhibitor. Paralleling the APH(3\textsuperscript{9})-IIa result, CKI-7 was found to inhibit APH(9)-Ia ($K_i = 159\pm 11 \mu M$) in a competitive fashion with respect to ATP, although 2.5 times less effectively. These results suggest that the CKI-7 scaffold may be exploited for the development of broad-spectrum APH inhibitors. This possibility is further reinforced by the observation that a third APH enzyme, APH(2\textsuperscript{9})-Ia is similarly inhibited by this compound ($K_i = 87.1\pm 17.8 \mu M$) [9]. However, the inhibition constants for CKI-7 are high, especially when compared to that obtained for the ePK CK1, which is an order of magnitude lower ($K_i = 8.5 \mu M$) [22]. Nonetheless, at present CKI-7 is the only compound identified that is able to inhibit the ATP-binding site of several APH enzymes.

Crystal structures of APH+CKI-7 complexes

To further examine the suitability of the CKI-7 scaffold for inhibitor development, we determined the crystal structures of APH(3\textsuperscript{9})-IIa and APH(9)-Ia in complex with CKI-7 (Figure 1A,B). The APH(3\textsuperscript{9})-IIa-CKI-7 inhibitor complex was crystallized in the space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} with two inhibitor-bound enzyme molecules in the asymmetric unit, analogous to the nucleotide-bound enzyme complexes [8,20]. The structure has been refined to 2.15 Å with an $R_{	ext{cryst}}$ of 0.189 and $R_{	ext{free}}$ of 0.235. The CKI-7-bound APH(9)-Ia complex was crystallized in space group P3\textsubscript{1}2\textsubscript{1}1 with one molecule per asymmetric unit. This structure has been refined to 2.9 Å and a final $R_{	ext{cryst}}$ and $R_{	ext{free}}$ of 0.222 and 0.279, respectively. Comparison of these crystal structures with the apo, nucleotide-bound, and aminoglycoside and nucleotide-bound states, available for both APH(3\textsuperscript{9})-IIa and APH(9)-Ia, reveals that the CKI-7-bound enzyme conformations most closely resemble that of the binary nucleotide-bound states. This is to be expected, as CKI-7 is competitive with ATP. However, some differences can also be noted between the APH-CKI-7 bound states and that of the APH-nucleotide bound states, most notably in the region designated as the glycine-rich loop in ePKs. In APH(3\textsuperscript{9})-IIa the homolog of the glycine-rich loop are residues 21-27 (Figure 2C). In the nucleotide-bound enzyme structures, the loop is positioned above the phosphate moieties of the nucleotide [20], whereas, in the APH(3\textsuperscript{9})-IIa-CKI-7 structure, the tip of the loop points back into the phosphate-binding area, delimiting the nucleotide-binding pocket, reminiscent of what is seen in the apo state of APH(3\textsuperscript{9})-IIa [20]. For APH(9)-Ia, this same loop (residues 28-34) also adopts a different conformation in the APH(9)-Ia-CKI-7 structure compared to the nucleotide-bound state (Figure 2D). In fact, the conformation observed is comparable to what is observed for APH(3\textsuperscript{9})-IIa, i.e. the loop of APH(9)-Ia also dips into the phosphate-binding area of the nucleotide binding pocket.
either a tyrosine or phenylalanine among all APH(3'), APH(9), as well as APH(30) enzymes, and shown to be important for binding and catalysis [20]. Intriguingly, despite the conserved nature of this residue, the aromatic side chain of Tyr42 of APH(3')-IIIa points toward the linker region of the protein whereas the side chain of Phe50 in APH(9)-Ia points in the opposite direction toward the ribose- and phosphate-binding pockets (Figure 3A). Accordingly, the adenine ring of the bound nucleotides adopt distinct orientations, differing by a rotation of approximately 40° [11]. The corresponding 40° difference is also observed between the isoquinoline rings of CKI-7 bound to APH(3')-IIIa or APH(9)-Ia (Figure 3A).

The linker region between the N- and C-terminal lobes of the APH enzymes plays an important role in the binding of the isoquinoline or adenine ring. The N1 and N6 of the adenine ring form hydrogen bond interactions with the main chain amide of Ala93 (APH(3')-IIIa numbering) and carbonyl of Ser91, respectively [8]. Although the sole cyclic nitrogen in the isoquinoline, N2, and the adenine, N1, are located in different positions of the ring structure, CKI-7 is positioned in such a way that N2 of the isoquinoline overlays with N1 of the adenine ring in the nucleotide. Consequently, an interaction analogous to that between N1 of the adenine and the linker amide is observed between N2 of the isoquinoline and the amide of Ala93 in APH(3')-IIIa or Ile103 in APH(9)-Ia (Figure 3A). The CKI-7-binding sites are delineated by surface representation in their respective enzymes. CKI-7 binds to the same region and in an analogous manner as the nucleotide to each of the enzyme. The phosphates of the nucleotide are buried in the surface representing the homologous gly-rich loop in the presence of CKI-7.

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Figure 2. Nucleotide/inhibitor binding sites of APH(3')-IIIa and APH(9)-Ia. The CKI-7- and nucleotide-bound APH(3')-IIIa are shown in cartoon representation in panels A and C respectively. The CKI-7- and nucleotide-bound APH(9)-Ia are shown in cartoon representation in panels B and D, respectively. (A) CKI-7 bound to APH(3')-IIIa in magenta sticks and its simulated annealing F o-Fc omit map, contoured at 2.5σ, are shown. Tyr42, which forms stacking interactions with the isoquinoline, and Ala93, which hydrogen bonds with the inhibitor, are shown in sticks. (B) CKI-7 bound to APH(9)-Ia in dark blue sticks and its simulated annealing F o-Fc omit map, contoured at 2.5σ, are shown. Phe50 whose aromatic ring stacks with the isoquinoline, and Ile103, which forms hydrogen bond interactions with the inhibitor, are shown in sticks. (C,D) Comparison of the inhibitor- and nucleotide-bound APHs. The homologous ePK gly-rich loop is highlighted in green for nucleotide-bound APH(3')-IIIa (C) and red for APH(9)-Ia (D). Tyr42 in APH(3')-IIIa and Phe50 in APH(9)-Ia, that stacks the adenine ring of the nucleotide are shown in sticks and their carbon atoms are colored green for APH(3')-IIIa (C) and red for APH(9)-Ia (D). The CKI-7-binding sites are delineated by surface representation in their respective enzymes. CKI-7 binds to APH(3')-IIIa and APH(9)-Ia are colored as in panels A and B. CKI-7 binds to the same region and in an analogous manner as the nucleotide to each of the enzyme. The phosphates of the nucleotide are buried in the surface representing the homologous gly-rich loop in the presence of CKI-7.
two APH enzymes. In APH(3')-IIIa, the aminoethyl-amide adopts an extended conformation and it is situated just beyond the ribose-binding area, toward the solvent exposed opening of the ATP-binding pocket (Figure 2A and 3A). Alternatively, using the terminology of the different compartments in the ATP-binding site of ePK, the aminoethyl-sulfonamide lies adjacent to the ribose-binding pocket, bordering the specificity surface [23] or the entrance pocket [24]. This portion of the inhibitor is more flexible than the isoquinoline ring as reflected by the relatively higher thermal factors. Two hydrogen bonds are observed between this section of the CKI-7 and the APH(3')-IIIa (Figure 2A). One of which is found between one of the oxygen atoms of the sulfonamide group (O2S) and the hydroxyl group of Tyr42. The second hydrogen bond is formed between the terminal nitrogen of the aminoethyl tail, N2', and the main chain carbonyl of Ser194. An analogous interaction is observed in the APH(3')-IIIa-nucleotide complex between the carbonyl of Ser194 and the O3' of the ribose, which approximately overlaps the N2' of CKI-7. In APH(9)-Ia, the aminoethyl group of the inhibitor is positioned in the entrance pocket [24] or the solvent-exposed side of the adenine-binding pocket and it points back at itself forming an intramolecular interaction with the equatorial sulfonamide oxygen atom (O2S) (Figure 2B,3A). The inhibitor also makes a second interaction with the linker region of APH(9)-Ia via the N3 of the aminoethyl tail and the main chain carbonyl of Ile103. No interactions, direct or water-mediated, are observed between the linker of APH(3')-IIIa and the aminoethyl of CKI-7 since the linker is one residue longer in APH(3')-IIIa and situated over 7 Å away from the binding pocket compared to the equivalent in APH(9)-Ia (Figure 3A).

Comparison of CKI-7-bound APHs and casein kinase 1

The crystal structure CK1 in complex with CKI-7 has been determined [25] (Figure 1C). Here, CKI-7 also occupies the ATP-binding cleft of CK1, and the overall structures of the inhibitor-nucleotide-bound CK1 are the same, differing slightly in the glycine-rich loop, analogous to what is observed for APH(3')-IIIa and APH(9)-Ia. The isoquinoline ring of the inhibitor is coplanar with the adenine moiety of ATP and the aminoethyl-sulfonamide points away from the ribose toward the solvent accessible opening of the binding pocket. When the CKI-7-bound structures of APH(3')-IIIa, APH(9)-Ia, and CK1 are superposed using the coordinates of conserved active site residues, it is apparent that the plane of the isoquinoline ring in the CK1 structure differs from that observed in APH(3')-IIIa by a rotation of approximately 40° (Figure 3B) but is nearly coplanar to that in APH(9)-Ia (Figure 3C), analogous to the binding of the adenine ring in the nucleotide-bound enzymes [20]. Furthermore, the linker region of CK1, in particular the N-terminal section which form hydrogen bond contacts with the ring structure of the ligand, superpose well with that of APH(9)-Ia.

The hydrogen bond between the cyclic nitrogen and a main chain amide (Leu88 in CK1) in the linker of the enzyme is also present in the CKI-7-bound CK1 (Figure 3B,C). The equivalent hydrogen bond observed between N1 of adenine and the tethering segment in the three enzymes is conserved in all adenine-binding to ePKs [26]. This hydrogen bond is not unique to isoquinoline-sulfonamide type inhibitors binding to the three enzymes discussed here. A majority of ePK crystal structures complexed with an ATP-competitive inhibitor form at least one hydrogen bond with residues in the linker region, mimicking the ones between N1 and/or the exocyclic N6 of the adenine and the enzyme [23]. The significance of the hydrogen bond interaction is corroborated by a previous observation in which naphthalene sulfonamide molecules did not display selective inhibition against ePKs until the all-carbon napthalene ring is substituted with an isoquinoline [27].

In contrast to APH(3')-IIIa in which the aminoethyl tail adopts an extended conformation, this groups adopts the same conformation and is placed in the equivalent area as that in APH(9)-Ia. The aminoethyl tail found in the CK1 structure bends back toward the sulfonamide group and forms an intramolecular interaction between the terminal nitrogen atom and the equatorial sulfonamide oxygen atom. Deviating slightly from the binding mode of CKI-7 to APH(9)-Ia, the contact between the N3 of the aminoethyl and carbonyl of Leu88 located in the linker of the enzyme is achieved.
Implications for Inhibitor Design and Adjuvant Development

Previous studies, together with the present report, indicate that CKI-7 can be exploited as a lead compound for APH inhibitor development. Two requirements must be met, however, to make such an inhibitor potentially useful in drug-adjuvant therapy: (1) despite the structural homology between APHs and ePKs, an inhibitor should be selective for the bacterial resistance enzymes. (2) Given that resistant pathogens can harbor multiple APH enzymes, it is desirable that such an inhibitor has a broad-spectrum of activity against various APHs. It is encouraging to note that CKI-7 is only able to effectively inhibit a few ePKs [28] while it can modestly inhibit at least three different APHs. This suggests that the two requirements for an adjuvant application might be achievable. The structural data for the three kinase-CKI-7 complexes provides further information to assess feasibility.

With respect to achieving selectivity for APHs vs. ePKs, the three kinase-CKI-7 structures reveal ample differences in binding, as also reflected in the variance in the enzymes' $K_i$ values, to confidently suggest that inhibitors based on the isoquinoline scaffold can be developed for either APH(3')-IIIa or APH(9)-Ia that do not bind to CK1 and likely other ePKs. This confidence is fueled by the extensive expertise in the development of specific inhibitors to the ATP-binding pocket of ePKs, which has shown that sequence divergence in this part of the enzyme is a good predictor of whether selectivity can be obtained [24,29,30,31] The ATP-binding pocket of APH(3')-IIIa is distinct from CK1 and other ePKs as it possesses a large aromatic residue (Tyr42) that forms stacking interactions with the isoquinoline moiety. Additionally, the combination of a large nonpolar gatekeeper residue (Met90), a nonpolar residue in the entrance pocket (Leu97) and the absence of a glycine residue in the hinge region that can induce backbone conformation changes, results in a pocket that is predicted to be suitable for selective targeting. Similarly, the nucleotide-binding pocket in APH(9)-Ia shares many of these distinguishing features with APH(3')-IIIa, though it has a polar residue in the entrance pocket (Asn107), making this pocket somewhat less different from a few ePKs.

Regarding the feasibility of obtaining an inhibitor with broad-specificity against various APHs, the structural data presented here is less encouraging. The differences observed in the nucleotide-binding pocket between APH(3')-IIIa and APH(9)-Ia are similar in magnitude as those between APH(3')-IIIa and CK1. This in itself is not necessarily problematic as long as those differences do not compromise their distinctive features from ePKs. However, one of the most distinguishing features of many APHs, i.e. the aromatic residue that forms stacking interactions with the isoquinoline moiety, has differential impacts on inhibitor binding in the two APH structures studied. As a result, the orientation and conformation of CKI-7 observed in APH(9)-Ia mirrors what is seen in CK1. These observations illustrate the diversity in the architectures of the ATP-binding pocket present within the APH family of enzymes, which will undoubtedly complicate development of broad-spectrum inhibitors. On the other hand, it must be realized that the selection of APH(3')-IIIa and APH(9)-Ia for these studies impacts the conclusions with respect to the feasibility of adjuvant development. As mentioned, APH(9)-Ia is an atypical member of the APH family, and while it provides valuable insights into the extent of the diversity present in nucleotide-binding pockets of APH enzymes, it does not necessarily reflect the diversity of nucleotide-binding pockets likely encountered in clinical settings. Nonetheless, our observations lead us to contend that a universal inhibitor for all APHs targeting the nucleotide-binding pocket may not be feasible, but the contrasting details between APH(3')-IIIa, APH(9)-Ia and ePKs suggests that selective inhibitors that target a subset of APHs is attainable.
soaked briefly in a cryoprotectant solution containing 20% PEG 3350, 0.2 M calcium acetate and 1 M sodium formate. Processing of diffraction data for both crystals was performed using HKL2000 [34]. A summary of data collection and processing statistics is shown in Table 1.

Structure Determination and Refinement

Despite different crystal growth conditions, APH(3')-IIIa-CKI-7 crystal was isomorphous with APH(3')-IIIa-ADP crystals. Hence, the APH(3')-IIIa-ADP structure (PDB ID 1J7L) [8], excluding the ligand and water molecules, was used as the starting model for the refinement of APH(3')-IIIa-CKI-7 complex in the Crystallography and NMR System (CNS) program [35]. After rigid body refinement and an initial cycle of positional and grouped thermal factor refinement, one molecule of CKI-7 was modeled in each active site in the space where $S_2$-weighted difference maps ($2F_o-F_c$ and $F_o-F_c$) displayed positive electron density. The stereochemical parameters for CKI-7 used in subsequent refinement were based on the conformation of the inhibitor found in the crystal structure of casecin kinase 1 (PDB ID 2C8N) [25] in conjunction with values from the energy minimized conformation obtained from the molecular mechanics program MM2 [36,37] implemented in CambridgeSoft Chem3D software. Upon inspection, several regions required remodeling due to considerable deviations from the difference density maps. Cycles of positional and individual thermal factor refinement was alternated with manual retitling using the program O [38] and incorporation of solvent molecules. The process was continued in the programs PHENIX [39] and COOT [40] until no further improvement in model statistics could be obtained.

The structure of CKI-7-bound APH(9)-Ia was solved by molecular replacement using Phaser [41] as implemented in the CCP4 suite [42] and the apo structure of APH(9)-Ia (PDB ID 3I1A) as the starting model. A solution of the proper position and orientation of the inhibitor-bound molecule was obtained only when the search model was input as two entities – the N-terminal lobe (residues 3–199) and the C-terminal lobe (residues 200–330). Following several cycles of positional and grouped B-factor refinement, a molecule of CKI-7 was placed in the nucleotide-binding site according to the observed positive difference maps. The same stereochemical parameters used for APH(3')-IIIa-bound CKI-7 were applied to the APH(9)-Ia inhibitor for structure refinement. Iterative cycles of positional and individual thermal factor refinement using CNS are intercalated with manual adjustments along with the addition of water molecules. Several regions (residues 27–40, 53–60, 89–97, and 297–309) that display substantial difference between the model and electron density were manually rebuilt using the program O [38] according to difference density maps and simulated annealing omit maps. Final refinement statistics for the inhibitor-bound structures of both APH enzymes are summarized in Table 1.

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Author Contributions

Conceived and designed the experiments: DHF BX AMB. Performed the experiments: DHF BX JH. Analyzed the data: DHF. Wrote the paper: DHF AMB.

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