Structure-guided optimization of protein kinase inhibitors reverses aminoglycoside antibiotic resistance

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INTRODUCTION

Activity of the aminoglycoside phosphotransferase APH(3′)-Ia leads to resistance to aminoglycoside antibiotics in pathogenic Gram-negative bacteria, and contributes to the clinical obsolescence of this class of antibiotics. One strategy to rescue compromised antibiotics such as aminoglycosides is targeting the enzymes that confer resistance with small molecules. We demonstrated previously that ePK (eukaryotic protein kinase) inhibitors could inhibit APH enzymes, owing to the structural similarity between these two enzyme families. However, limited structural information of enzyme–inhibitor complexes hindered interpretation of the results. In addition, cross-reactivity of compounds between APHs and ePKs represents an obstacle to their use as aminoglycoside adjuvants to rescue aminoglycoside antibiotic activity. In the present study, we structurally and functionally characterize inhibition of APH(3′)-Ia by three diverse chemical scaffolds, anthrapyrazolone, 4-anilinoquinazoline and PP (pyrazolopyrimidine), and reveal distinctions in the binding mode of anthropyrazolone and PP compounds to APH(3′)-Ia compared with ePKs. Using this observation, we identify PP derivatives that select against ePKs, attenuate APH(3′)-Ia activity and rescue aminoglycoside antibiotic activity against a resistant Escherichia coli strain. The structures described in the present paper and the inhibition studies provide an important opportunity for structure-based design of compounds to target aminoglycoside phosphotransferases for inhibition, potentially overcoming this form of antibiotic resistance.

Key words: aminoglycoside phosphotransferase APH(3′)-Ia, antibiotic resistance, crystal structure, protein kinase inhibitor, structure-based drug design.

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INTRODUCTION

Antibiotic resistance is a serious global public health problem [1]. Resistance is an issue with all classes of currently used antibiotics, resulting in the emergence of pathogens untreatable using the available therapeutic arsenal. The genetic source of the problem is the ‘antibiotic resistome’, the reservoir of bona fide resistance genes plus genes capable of evolving into resistance-conferring function through antibiotic selection [2].

Within this context of the need for new antimicrobials, industry is increasingly retreating from this space with a resultant atrophy of discovery pipeline output [3]. New approaches to circumvent difficulties in antimicrobial discovery include ‘re-purposing’ of compounds not used previously as antimicrobials [4]. Similarly, non-antimicrobials have been shown to potentiate the activity of antibiotics, although their cellular targets may not be known [5,6]. In this approach, ideally the adjuvant compound would be targeted against the resistance mechanism, freeing the established antimicrobial to affect its cellular target.

Enzyme-mediated antibiotic resistance is especially amenable to this combinatory approach, as exemplified by the clinical application of β-lactam-β-lactamase inhibitor combinations [7]. Fitting this model, aminoglycoside antibiotics offer a class of potent antimicrobials that have lost clinical efficacy as a result of enzyme-catalysed modification [8]. Importantly, aminoglycosides are one of the few antibiotic classes that show good efficacy against Gram-negative pathogens, which can cause infections that are especially in need of new therapeutic options [9]. We are interested in investigating compounds targeted against aminoglycoside-modifying enzymes, which include AACs (aminoglycoside N-acetyltransferases), ANT(aminoglycoside nucleotidytransferases; O-nucleotidytransferases) or APHs (aminoglycoside phosphotransferases; O-phosphotransferases), also known as AKs (antibiotic kinases).

AK enzymes are one of the most common sources of aminoglycoside antibiotic resistance. They catalyse the transfer of the γ-phosphate group from ATP or GTP [10–12] in a regiospecific manner to the antibiotic substrates and thereby inactivate the drug. AK enzymes vary significantly in sequence, in substrate profile and in the modification site on the antibiotic substrate. Many studies have focused on detailed molecular characterization of AK enzymes and their interactions with aminoglycoside and nucleotide substrates [13–22]. These structural analyses demonstrated that, despite sequence variation, AK enzymes adopt a common ePK (eukaryotic protein kinase)-like fold [22]. These efforts also demonstrated that the antibiotic-binding site contains a high degree of functional and structural diversity, consistent with the chemical diversity of aminoglycoside substrates. In contrast with diversity in the structure of the antibiotic-binding site, the NTP (nucleotide triphosphate)-binding site has a higher degree of structural conservation. This site contains structural similarity with ePKs by virtue of its location at the interface between the

Abbreviations used: AK, antibiotic kinase; APH, aminoglycoside phosphotransferase; ARF, antibiotic rescue factor; CDPK1, calcium-dependent protein kinase 1; ePK, eukaryotic protein kinase; JNK, c-Jun N-terminal kinase; MIC, minimal inhibitory concentration; MR, molecular replacement; NPL, nucleotide-positioning loop; NTP, nucleotide triphosphate; PKI, protein kinase inhibitor; PP, pyrazolopyrimidine; SAD, single anomalous dispersion; SP600125, anthra[1,9-cd]pyrazol-6(2H)-one; TLS, Translation–Libration–Screw-rotation.

The structural co-ordinates reported will appear in the PDB under accession codes 4EJ7, 4FEU, 4FEV, 4FEX, 4GKH and 4GK1.

1 To whom correspondence should be addressed (email alexei.savchenko@utoronto.ca).
N- and C-terminal lobes of the bilobal fold, the NTP contacting both lobes, the presence of a short inter-domain linker sequence (also known as the hinge) and the conservation of critical residues.

The NTP-binding site of the ePK catalytic domain is a well-characterized drug target [23]. The similarity between the NTP-binding site of ePKs and AKs, along with extensive libraries of small-molecule ePK inhibitors (PKIs; protein kinase inhibitors), prompted us to test the inhibition potential of PKIs against AKs [24]. This produced a matrix of inhibitory activity of 80 chemically diverse PKIs against 14 representative AKs; the compounds spanned five orders of magnitude in affinity for APHs and the study found broad and narrow spectrum inhibitors [24]. The findings confirmed that the NTP-binding site of AKs can be exploited for inhibition and also demonstrated that PKIs are able to select for structural differences in AK enzymes. Missing from that study was significant structural analysis of the many enzyme–inhibitor pairs to rationalize the patterns of selectivity; we determined the structure of only one enzyme–general inhibitor pair [APH(2′)-I a and quercetin].

One of the AK enzymes for which multiple, diverse and specific inhibitors were identified is APH(3′)-I a. The gene encoding this enzyme (apha1) was originally found on the transposable element Tin903 in Escherichia coli [25] and is now widely distributed across Gram-negative bacterial pathogens responsible for clinical antibiotic resistance outbreaks (reviewed in [26]). The enzyme has high catalytic efficiency and activity against a broad spectrum of antibiotics [26,27]. Furthermore, APH(3′)-I a demonstrates plasticity for its nucleotide substrate and can utilize both GTP and ATP as a phosphate donor [27].

In the present study, we present the 3D structure of APH(3′)-I a and examine the structural basis of inhibition by three distinct PKI scaffolds. This analysis reveals the specific features of the enzyme–inhibitor interface that can be exploited in the development of AK-specific inhibitors. Guided by the findings in the present study, we further studied APH(3′)-Ia inhibition by the PP (pyrazolopyrimidine) scaffold, identifying variants that are inactive against ePKs. We show that these PP derivatives are capable of attenuating APH(3′)-Ia activities in vitro and efficiently rescue aminoglycoside antibiotic action against an aminoglycoside-resistant E. coli strain. These results strengthen the possibility of re-purposing PKI molecules and combining them with aminoglycosides as a strategy to overcome this type of antibiotic resistance.

**EXPERIMENTAL**

**Protein expression and purification**

APH(3′)-I a was purified as described previously for APH(4)-I a [14].

**Crystallization and structure determination**

APH(3′)-Ia · Ca²⁺ · ATP complex crystals were grown at room temperature (23 °C) using hanging-drop vapour diffusion by mixing 14 mg/ml protein with reservoir solution containing 0.1 M calcium acetate, 20% PEG-3350 and 2 mM ATP. Working inhibitor solutions were prepared by dissolving inhibitor stock solutions (in 100% DMSO) into the following buffer: 0.6 M NaCl, 20 mM sodium malonate (pH 7), 2.5 mM MgCl₂, 0.5 mM CaCl₂ and 0.5 mM TCEP [tris-(2-carboxyethyl)phosphine], such that final DMSO concentration was between 2 and 5% and final inhibitor concentration was between 0.05 and 0.3 mM (final concentration of compounds could only be estimated as volume was adjusted to maintain solubility). Working inhibitor solutions were mixed with 0.5–2 mM kanamycin A in water, 4–8 mg of protein dissolved in the above buffer and incubated for 1.5–2 h at 4 °C. The mixtures were concentrated to a final protein concentration of no less than 15 mg/ml, and final inhibitor concentrations between 1 and 6 mM were then centrifuged to remove insoluble components. Hanging drops were set up at room temperature and reservoir solutions that resulted in ternary complex crystals each contained 0.1 M sodium acetate (pH 4.5) plus the following to yield their respective complexes: SP600125 {anthra[1,9-cd]pyrazol-6(2H)-one} (8%) PEG-3350, 0.2 M NDSB-221; tyrphostin AG 1478 [N-(3-cholorophenyl)-6,7-dimethoxyquinazolin-4-amine] (14%) PEG-3350, 0.3 M NDSB-221; PP1 {1-t-butyl-3-(4′-methylphenyl)pyrazolo[3,4-d]pyrimidin-4-amine} (18%) PEG-3350; PP2 {1-t-butyl-3-(4′-chlorophenyl)pyrazolo[3,4-d]pyrimidin-4-amine} (14%) PEG-3350; 1-NA-PP1 (1-t-butyl-3-naphthalen-2-yl-1H-pyrazolo[3,4-d]pyrimidin-4-amine) (7%) PEG-3350; and 1-NM-PP1 {1-(1-methylethyl)-3-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine} (8%) PEG-3350. All crystals were cryo-protected with paratone oil before shipment for diffraction data collection. PKIs were purchased from Cayman Chemical.

**X-ray diffraction data collection**

Diffraction data for the APH(3′)-I a · ATP complex was collected at 100 K, selenomethionine peak absorption wavelength 0.97940 Å (1 Å = 0.1 nm), at beamline 19ID at the Structural Biology Centre, Advanced Photon Source. Diffraction data for each ternary complex were collected at 100 K, selenomethionine peak absorption wavelength 0.97856 Å, at beamlines 21-ID-F or 21-ID-G at Life Sciences Collaborative Access Team, Advanced Photon Source. All diffraction data were reduced with HKL-3000 [28], except for APH(3′)-I a · kanamycin · 1-NA-PP1 and 1-NM-PP1 ternary complexes, which were reduced with XDS [29] and Scala [30].

**Structure determination and refinement**

The structure of the APH(3′)-Ia · Ca²⁺ · ATP complex was determined by SAD (single anomalous dispersion) using HKL-3000. Matthew’s coefficient calculation suggested three copies in the asymmetric unit, and 21 total selenomethionine sites; 18 were located. Initial model building and refinement was performed with ARP/wARP [31] and Refmac [32], with later stages of refinement with PHENIX [33]. TLS (Translation–Libration–Screw-rotation) parameterization groups were residues 1–24, 25–103 and 104–271 for each chain, as determined by the TLSMD server [34]. ATP, Ca²⁺ and solvent molecules were built into positive o–Fc density. These results strengthen the possibility of re-purposing PKI molecules and combining them with aminoglycosides as a strategy to overcome this type of antibiotic resistance.

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into residual positive $F_c - F_e$ density. Occupancies of PP1, PP2, tyrophostin AG 1478, 1-NA-PP1 and 1-NM-PP1 molecules were refined while occupancies of SP600125 molecules were fixed at 1.0. The geometry of all structures were verified using PHENIX and the RCSB PDB Validation Server. Ramachandran statistics were verified: all residues fit favoured regions.

**Structural analysis**

Structure alignments were performed with PyMOL (http://www.pymol.org) or Coot. Structure images produced with PyMOL.

The following structures of ePK–anthrapyrazolone complexes were used for comparisons: PDB codes 1UK1, 1PMV and 2ZMD. Structures of ePK-4-anilinoquinazoline complexes used for comparisons were: PDB codes 3NYV, 1D18, 1D19, 1M17, 1XKK and 2ITY. The following structures of ePK–PP complexes were used for comparisons: PDB codes 1QCF, 2IVV, 2V4L, 3EN4, 3EN5, 3EN6, 3EN7, 3ENE, 2WEI, 317B, 317C, 3EL8, 1QPE, 2ZV9, 3EGQ, 3NCG, 3MWU, 3N51, 3MA6, 3NZS, 3NZU, 2WXK and 2WXM. Modelling of 1-NA-PP1/1-NM-PP1 into ePK nucleotide-binding sites as performed by manually aligning the purine cores of these compounds with those of the PP inhibitors in the structures of *Homo sapiens* Hck bound to PP1, PDB code 1QCF [37] and *H. sapiens* PI3K (phosphoinositide 3-kinase) bound to compound S2, PDB code 3ENE [38], which were bound to the ePK enzymes in the ‘C3 up’ conformation, followed by flipping 1-NA-PP1/1-NM-PP1 180° around the plane of the purine core such that they conform to the ‘C3 down’ conformation in the active site. Amino acids from the ePK enzymes that overlapped in space with 1-NA-PP1/1-NM-PP1 were considered to be sterically clashing residues. The following structures of PP compounds with large C3 substituents bound to protozoan kinases were used for comparisons: PDB codes 2WEI, 317B, 317C, 3MA6, 3MWU, 3N51 and 3NCG. Shape complementarity was calculated using Sc [39]. Buried surface area was calculated by PDBePISA server [40].

**Chemical synthesis**

Compounds NT6-66 and NT6-67 were synthesized according to the scheme in Figure 5(C) and characterized by NMR and MS according to [41].

**Antibiotic rescue factor determinations**

Growth curves were measured with *E. coli* ΔtolC ΔbamB containing pET22-aphA1 or pGPD4-aac(3)-Ia (pBR322 backbone with lacI promoter) set up according to the CLSI broth microdilution method (CLSI document M7-A5). Inhibitors were dissolved in DMSO, diluted and added at a final concentration of 5% (v/v) while aminoglycoside concentration was held constant at one-quarter of the MIC (minimal inhibitory concentration) (32 μg/ml). Plates were incubated at 37°C with shaking in a Tecan Sunrise™ plate reader over a 24-h period. Growth was monitored every 15 min by $D_{600}$ measurements and corrected for background (no growth control). ARFs (antibiotic rescue factors) were calculated with kanamycin at one-quarter of the MIC at 20 h incubation using the following formula [24]:

$$A - D - [(A - B) + (A - C)]$$

Where $A$ = growth control, $B$ = no aminoglycoside control, $C$ = no APH(3′)-Ia inhibitor control, $D$ = growth in the presence of aminoglycoside and APH(3′)-Ia inhibitor.

**Inhibition kinetics**

The *in vitro* activity of APH(3′)-Ia was monitored by coupling the release of GDP with pyruvate kinase/lactate dehydrogenase as described previously [24]. IC$_{50}$ values were determined in duplicate and inhibitors added at a final concentration of 2% (v/v) DMSO. $K_i$ values were determined from calculated IC$_{50}$ values [42]. All inhibitors were competitive, as indicated by reversible inhibition behaviour (dose dependence, slope factor ~ 1) as shown previously for PP1, PP2, SP600125, tyrophostin AG 1478, and further identified for 1-NA-PP1 and 1-NM-PP1.

**Accession numbers**

Co-ordinates and structure factors have been deposited in the Protein Data Bank with the following accession numbers: PDB 4EJ7 [APH(3′)-Ia·Ca$^{2+}$·ATP complex]; PDB 4FEU [APH(3′)-Ia·kanamycin·SP600125 complex]; PDB 4FEV [APH(3′)-Ia·kanamycin·PP1 complex]; PDB 4FEW [APH(3′)-Ia·kanamycin·PP2 complex]; PDB 4FEX [APH(3′)-Ia·kanamycin·tyrophostin AG 1478 complex]; PDB 4GKH [APH(3′)-Ia·kanamycin·1-NA-PP1 complex]; and PDB 4GKI [APH(3′)-Ia·kanamycin·1-NM-PP1 complex].

**RESULTS AND DISCUSSION**

**Structural analysis of the APH(3′)-Ia·ATP complex**

In order to gain a molecular understanding of the active site of APH(3′)-Ia, we first determined the structure of this enzyme in complex with a nucleoside triphosphate. In our activity assays, the $k_{cat}$ for modification of kanamycin by APH(3′)-Ia using GTP is 10-fold higher than with ATP (T. Shaka, P. Spanogiannopoulos and G. Wright, unpublished work), indicating that GTP is a superior substrate. However, we could obtain crystals only of the APH(3′)-Ia·ATP complex, and this structure was determined by SAD phasing using crystals grown from selenomethionine-derivatized protein (Table 1). In this structure the APH(3′)-Ia protein adopted the typical bi-lobe ePK-like fold that is distinctive of all previously characterized AKs [13–22]. The nucleotide-binding sites located at the hinge connecting the two lobes contained unambiguous additional density corresponding to the ATP molecule (Figure 1A). The three ATP molecules in the asymmetric unit were identical in conformation except for a slight rotation of the terminal phosphate about the Py–O3B bond. Each ATP molecule adopted a bidentate chelating conformation with respect to two ions, which were modelled as Ca$^{2+}$ due to the prevalence of these ions in characterizations of this enzyme [17,21]. The nucleotide-binding sites located at the hinge connecting the two lobes contained unambiguous additional density corresponding to the ATP molecule (Figure 1A). The three ATP molecules in the asymmetric unit were identical in conformation except for a slight rotation of the terminal phosphate about the Py–O3B bond. Each ATP molecule adopted a bidentate chelating conformation with respect to two ions, which were modelled as Ca$^{2+}$ due to the prevalence of these ions in characterizations of this enzyme [17,21]. The nucleotide-binding sites located at the hinge connecting the two lobes contained unambiguous additional density corresponding to the ATP molecule (Figure 1A). The three ATP molecules in the asymmetric unit were identical in conformation except for a slight rotation of the terminal phosphate about the Py–O3B bond. Each ATP molecule adopted a bidentate chelating conformation with respect to two ions, which were modelled as Ca$^{2+}$ due to the prevalence of these ions in characterizations of this enzyme [17,21]. The nucleotide-binding sites located at the hinge connecting the two lobes contained unambiguous additional density corresponding to the ATP molecule (Figure 1A). The three ATP molecules in the asymmetric unit were identical in conformation except for a slight rotation of the terminal phosphate about the Py–O3B bond. Each ATP molecule adopted a bidentate chelating conformation with respect to two ions, which were modelled as Ca$^{2+}$ due to the prevalence of these ions in characterizations of this enzyme [17,21].

The adenine moiety of ATP occupied a hydrophobic pocket formed by Phe$^{51}$, Ile$^{102}$ and Ile$^{110}$ and establishes a number of hydrogen bonds with backbone atoms of the hinge, specifically between N6 and the carbonyl oxygen of Thr$^{86}$, between N1 and the amide nitrogen of Ile$^{101}$, and a weaker bond between the C2–H and the carbonyl oxygen of Ile$^{101}$ (Figure 1B). Overall, the binding mode of ATP to APH(3′)-Ia was comparable with those of adenosine-based nucleoside phosphates or non-hydrolysable analogues in complex with other APH structures. Given that ATP is also a substrate of this enzyme and that the adenine and guanine bases have been shown to form distinct interactions with the hinge region of one APH enzyme [12], it should be noted that the

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Table 1  X-ray diffraction data collection and refinement statistics

\[
R_{sym} = \frac{\sum_i |I_i(h) - \langle I(h) \rangle| / \sum_i |I_i(h)|}{\sum_i |I_i(h)| / \sum_i |F_{calc}(h)|},
\]

where \(I_i(h)\) and \(\langle I(h) \rangle\) are the ith and mean measurement of the intensity of reflection \(h\). Figures in parentheses indicate the values for the outer shells of the data. R-factor = \(\frac{\sum |F_{obs}(h) - F_{calc}(h)| / \sum F_{obs}(h)}{\sum F_{obs}(h)}\), where \(F_{obs}(h)\) and \(F_{calc}(h)\) are the observed and calculated structure factor amplitudes respectively. N/A, not applicable.

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Figure 1  Overview of APH(3′)-Ia structures

(A) Structure of the APH(3′)-Ia · Ca\(^{2+}\) · ATP complex. APH(3′)-Ia hinge region is coloured green, NPL is coloured blue and Ca\(^{2+}\) ions are shown in black spheres. N- and C-termini are labelled. (B) Interactions between APH(3′)-Ia and ATP. (C) Superimposition of APH(3′)-Ia · ATP, APH(3′)-Ia · kanamycin · SP600125, APH(3′)-Ia · kanamycin · PP2 [representative of APH(3′)-Ia · kanamycin · PP1] and APH(3′)-Ia · kanamycin · tyrphostin AG 1478 complexes. As the hinge region of APH(3′)-Ia is nearly identical across all inhibitor complexes, only one copy of the enzyme is shown.

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APH(3′)-Ia · ATP complex structure presented may not represent the binding conformation of the guanine-containing NTP.

**APH(3′)-Ia · kanamycin · inhibitor ternary complex structures**

To characterize inhibition of APH(3′)-Ia at the molecular level, we selected PP1, PP2, SP600125 and tyrphostin AG 1478, representing three distinct chemical scaffolds, from the broad range of APH(3′)-Ia inhibitors [24]. These compounds were initially designed as ATP-competitive inhibitors, but these are all also GTP-competitive inhibitors of APH(3′)-Ia (T. Shaky, P. Spanogiannopoulos and G. Wright, unpublished work). We determined crystal structures of the APH(3′)-Ia · kanamycin · SP600125, APH(3′)-Ia · kanamycin · PP1, APH (3′)-Ia · kanamycin · PP2 and APH(3′)-Ia · kanamycin · tyrphostin AG 1478 ternary complexes to 2.37 Å, 2.69 Å, 1.89 Å and 1.97 Å respectively (Figure 1C and Figure 2). All four ternary complex structures were solved by MR (molecular replacement) using the APH(3′)-Ia · ATP structure as a model. In all four inhibitor complex structures, the conformation of the APH(3′)-Ia hinge was highly conserved and nearly identical to that observed in the APH(3′)-Ia · ATP complex, indicating that this is a stable interaction site. In contrast, the position of the NPL (nucleotide-positioning loop) was variable or disordered in some copies of the enzyme in the crystals. A similar phenomenon has been described previously for other structurally characterized members of the APH(3′) subgroup of enzymes and was attributed to structural flexibility of the NPL [43].

In all APH(3′)-Ia ternary complex structures, electron density corresponding to the inhibitor molecules was found at the ATP-binding site near the hinge (Figure 1C). We conducted a detailed analysis of the APH(3′)-Ia interactions with these four inhibitors and compared their interactions with those of ePKs.

**First observation of a distinct binding mode of a PKI to an AK: inhibition of APH(3′)-Ia by the anthrapyrazolone SP600125**

The SP600125 compound has been extensively used as an inhibitor of the JNK (c-Jun N-terminal kinase) family and the serine/threonine kinase Mps1 [44–46]. SP600125 was identified to be a potent inhibitor of APH(3′)-Ia and a general inhibitor of the APH(3′), APH(2′), APH(4) and APH(9) subfamilies [24]. In the APH(3′)-Ia · kanamycin · SP600125 complex structure, the inhibitor molecule wedged into a hydrophobic pocket formed by the side chains of Phe101, Ile305 and Ile415 (Figure 3A). The planar nature of the compound enabled it to form a π–stacking interaction with Phe101. SP600125 formed two hydrogen bonds with backbone atoms in the hinge region, specifically between N1 and the Ile101 amide hydrogen, and between N2 and the carbonyl oxygen of Ile305. Additional bonds were formed between the C10–H and the carbonyl oxygen of Thr99, and the C9–H and the carbonyl oxygen of Thr98.

As SP600125 is a well-studied ePK inhibitor, we compared the binding mode of this compound with APH(3′)-Ia and ePKs (Supplementary Figure S1A at http://www.biochemj.org/bj/454/bj4540191add.htm). According to our analysis, SP600125 adopted a conformation in the APH(3′)-Ia active site that was rotated 180° relative to its orientation in the active site of all three structurally characterized complexes of this compound with ePKs [44–46]. As a result, the inhibitor’s C7–C10 ring was positioned to face deeper into the APH(3′)-Ia active site, whereas in its interactions with ePKs, the C3–C5 ring faces into the deepest region of the active site. Thus the structure of APH(3′)-Ia in complex with SP600125 provided the first example of a distinct mode of binding of a PKI to an antibiotic resistance enzyme.

We could rationalize the broad inhibition profile of SP600125 against multiple AK subgroups using the crystallographic data: SP600125 is anchored to the APH(3′)-Ia enzyme through hydrogen bonds with the main chain atoms of the hinge region and the only interactions involving side chains were with highly conserved positions. In this regard, the interactions mediated by SP600125 resemble those we observed previously in the binding of another AK general inhibitor, quercetin, in binding to APH(2′)-IVA [24]. On the basis of these structural observations, we suggest the anthrapyrazolone scaffold as a source of effective general inhibitors of AK enzymes, and could be useful against bacterial strains carrying multiple aph genes. Although the SP600125 molecule has been shown to bind ePK enzymes such as JNK family kinases, its interaction mode with ePKs is significantly different from that revealed in the APH(3′)-Ia · kanamycin · SP600125 complex structure, therefore retaining the possibility for development of AK-specific inhibitors.

**4-Anilinoquinazoline tyrphostin AG 1478 occupies a deeper binding pocket in APH(3′)-Ia that is also found in ePKs**

4-Anilinoquinazoline-based inhibitors, in general, have been optimized to be specific and potent inhibitors of ePKs [47]. Our data demonstrated previously that tyrphostin AG 1478 shows inhibitory activity with high specificity for the APH(3′) subgroup [24]. According to the APH(3′)-Ia · kanamycin · tyrphostin AG 1478 structure, the inhibitor molecule was held in the NTP-binding

![Figure 2](306x610 to 550x721) **Figure 2 Chemical structures of PKIs analysed**

![Figure 3](43x356 to 343x469) **Figure 3 Structures of anthrapyrazolone and 4-anilinoquinazoline inhibitors bound to APH(3′)-Ia**

(A) APH(3′)-Ia · kanamycin · SP600125 and (B) APH(3′)-Ia · kanamycin · tyrphostin AG 1478 complexes. The electron density shown is simulated annealing F₀–Fᵣ omit density at 2.5 and 2.0 σ for SP600125 and tyrphostin AG 1478 respectively.
site through hydrophobic interactions with the side chains of Phe\textsuperscript{53}, Ile\textsuperscript{86} and Ile\textsuperscript{101} (Figure 3B). In addition, through its N\textsubscript{1} atom, tyrphostin AG 1478 formed a hydrogen bond with the amide of Ile\textsuperscript{101}. The 7-methoxy group of tyrphostin AG 1478 was accommodated into a cleft formed by Pro\textsuperscript{102} and the 6-methoxy group occupied an area near the deoxyribose-binding site for ATP. The quinazoline core and the 4-N-(3'-chloroanilino) groups were roughly co-planar, with rotations of 4–9° about the N4–C1, bond among the four of the five observed copies of the molecule in the asymmetric unit of the crystal. Notably, the 4-N-(3'-chloroanilino) group of tyrphostin AG 1478 wedged into a deeper pocket formed by APH(3')-Ia and the 6-methoxy group occupied an area near the deoxyribose-binding site for ATP. The quinazoline core and the 4-N-(3'-chloroanilino) groups were roughly co-planar, with rotations of 4–9° about the N4–C1, bond among the four of the five observed copies of the molecule in the asymmetric unit of the crystal. Notably, the 4-N-(3'-chloroanilino) group of tyrphostin AG 1478 wedged into a deeper pocket formed by APH(3')-Ia and the 6-methoxy group occupied an area near the deoxyribose-binding site for ATP.

Figure 4  Binding mode of PP-based compounds to APH(3')-Ia and comparison with PP-based compounds bound to ePKs

(A) Structure of APH(3')-Ia · kanamycin · PP1 complex. (B) Structure of APH(3')-Ia · kanamycin · PP2 complex. Electron density shown for both inhibitors is simulated annealing F\texttextsubscript{o}–F\texttextsubscript{c} omit density at 3.0 σ. (C) Binding modes of PP-based inhibitors to ePKs. Left-hand panel, PP1 bound to H. sapiens Hck (PDB 1QCF); middle panel, S2 bound to H. sapiens PI3K (PDB 3ENE); right-hand panel, BK3 bound to T. gondii CDPK1 (PDB 3N51). Gatekeeper residues are coloured yellow and hinge residues are cyan. Sticks of conserved Lys–Asp/Glu salt bridge in active site are shown.

Inhibition of APH(3')-Ia by PP-based compounds takes place through a binding mode distinct from that observed for ePKs

The PPs are a well-known scaffold active against human protein kinases [37,38,49,50], as well as against protozoan CDPK1 (calcium-dependent protein kinase) (PDB code 3MA6 and [51]). Our previous study showed that the PP1 and PP2 representatives of this class were specific inhibitors of the APH(3')-Ia subfamily [24]. The orientation of PP1 and PP2 in the APH(3')-Ia · kanamycin · PP1 and APH(3')-Ia · kanamycin · PP2 ternary complex structures was such that the 3-(4'-methylphenyl or 4'-chlorophenyl) group points towards the C-terminal domain of the enzyme (Figure 4); we term this the C3-substituent ‘down’ orientation. The PP compounds were anchored to APH(3')-Ia primarily through hydrophobic interactions with the side chains of Ile\textsuperscript{101}, Ile\textsuperscript{215} and Phe\textsuperscript{53} and two hydrogen bonds to main chain atoms in the hinge region: the N4 group with the carbonyl oxygen of Ile\textsuperscript{101} and N5 with the amide of Ile\textsuperscript{101}. The larger residues in the deepest region of the binding pocket of APH(2')-subfamily enzymes would obstruct the binding of PP1/PP2, and thus explain the subfamily specificity of these compounds.
To validate the interactions of PP compounds with APH(3′)-Ia, we investigated the effect of additional groups at the N1, C3 and N4 positions of the PP scaffold on in vitro inhibition of APH(3′)-Ia. PP variants with modifications at the N1 position ranging in size from isobutyl to hexyl [41] demonstrated 2–25-fold reduced inhibition of APH(3′)-Ia activity relative to PP1 (Figure 5). Modifications at C3 with large substituents including indole or naphthalen-1-ylmethyl also adversely affected APH(3′)-Ia activity (3–9-fold decrease relative to inhibition by PP1). Given the comparatively larger sizes of the tested modifications at C3, these results suggest that APH(3′)-Ia is more sensitive to substitutions at N1 and are consistent with a C3 ‘down’ binding orientation of PP compounds to the enzyme.

Next, we compared the molecular details of interactions of PP compounds and APH(3′)-Ia with those interactions formed between this class of compounds and ePKs. Through a search of the PDB, we identified 18 distinct complex structures between ePKs and PP-type compounds. Despite the diverse chemical nature of the PP compounds, the comparative analysis demonstrated a common binding position and orientation, with C3 substituents occupying deeper regions of the NTP sites (C3 ‘up’) (Figure 4C). This analysis showed that PP compounds adopt different binding orientations to APH(3′)-Ia and ePKs. On the basis of this analysis, we hypothesized that PP compounds with large C3 substituents would clash with the enzyme if bound in this C3 ‘up’ binding orientation; this is consistent with the data presented in Figure 5(C) and with a C3 ‘down’ binding orientation instead.

### Structural basis of inhibition of APH(3′)-Ia by C3-substituted PP compounds

To validate this hypothesis, we determined the crystal structures of APH(3′)-Ia in complex with kanamycin and 1-NA-PP1 or 1-NM-PP1, two PP variants with large aromatic substituents at the C3 position. These ternary complex structures were solved by MR to resolutions of 1.96 Å and 1.88 Å respectively. In these structures, both compounds bind to APH(3′)-Ia in the C3 ‘down’ binding orientation (Figure 6). The 3-naphthalen-2-yl group of 1-NA-PP1 was rotated $\sim 45^\circ$ relative to the plane of the PP which provides for substantial hydrophobic interaction with Phe53. Similarly, the naphthalen-1-yl group of 1-NM-PP1 packed against one face of Phe53, with the additional methyl group linking the PP core and
the napthalen-1-yl group facilitating a more acute packing angle against this residue. In addition, the 1-NA-PP1 and 1-NM-PP1 compounds formed hydrophobic interactions with the methyl group of Thr105 and the side chains of Val33, Ile40, Ile205 and Ile215.

In order to provide a quantitative comparison of APH(3′)-Ia interaction with PP compounds, we computed the shape complementarity between these inhibitors and the enzyme [39]. We calculated complementarity values of 0.740 and 0.761 (1.0 would indicate complete shape complementarity) for 1-NA-PP1 and 1-NM-PP1 respectively. In comparison, the corresponding values for PP1 and ATP are 0.691 and 0.754 respectively.

Interestingly, the buried surface area between PP1 and the larger 1-NA-PP1 and 1-NM-PP1 was nearly identical (432, 427 and 422 Å² buried respectively). Although overall the buried surface area of 1-NA-PP1, 1-NM-PP1 and the parent compound PP1 on binding to APH(3′)-Ia were not significantly different, the increased shape complementarity and large aromatic substituents at the C3 atom of 1-NA-PP1 and 1-NM-PP1 may provide for additional favourable hydrophobic packing interactions with APH(3′)-Ia, especially residue Phe53.

To the best of our knowledge, the complexes between PP compounds with large C3 substituents bound to CDPK1 from Toxoplasma gondii and Cryptosporidium parvum represent the only structurally characterized examples of interactions of this type of PP derivative with a kinase catalytic domain [50]. Therefore we compared the binding mode of these compounds observed in CDPK1 complexes with their binding to APH(3′)-Ia. In each of the seven retrieved structures, the inhibitors bound in the C3 ‘up’ orientation to the protozoan kinases (Supplementary Figure S1C). In these complexes, the aromatic substituents at C3 were able to access the deeper pocket of the ATP-binding site due to the presence of smaller residues (glycines) in this area. This confirmed the prominent contrast with the C3 ‘down’ orientation that we observed for 1-NA-PP1 and 1-NM-PP1 bound to APH(3′)-Ia.

We modelled 1-NA-PP1 and 1-NM-PP1 into representative ePK active sites in the C3 ‘up’ as well as in C3 ‘down’ conformations (Figure 6C). This exercise demonstrates that 1-NA-PP1 and 1-NM-PP1 would clash with ePK residues in the N-terminal β-sheet or glycine-rich loop that form the ‘ceiling’ of the NTP-binding site. The ePKs analysed appear to have a more constricted NTP-binding site that would preclude the binding of 1-NA-PP1 and 1-NM-PP1 in the C3 ‘down’ orientation.

**Structure-selected APH(3′)-Ia inhibitors rescue aminoglycoside resistance**

Having established the structural basis of APH(3′)-Ia inhibition by PKIs and identified the specific scaffolds with distinct binding to APH(3′)-Ia and ePKs, we then tested whether these compounds could re-sensitize kanamycin-resistant bacteria to the antibiotic. For these experiments a hyperpermeable efflux mutant strain of *E. coli* (ΔtolC ΔbamB) expressing *aphA1* was grown in the presence of PKIs alone or in combination with kanamycin at one-quarter of the MIC (Figure 7, and Supplementary Figure S2 at http://www.biochemj.org/bj/454/bj4540191add.htm). The bacterial growth curves under these conditions were quantitatively compared using the ARF, a measurement of the effectiveness of a compound for recovering antibiotic activity (a value of 1 would indicate complete restoration of antibiotic activity) [24]. All inhibitors had a minimal impact on cell growth. The inhibitors tyrphostin AG 1478 or SP600 125 in combination with kanamycin demonstrated only modest antibiotic rescue activity. In contrast, PP1 or PP2 in...
Inhibition of an antibiotic resistance kinase

Figure 7 C3-substituted PP compounds rescue activity of kanamycin-resistant E. coli

Shown are growth curves of E. coli ΔtolC ΔbamB expressing APH(3′)-Ia grown in the presence of 1-NA-PP1 alone (64 μM, grey dotted line), 1-NA-PP1 (64 μM) in combination with kanamycin at one-quarter of the MIC (black dotted line), 1-NM-PP1 alone (32 μM, grey dashed line) and 1-NM-PP1 (32 μM) in combination with kanamycin at one-quarter of the MIC (black dashed line). Also shown are normal growth control (continuous black line) and kanamycin at one-quarter of the MIC only (continuous grey line). Inset, corresponding ARF [24] values. OD600, attenuation measured at 600 nm.

combination with the antibiotic were able to decrease bacterial growth and displayed ARF values of 0.33 and 0.18 respectively. Most importantly, the addition of 1-NA-PP1 or 1-NM-PP1 in combination with the antibiotic led to bacterial growth suppression with ARF values of 0.98 and 0.95 respectively, thus nearly completely re-sensitizing this antibiotic-resistant strain to kanamycin.

To test for specificity, the PPs PP1, PP2, 1-NA-PP1 and 1-NM-PP1 were also tested against E. coli carrying a structurally unrelated aminoglycoside resistance gene, aminoglycoside acetyltransferase aac(3)-Ia, which we predicted would not show synergy with APH inhibitors. All PPs tested failed to re-sensitize this resistant strain to the aminoglycoside antibiotic gentamicin, consistent with our structural data that these compounds are specific to the APH(3′)-Ia enzyme (Supplementary Figure S3 at http://www.biochemj.org/bj/454/bj4540191add.htm).

Antibiotic susceptibility tests with the hyperpermeable E. coli strain have demonstrated increased sensitivity to various antibiotics (T. Shakya, P. Spanogiannopoulos and G. Wright, unpublished work and [52]). We have noticed that this strain is particularly more sensitive to larger hydrophobic antibiotics (i.e. rifampin and erythromycin). Although 1-NA-PP1 and 1-NM-PP1 possess a marginally lower Ki compared with the parent compound PP1, their enhanced in vivo activity (in the absence of the major efflux component ToC) may be attributed to this increased sensitivity. Illustrating the importance of permeability, all PPs failed to re-sensitize wild-type E. coli expressing apfa1 (Supplementary Figure S4 at http://www.biochemj.org/bj/454/bj4540191add.htm). However, SP600125 and tyrphostin AG 1478 are also large hydrophobic compounds, but do not rescue aminoglycoside resistance, therefore compound size is not the only criterion for successful in vivo activity of PKIs; specific structural features shown by 1-NA-PP1 and 1-NM-PP1, particularly shape complementarity with the APH(3′)-Ia, are essential.

The compounds 1-NA-PP1 and 1-NM-PP1 were previously termed BKIs (bumped kinase inhibitors) and were shown to be inactive against wild-type mammalian kinases [53]. In these kinases, a ‘gatekeeper’ residue, typically a methionine, phenylalanine or tyrosine residue, restricts the depth of the NTP-binding site in ePKs and prevents such PP compounds with large C3 substituents from binding in the ‘up’ orientation. Using the insight that 1-NA-PP1 and 1-NM-PP1 interact with APH(3′)-Ia in the C3 ‘down’ orientation, modelling of these compounds into ePKs in the C3 ‘down’ orientation suggested that these compounds would clash with residues in ePKs in the ribose-binding area of the binding site. This suggested that PP compounds with large C3 substituents are unable to bind ePKs in either the C3 down orientation or in the C3 up orientation and are therefore ineffective against wild-type ePKs. On the basis of this analysis, our identification of the C3 down orientation of PP compounds to APH(3′)-Ia was an important advance in the identification of APH-specific inhibitors.

Whereas the NTP-binding site of APH(3′)-Ia shares enough structural features with ePK ATP-binding sites to enable the use of PKI molecules as initial leads for APH inhibitors, we demonstrated in the present study that multiple classes of PKIs bind to this bacterial enzyme in a distinct orientation from their binding mode to ePKs. This observation allowed us to optimize compounds that select for binding to APH(3′)-Ia over ePKs. Keeping in mind the permeability barrier, which may be overcome with compound optimization, the APH inhibitor molecules investigated in the present study thus represent privileged chemical matter for design of the next generation of AK-specific inhibitors. We believe that these findings provide a significant advance toward addressing antibiotic resistance by restoration of aminoglycoside antibiotic activity.

AUTHOR CONTRIBUTION

Peter Stogios determined the crystal structures, performed all structural analysis, interpreted the data and wrote the paper. Peter Spanogiannopoulos designed and performed the enzyme kinetics and growth curve experiments, interpreted the data and wrote the paper. Elena Evdokimova performed the crystallography. Olga Egorova performed the protein purification and preliminary crystallography. Tushar Shyaka designed and performed the enzyme kinetics experiments. Nick Todorovic designed and performed chemical synthesis and wrote the paper. Alfredo Capretta interpreted the data and wrote the paper. Gerard Wright interpreted the data and wrote the paper.Alexei Savchenko interpreted the data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Structure-guided optimization of protein kinase inhibitors reverses aminoglycoside antibiotic resistance

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Figure S1 Comparison of binding mode of SP600125, 4-anilinoquinazoline compounds, and pyrazolopyrimidine compounds between ePKs and APH(3′)-Ia

(A) Structures of SP600125 complexes. (B) Structures of 4-anilinoquinazoline inhibitor complexes. (C) Structures of C3-derivatized PP inhibitor complexes. For each of the three inhibitor classes, structures shown are representative of all complexes found in search of PDB in that the inhibitor is bound in the same orientation and interactions between the inhibitor and the hinge region of the ePKs are conserved within the inhibitor class.

The structural co-ordinates reported will appear in the PDB under accession codes 4EJ7, 4FEU, 4FEV, 4FEW, 4FEX, 4GKH and 4GK1.

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Figure S2  Growth of *E. coli* expressing *aphA1* in the presence of PP1, PP2, tyrphostin AG 1478 and SP600125

(A) Growth curves of *E. coli*ΔtolC ΔbamB containing pET22-aphA1 grown in the presence of PP1 alone (64 μM; light blue triangles), PP1 (64 μM) in combination with kanamycin A at one-quarter of the MIC (dark blue triangles), PP2 alone (64 μM; tan diamonds) and PP2 (64 μM) in combination with kanamycin A at one-quarter of the MIC (brown diamonds). (B) Growth curves of *E. coli*ΔtolC ΔbamB containing pET22-aph(3)′-Ia grown in the presence of tyrphostin AG 1478 alone (64 μM; light blue triangles), tyrphostin AG 1478 (64 μM) in combination with kanamycin A at one-quarter of the MIC (dark blue triangles), SP600125 alone (64 μM; tan diamonds) and SP600125 (64 μM) in combination with kanamycin A at one-quarter of the MIC (brown diamonds). For both plots, normal growth control (black squares) and kanamycin A at one-quarter of the MIC control (green squares) are shown. Inset, corresponding ARF values for compounds. OD$_{600}$, attenuance measured at 600 nm.

Figure S3  Growth of *E. coli* expressing aminoglycoside acetyltransferase *aac(3)-Ia* in the presence of pyrazolopyrimidines

(A) Growth curves of *E. coli*ΔtolC ΔbamB containing pGDP4-aac(3)-Ia grown in the presence of 1-NA-PP1 alone (64 μM; light blue triangles), 1-NA-PP1 (64 μM) in combination with gentamicin at one-quarter of the MIC (dark blue triangles), 1-NM-PP1 alone (32 μM; pink diamonds) and PP2 (64 μM) in combination with gentamicin at one-quarter of the MIC (red diamonds). (B) Growth curves of *E. coli*ΔtolC ΔbamB containing pGDP4-aac(3)-Ia grown in the presence of PP1 alone (64 μM; light blue triangles), PP1 (64 μM) in combination with gentamicin at one-quarter of the MIC (dark blue triangles), PP2 alone (64 μM; tan diamonds), and PP2 (64 μM) in combination with gentamicin at one-quarter of the MIC (brown diamonds). For both plots the normal growth control is shown (black squares) and in the presence of gentamicin at one-quarter of the MIC control (green squares). OD$_{600}$, attenuance measured at 600 nm.
Figure S4  Growth of wild-type *E. coli* expressing aphA1 in the presence of pyrazolopyrimidines

(A) Growth curves of wild-type *E. coli* containing pET22-aphA1 grown in the presence of 1-NA-PP1 alone (64 μM; light blue triangles), 1-NA-PP1 (64 μM) in combination with kanamycin A at one-quarter of the MIC (dark blue triangles), 1-NM-PP1 alone (32 μM; pink diamonds) and 1-NM-PP1 (64 μM) in combination with kanamycin A at one-quarter of the MIC (red diamonds). (B) Growth curves of *E. coli* containing pET22-aphA1 grown in the presence of PP1 alone (64 μM; light teal triangles), PP1 (64 μM) and in combination with kanamycin A at one-quarter of the MIC (dark teal triangles), PP2 alone (64 μM; tan diamonds), and PP2 (64 μM) in combination with kanamycin A at one-quarter of the MIC (brown diamonds). For both plots also shown is normal growth control (black squares) and in the presence of kanamycin A at one-quarter of the MIC control (green squares). OD$_{600}$, attenuance measured at 600 nm.

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