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Research Paper

Active site labeling of the gentamicin resistance enzyme AAC(6′)-APH(2″) by the lipid kinase inhibitor wortmannin

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Abstract

Background: Aminoglycoside antibiotic resistance is largely the result of the production of enzymes that covalently modify the drugs including kinases (APHs) with structural and functional similarity to protein and lipid kinases. One of the most important aminoglycoside resistance enzymes is AAC(6′)-APH(2″), a bifunctional enzyme with both aminoglycoside acetyltransferase and kinase activities. Knowledge of enzyme active site structure is important in deciphering the molecular mechanism of antibiotic resistance and here we explored active site labeling techniques to study AAC(6′)-APH(2″) structure and function.

Results: AAC(6′)-APH(2″) was irreversibly inactivated by wortmannin, a potent phosphatidylinositol 3-kinase inhibitor, through the covalent modification of a conserved lysine in the ATP binding pocket. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine, an electrophilic ATP analogue and known inactivator of other APH enzymes such as APH(3′)-IIIa, did not inactivate AAC(6′)-APH(2″), and reciprocally, wortmannin did not inactivate APH(3′)-IIIa.

Conclusions: These distinct active site label sensitivities point to important differences in aminoglycoside kinase active site structures and suggest that design of broad range, ATP binding site-directed inhibitors against APHs will be difficult. Nonetheless, given the sensitivity of APH enzymes to both protein and lipid kinase inhibitors, potent lead inhibitors of this important resistance enzyme are likely to be found among the libraries of compounds directed against other pharmacologically important kinases.

Keywords: Antibiotic resistance; Aminoglycoside; Active site label

1. Introduction

Clinical resistance to aminoglycoside antibiotics is primarily the result of enzymes that covalently modify the antibiotics by *N*-acetylation, *O*-adenylation or *O*-phosphorylation. Aminoglycoside resistant strains of Gram-positive pathogens such as *Enterococcus*, *Staphylococcus* and *Streptococcus* commonly harbor plasmid-encoded kinases that phosphorylate important hydroxyl groups on the antibiotic (for review see [1]). The basis for resistance lies in the fact that the phosphorylated aminoglycoside no longer binds with high affinity to its target, the aminoacyl tRNA recognition site of the bacterial ribosome, thereby rendering the antibiotic ineffective. The most studied and best understood aminoglycoside kinase is APH(3′)-IIIa. The structure of APH(3′)-IIIa is homologous to serine/threonine kinases, despite very low amino acid sequence similarity ($<10\%$) [2]. This three-dimensional similarity parallels studies that have shown that aminoglycoside kinases (APHs) can phosphorylate peptides and proteins on Ser residues [3], and a growing body of evidence indicates that the molecular mechanism of phosphoryl transfer is similar in both classes of enzymes [4]. The structural and functional homology between APH(3′)-IIIa and protein kinases, and by inference all of the APHs, prompted a screen of protein kinase inhibitors to identify potential leads in the design of inhibitors against the aminoglycoside resistance enzymes. Indeed, several protein kinase inhibitors were identified for APH(3′)-IIIa and another important aminoglycoside kinase, AAC(6′)-APH(2″) [5].

AAC(6′)-APH(2″) is a bifunctional enzyme incorporating an N-terminal aminoglycoside acetyltransferase domain (AAC) and a C-terminal aminoglycoside kinase domain, and is responsible for the majority of clinically

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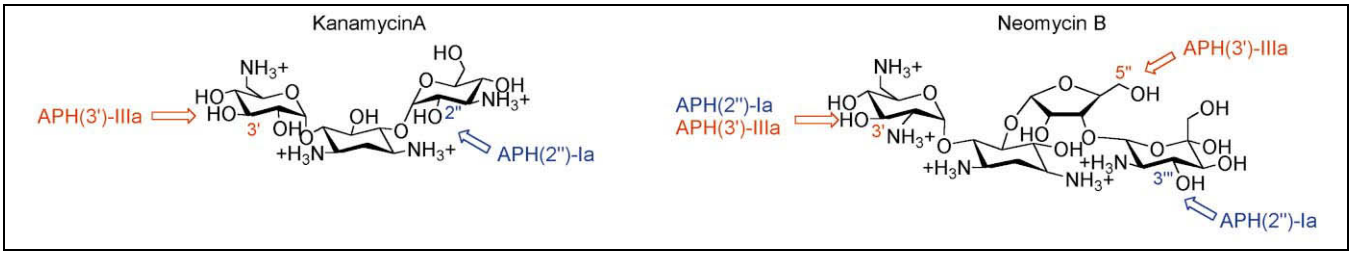

Fig. 1. Regiospecificities of phosphoryl transfer by APH(3′)-IIIa and APH(2″)-Ia, the phosphotransferase domain of AAC(6′)-APH(2″). APH(3′)-IIIa catalyzes phosphoryl transfer onto 4,6-disubstituted aminoglycosides exclusively on 3′-OHs, whereas it can bisphosphorylate 3′-OHs and 5′-OHs on 4,5-disubstituted aminoglycosides [7,10]. Phosphorylation by APH(2″)-Ia is highly dependent upon the individual aminoglycoside, where the enzyme can phosphorylate similar and different sites than APH(3′)-IIIa [8,9].

relevant aminoglycoside resistance in Gram-positive bacteria [6].

Although protein kinase inhibitors were identified for both APHs tested, the two enzymes showed definite selectivity towards specific compounds [5] indicating that the kinase active sites have important geometrical differences. This is consistent with the fact that the enzymes also have different aminoglycoside substrate profiles [7,8], with the kinase domain of AAC(6′)-APH(2″) (referred to as APH(2″)-Ia herein) having a broader substrate specificity than APH(3′)-IIIa; an observation that implies that APH(2″)-Ia has a greater ability to accept more diverse structures into its active site. In addition to a broader aminoglycoside specificity, APH(2″)-Ia has a more expansive kinase regiospecificity with the capacity to phosphorylate a number of hydroxyl groups around the aminoglycoside core, including positions 3′, 5″, 2″ and 3‴ depending on the aminoglycoside [8,9]. APH(3′)-IIIa, in contrast, phosphorylates either 3′- and/or 5″-hydroxyls [7,10] (Fig. 1). The kinetic mechanisms of the two enzymes are also different: APH(3′)-IIIa follows a Theorell–Chance mechanism where ADP release is solely rate limiting [11,12], while APH(2″)-Ia proceeds through a random rapid equilibrium mechanism [13] with the catalytic step making an important contribution to the turnover rate (Boehr and Wright, unpublished observations).

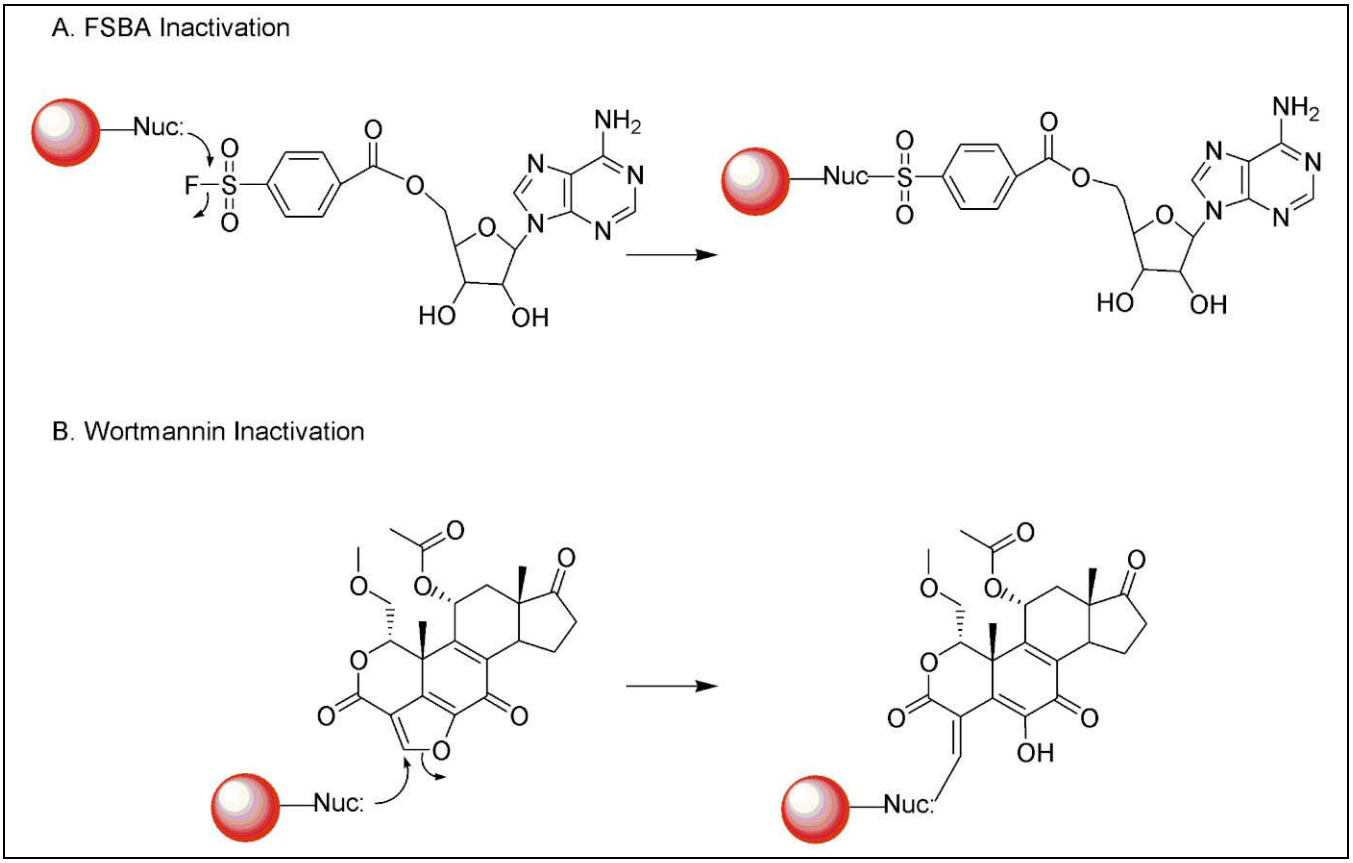

Fig. 2. Structure and reactivity of kinase active site labeling compounds FSBA (A) and wortmannin (B). A nucleophile in the active site, generally a lysine, attacks the electrophilic position on the respective compound as indicated resulting in an inactivated enzyme.

The rational design of selective inhibitors of aminoglycoside kinases requires a structural understanding of the differences between these enzymes, however while much information has been gleaned from the structural determination of APH(3′)-IIIa, similar information is lacking for APH(2″)-Ia. As a first step in elaborating the structural differences between these enzymes, we elected to explore active site labeling techniques.

One candidate active site label is the electrophilic ATP analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), which was previously shown to inactivate APH(3′)-IIIa by modifying the invariant Lys44 in the ATP binding pocket [14]. Another candidate is the lipid kinase inhibitor wortmannin (see Fig. 2 for structures). Lipid kinases also share a similar fold with aminoglycoside and protein kinases, as seen with the three-dimensional structure of phosphatidylinositol phosphate kinase type IIB [15], and therefore, lipid kinase inhibitors have the potential to inhibit aminoglycoside kinases as well. Wortmannin was originally discovered to be an inhibitor of myosin light chain kinase with an $IC_{50} \sim 0.2$ μM [16]. Later studies showed wortmannin to be a 100-fold more potent irreversible inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase) with an $IC_{50} \sim 2$ nM [17], and is now commonly used to study the role of PI 3-kinase in signal transduction cascades (for review see [18]). Wortmannin acts by covalently binding Lys802 in the ATP binding pocket of PI 3-kinase [19]. A study of a series of structural analogues of wortmannin concluded that PI 3-kinase is irreversibly inactivated through nucleophilic attack of the Lys amino group at the C21 position of wortmannin generating the substituted α–β unsaturated lactone (Fig. 2) [20].

In the present study, we show that wortmannin is an inactivator of APH(2″)-Ia but not APH(3′)-IIIa. This contrasts with the ATP analogue FSBA which did not inactivate APH(2″)-Ia, but was previously shown to covalently modify APH(3′)-IIIa and the spectinomycin kinase APH(9)-Ia [14,21]. These different active site labeling sensitivities provide a chemical means of discriminating between aminoglycoside kinases and highlight the geometrical differences in the active sites of the two enzymes.

2. Results

2.1. Kinetic analysis of APH(2″)-Ia domain and N-terminal 6-histidine (6-His)-tagged AAC(6′)-APH(2″)

We have previously reported the purification of the aminoglycoside resistance determinant AAC(6′)-APH(2″) in *Bacillus subtilis* using an expression plasmid under the control of the constitutive *vegII* promoter [8]. In this study, we created *Escherichia coli* expression constructs that introduce an N-terminal hexa His tag on AAC(6′)-APH(2″) (HisAAC(6′)-APH(2″)) for ease in purification and a construct that expresses a C-terminal truncated ver-

Table 1
Enzymatic properties of aminoglycoside modifying enzymes

Construct	APH activity						AAC activity					
	Kanamycin A[a]			ATP[b]			Kanamycin A[c]			Acetyl CoA[d]		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
B. subtilis-expressed AAC(6′)-APH(2″)	7.0 ± 0.9	0.41 ± 0.02	5.8×10^4	64 ± 9	0.17 ± 0.02	2.7×10^3	11 ± 1	0.69 ± 0.02	6.3×10^4	7.2 ± 1.4	0.24 ± 0.04	3.4×10^4
HisAAC(6′)-APH(2″)	4.7 ± 0.5	0.32 ± 0.03	6.8×10^4	135 ± 9	0.32 ± 0.03	2.4×10^3	31 ± 3	1.7 ± 0.2	5.6×10^4	38 ± 5	1.2 ± 0.3	3.1×10^4
C-terminal truncate APH(2″)-Ia domain	6.0 ± 1.1	0.24 ± 0.01	4.0×10^4	90 ± 3	0.21 ± 0.01	2.3×10^3	–	–	–	–	–	–

[a]ATP held at 1 mM.
[b]Kanamycin A held at 100 μM.
[c]Acetyl CoA held at 100 μM for *B. subtilis*-expressed AAC(6′)-APH(2″) and 300 μM for HisAAC(6′)-APH(2″).
[d]Kanamycin A held at 100 μM for *B. subtilis*-expressed enzyme AAC(6′)-APH(2″) and 300 μM for HisAAC(6′)-APH(2″).

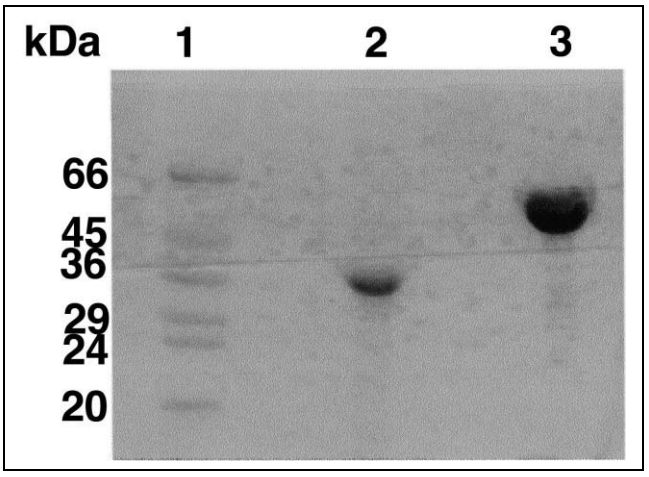

Fig. 3. Purity of APH(2″) enzymes. The sodium dodecyl sulfate–polyacrylamide gel was stained with Coomassie blue. Lane 1, molecular mass standards; lane 2, APH(2″)-Ia fragment beginning at Met175 of AAC(6′)-APH(2″); lane 3, His6-AAC(6′)-APH(2″).

sion that encodes only the APH(2″)-Ia domain of the bifunctional enzyme (Fig. 3).

The N-terminal His tag did not have a significant effect on the activity of AAC(6′)-APH(2″). Although there were modest changes to K_M and k_{cat} for some substrates, the catalytic efficiencies (k_{cat}/K_M) for these substrates remained comparable to those determined for the *B. subtilis*-expressed enzyme (Table 1). Likewise, the C-terminal truncated version encoding only the APH(2″) domain did not display significantly different aminoglycoside modification properties (Table 1).

Most of the subsequent studies utilized the C-terminal truncated version of APH(2″), except where indicated. The *B. subtilis*-expressed AAC(6′)-APH(2″), HisAAC(6′)-APH(2″) and the truncated APH(2″)-Ia domain all showed identical sensitivities to the active site labeling compounds FSBA and wortmannin (data not shown).

2.2. *APH(2″)-Ia and APH(3′)-IIIa have different sensitivities to the covalent modifiers FSBA and wortmannin*

FSBA, an inactivator of APH(3′)-IIIa [14], did not have an effect on either the acetyltransferase or phosphotransferase activity of HisAAC(6′)-APH(2″) at concentrations of 2 mM (data not shown). In contrast, wortmannin did not affect the activity of APH(3′)-IIIa up to a concentration of 2 mM, but did efficiently inactivate the phosphotransferase domain of HisAAC(6′)-APH(2″) (Fig. 4). The reaction appeared to be irreversible as extensive dialysis or gel filtration could not restore APH activity. The acetyltransferase activity of the bifunctional enzyme was not affected (Fig. 4). Thus, FSBA is a specific inactivator of APH(3′)-IIIa, whereas wortmannin is specific to APH(2″)-Ia.

Wortmannin inactivation of APH(2″) was accompanied by a marked change in color of the reaction mixture from colorless to yellow, which permitted ready monitoring of enzyme inactivation by monitoring of the increase in absorbance at 440 nm (Fig. 5). The presence of an isosbestic point is consistent with formation of a single wortmannin species.

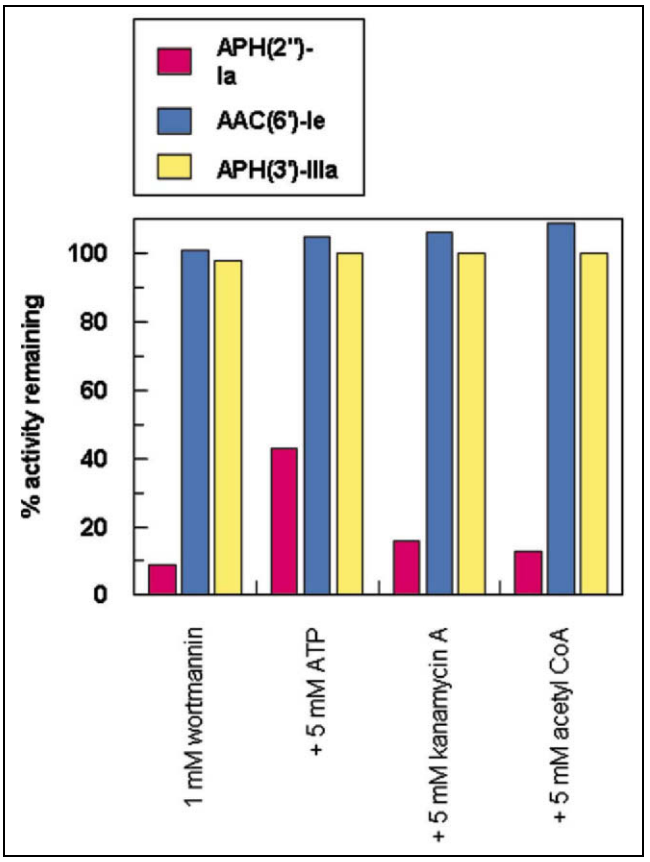

Fig. 4. Wortmannin inactivates the aminoglycoside kinase (APH(2″)) activity of AAC(6′)-APH(2″). Inactivation assays were performed as described in Section 5 following a 60 min incubation at 22°C.

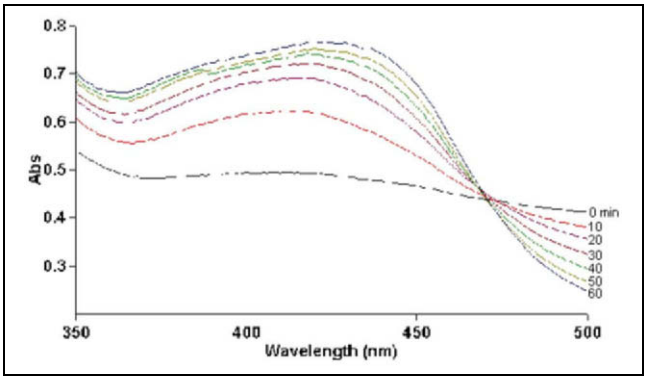

Fig. 5. Reaction of wortmannin with HisAAC(6′)-APH(2″) as followed by absorbance spectrum scans with a Cary 3E UV/Vis spectrophotometer.

Table 2
Peptides labeled by wortmannin as identified by mass spectral analysis of trypsin-digested and inactivated APH(2″)-Ia

Peptide	Predicted modified residue
YDDNATVK*AMK	Lys13
LVNNEYIFK*TK	Lys52
STMSEEEQNLLK*R	Lys125
NIK*QEFIENGR	Lys287

2.3. *ATP protection of APH(2″)-Ia activity from inactivation by wortmannin*

To validate the specificity of the reaction and to localize the site of modification, substrate protection experiments were performed. Neither kanamycin A nor acetyl CoA afforded any protection to APH(2″)-Ia, however, ATP was able to protect APH(2″)-Ia from inactivation (Fig. 4), suggesting that wortmannin binds at or near the ATP binding pocket of APH(2″)-Ia and interacts with an important active site residue.

2.4. *Kinetics of inactivation of APH(2″)-Ia by wortmannin*

Wortmannin inactivated APH(2″)-Ia in both a concentration- and time-dependent manner following pseudo first order kinetics (Fig. 6A), consistent with the following model:

$$E + I \overset{K_i}{\rightleftharpoons} EI \overset{k_{max}}{\longrightarrow} EI^*$$

where K_i is the apparent binding constant, k_{max} is the rate of inactivation at saturating inactivator (I), and EI* is the modified enzyme. Replot of the pseudo first order constants, k_{obs}, versus wortmannin concentration to determine K_i and k_{max} revealed the predicted saturation kinetics typical of enzyme affinity reagents (Fig. 6B). The dissociation constant, K_i, and the maximal rate of inactivation, k_{max}, were calculated to be 1.05 ± 0.1 mM and $1.51 \pm 0.07 \times 10^{-2}$ min^{-1} respectively. Thus wortmannin was shown to be a specific inactivator of APH(2″)-Ia likely functioning by covalently labeling the enzyme in a region adjacent or overlapping the ATP binding site.

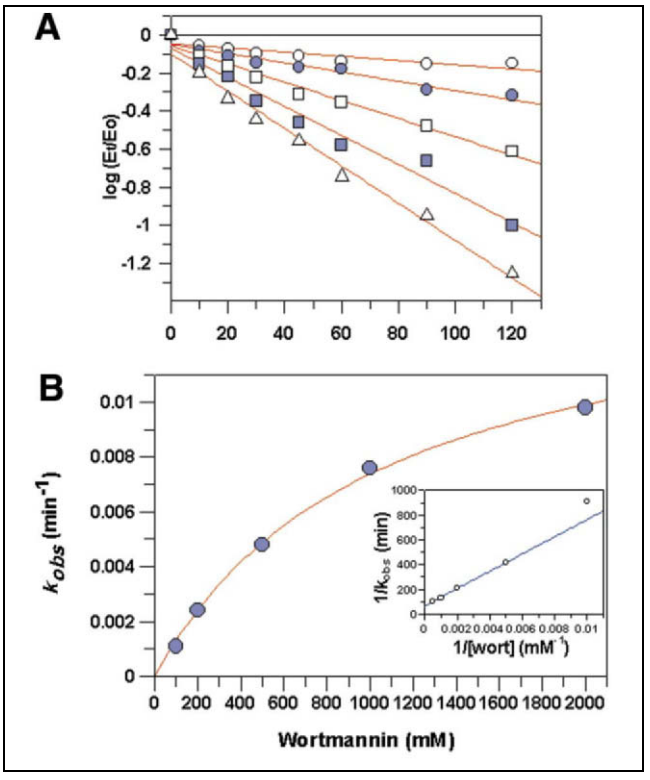

Fig. 6. Time- and concentration-dependent inactivation of APH(2″)-Ia by wortmannin. (A) APH(2″)-Ia (0.4 nmol) in 50 mM HEPES-NaOH pH 7.5 and DMSO (5% v/v) inactivated with wortmannin at a final concentration of 100 (○), 200 (●), 500 (□), 1000 (■) and 2000 (△) μM. (B) Plot of first order rates of inactivation (k_{obs}) vs. inactivator concentration. The inset is a double reciprocal replot to determine K_i and k_{max}, which were calculated to be 1.05 ± 0.1 mM and $1.51 \pm 0.07 \times 10^{-2}$ min^{-1} respectively.

Table 3
Kinetic analysis of APH(2″)-Ia Lys to Ala mutants

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (WT)/k_{cat} (mut)	k_{cat}/K_M (WT)/k_{cat}/K_M (mut)
Lys13Ala					
Kanamycin A[a]	10.6 ± 2.0	0.28 ± 0.02	2.6×10^4	0.86	1.5
ATP[b]	113 ± 12	0.33 ± 0.02	2.9×10^3	0.64	0.79
Lys52Ala					
Kanamycin A[c]	2.07 ± 0.86	0.017 ± 0.001	8.2×10^3	14	4.9
ATP[b]	227 ± 36	0.015 ± 0.001	6.6×10^1	14	35
Lys125Ala					
Kanamycin A[a]	6.53 ± 1.13	0.20 ± 0.01	3.1×10^4	1.2	1.3
ATP[b]	100 ± 12	0.24 ± 0.02	2.4×10^3	0.88	0.96
Lys287Ala					
Kanamycin A[a]	8.80 ± 1.03	0.30 ± 0.01	3.4×10^4	0.80	1.2
ATP[b]	94 ± 13	0.23 ± 0.02	2.4×10^3	0.88	0.96

[a]ATP held at 1 mM.
[b]Kanamycin A held at 100 μM.
[c]ATP held at 2 mM.

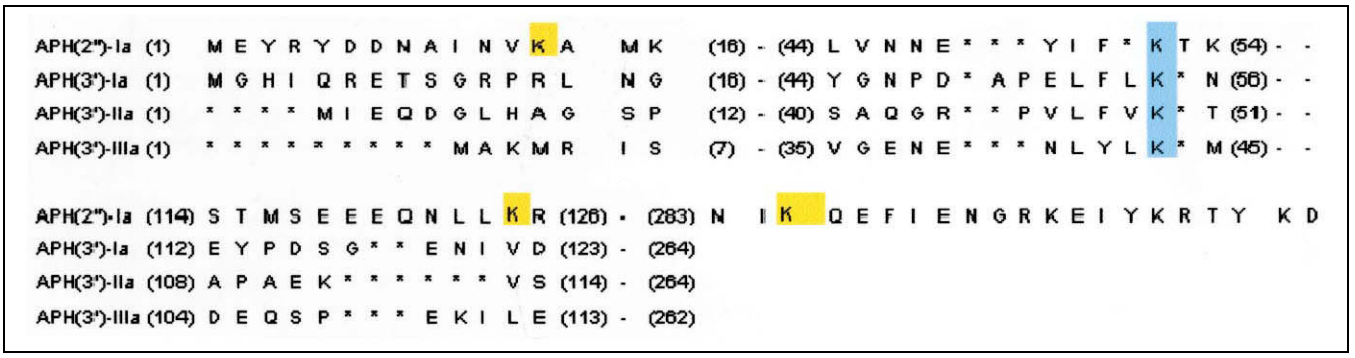

Fig. 7. Primary sequence alignment of aminoglycoside phosphotransferases. Lysines modified in APH(2″)-Ia by wortmannin are highlighted. Only Lys52 is conserved among the APHs (highlighted in blue) and is homologous to Lys44 in APH(3′)-IIIa which is known to interact with the α- and β-phosphates of ATP.

2.5. *Wortmannin labels Lys residues in APH(2″)-Ia*

Identification of covalently labeled amino acids was accomplished using a combination of tryptic peptide mapping and mass spectral analysis. Four Lys residues (Lys13, Lys52, Lys125 and Lys287) were tentatively identified as being modified under the conditions used to perform the experiment (Table 2). Efforts to establish stoichiometry of labeling using mass spectrometry (MS) failed.

The role of each of the identified Lys residues in the aminoglycoside modifying activity of APH(2″)-Ia was probed by mutating each Lys to Ala separately and characterizing the mutant enzymes. Mutagenesis of Lys13, Lys125 and Lys287 had little to no effect on the activity of APH(2″)-Ia (Table 3). However, mutagenesis of Lys52 had a dramatic effect on the aminoglycoside modifying ability of APH(2″)-Ia with significant changes on both the ATP and kanamycin A kinetic parameters (Table 3). The effect was most pronounced with respect to ATP where there was a 35-fold decrease in the catalytic efficiency (Table 3) which is consistent with this residue having a role in ATP capture, and demonstrates that this residue is homologous to Lys44 in APH(3′)-IIIa as suggested by the primary sequence alignment (Fig. 7). An equivalent Lys44Ala mutation in APH(3′)-IIIa had a similar 31-fold effect on catalytic efficiency [23].

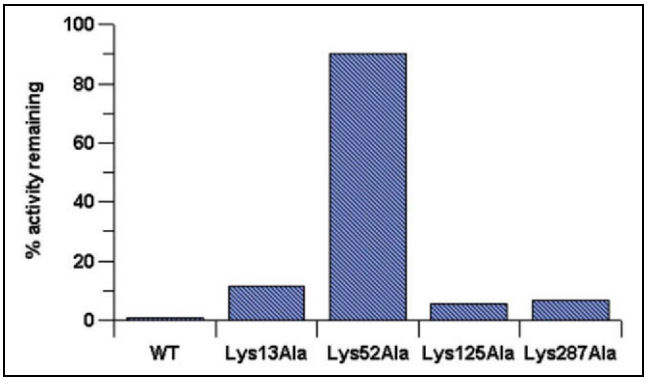

Fig. 8. Wortmannin inactivation of Lys→Ala mutants of APH(2″)-Ia. Only the Lys52Ala mutant is protected against inactivation.

The impact of mutating Lys52 on ATP capture is consistent with the conclusion that wortmannin inactivates APH(2″)-Ia by covalently modifying this residue. Additional support for this conclusion comes from the observation that wortmannin did not efficiently inactivate the Lys52Ala mutant (>90% activity remaining) under conditions where the wild type enzyme was completely inactivated (<1% activity remaining) (Fig. 8). The other Lys mutants remained susceptible to wortmannin inactivation (Fig. 8).

3. Discussion

AAC(6′)-APH(2″) and APH(3′)-IIIa are major aminoglycoside antibiotic resistance determinants in Gram-positive pathogens such as *Enterococcus* and *Staphylococcus* (for review see [22]). The two enzymes share a common active site motif, which is similar to that of protein and lipid kinases. The structure of APH(3′)-IIIa confirmed the similarity with protein and lipid kinases [23], and further functional studies showed that both AAC(6′)-APH(2″) and APH(3′)-IIIa have serine protein kinase activity [3] and are sensitive to protein kinase inhibitors [5]. Although the phosphotransferase domain of the bifunctional enzyme is likely to be structurally homologous to APH(3′)-IIIa, APH(3′)-IIIa and APH(2″)-Ia have important functional differences, including different aminoglycoside substrate specificities [7,8], phosphoryl transfer regiospecificities [7–10], kinetic mechanisms [11–13], and inhibitor sensitivities [5]. The structure of APH(3′)-IIIa has provided much valuable information that has directed our search for inhibitors of aminoglycoside kinases, but considering the important differences with APH(2″)-Ia, we also sought structural information concerning this important aminoglycoside resistance determinant.

3.1. *Inactivation of AAC(6′)-APH(2″) with wortmannin*

FSBA had previously been shown to inactivate APH(3′)-IIIa by covalently modifying a conserved lysine

that is important in binding ATP [14], but this compound had no effect on APH(2″)-Ia. In contrast, wortmannin, a potent PI 3-kinase inactivator, was able to inhibit APH(2″)-Ia but had no effect on APH(3′)-IIIa. Peptide mapping of APH(2″)-Ia following inactivation by wortmannin under saturating conditions and after prolonged incubation identified four candidate peptides containing Lys residues, which have the potential to react with the compound. Mutagenesis of only Lys52 substantially altered the aminoglycoside modifying ability of APH(2″)-Ia and wortmannin did not inactivate the Lys52Ala mutant but was still able to inactivate the other Lys to Ala mutants, demonstrating that wortmannin inactivation of APH(2″)-Ia requires Lys52 but not the other lysines identified in the mapping experiment. ATP protection of APH(2″)-Ia is consistent with modification of Lys52, where primary sequence alignment has shown this residue to be homologous to Lys44 in APH(3′)-IIIa (Fig. 7), a residue known to interact with the α- and β-phosphates of ATP [23]. We conclude that wortmannin inactivates APH(2″)-Ia by covalently modifying Lys52 in the ATP binding pocket. This conclusion is consistent with experiments on PI 3-kinase where wortmannin was shown to inactivate this lipid kinase by covalent modification of Lys802 [19], a residue homologous to Lys52 and Lys44 in APH(2″)-Ia and APH(3′)-IIIa respectively. Modification of the three other lysines in APH(2″)-Ia was likely an artifact of the prolonged incubation conditions used and to the non-specific reactivity of this electrophilic compound.

3.2. Differences in the ATP binding pockets of aminoglycoside phosphotransferases and implications for inhibitor design

It is intriguing that FSBA and wortmannin modify the homologous residue in the aminoglycoside phosphotransferases, and yet FSBA is specific for APH(3′)-IIIa and wortmannin is specific for APH(2″)-Ia. This suggests that there are fundamental differences in the ATP binding pockets of these two enzymes, in agreement with our previous studies showing that the enzymes have different sensitivities to certain protein kinase inhibitors which are generally targeted towards the ATP binding pocket [5]. Even among very similar protein kinases, there are differences in inhibitor sensitivities, and many potent and specific inhibitors have been directed against the ATP binding pocket of important pharmacological targets (reviewed in [24]). However, in the case of aminoglycoside kinases, it would be more desirable to develop broad range inhibitors that could inhibit a number of enzymes. The results of this study suggest that it would be challenging to develop broad range and potent inhibitors of aminoglycoside kinases by directing compounds against the ATP binding pocket. A broad range inhibitor would likely need to take advantage of other features common to the APHs such as the aminoglycoside binding pocket, but even then, design would be difficult considering that APHs show a diverse range of aminoglycoside substrate profiles and regiospecificities.

More potent and specific inactivators of APH(2″)-Ia based on the wortmannin structure would likely require modifications around the C and D rings, as studies with PI 3-kinase have identified these rings as critical in initial binding efficiency [20,25]. In the case of APH(2″)-Ia, positively charged substitutions might be favored as a negatively charged active site is a common motif in the structures of aminoglycoside modifying enzymes [23,26–28].

4. Significance

The most clinically relevant mode of resistance to aminoglycosides is the enzymatic modification of these antibiotics. A structural understanding of the resistance enzymes will aid in the development of potent inhibitors, where active site labeling techniques provide a means of identifying important enzymatic residues. Wortmannin, a potent PI 3-kinase inhibitor, inactivates the phosphotransferase domain of AAC(6′)-APH(2″) (APH(2″)-Ia) by covalently modifying an absolutely conserved lysine in the ATP binding pocket, however, it does not inactivate APH(3′)-IIIa. Likewise, FSBA, an electrophilic ATP analogue inactivates APH(3′)-IIIa but does not affect APH(2″)-Ia activity, suggesting that the APHs have important differences in their ATP binding pockets. Considering these differences, the design of broad range inhibitors against APHs will require interactions with other sites common to APHs, such as the aminoglycoside binding pocket. Wortmannin inactivation of APH(2″)-Ia complements other functional and structural studies demonstrating that protein, lipid and aminoglycoside kinases all share a common structural motif and suggests that these classes of enzymes all have a common ancestor. This finding augurs well for the discovery of APH inhibitors among the libraries of compounds developed for other pharmacologically important kinases.

5. Materials and methods

5.1. Chemicals

ATP, acetyl CoA, dithiodipyridine, β-NADH, phosphoenol pyruvate, pyruvate kinase/lactate dehydrogenase enzymes, and wortmannin were from Sigma (St. Louis, MO, USA). Kanamycin A, gentamicin and IPTG (isopropyl-β-D-thiogalactopyranoside) were from Bioshop (Burlington, ON, Canada). Restriction endonucleases were from MBI Fermentas (Hamilton, ON, Canada). All oligonucleotide primers were synthesized at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University.

5.2. Subcloning of aph(2″)-Ia into pET22b(+) and aac(6′)-aph(2″) into pET15b(+)

Plasmid pETBFAPH was constructed by the ligation of a 1 kb *aph(2″)-Ia* fragment of the bifunctional *aac(6′)-aph(2″)* gene beginning at position 525 of the gene, corresponding to Met175 (D. Daigle and G. Wright, unpublished) digested with *Nde*I and *Hin*dIII into pET22b(+), and likewise, plasmid pET15AACAPH was constructed by the ligation of the 1.5 kb *aac(6′)-aph(2″)* insert from pBF9 [8] digested with *Nde*I and *Bam*HI into pET15b(+), which generates the N-terminal 6-His-tagged enzyme. The new constructs were transformed into $CaCl_2$-competent *E. coli* BL21(DE3) for subsequent overexpression and purification.

5.3. Purification of enzymes

APH(3′)-IIIa was purified by previously described methods [7].

Overexpression of APH(2″)-Ia and the N-terminal His-tagged AAC(6′)-APH(2″) was similar to that for APH(3′)-IIIa, where 1 l of Luria broth supplemented with 50 μg/ml kanamycin A was inoculated with 10 ml of an overnight culture of *E. coli* BL21(DE3) containing the appropriate constructs and grown at 37°C for 3 h until $OD_{600} \sim 0.6$. Enzyme production was induced by the addition of 1 mM IPTG and grown for a further 3 h at 37°C. The cells were collected by centrifugation at $5000 \times g$ for 10 min, resuspended and washed with 0.85% NaCl, before storing them overnight at −20°C.

The purification of APH(2″)-Ia followed the same procedure as that used to purify the full length, non-His-tagged enzyme as outlined in [8]. For the purification of the N-terminal His-tagged version of AAC(6′)-APH(2″), the cells were resuspended in 15 ml 50 mM Na_2HPO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole, lysed using a French pressure cell at 20 000 psi and the supernatant cleared by centrifugation (10 000 × *g* for 20 min). The 15 ml of crude lysate was gently mixed with 5 ml Ni NTA agarose for 1 h at 4°C, and then loaded onto a column (9 × 3 cm). The resin was washed with 20 ml 50 mM Na_2HPO_4 pH 8.0, 300 mM NaCl, 20 mM imidazole and 20 ml 50 mM Na_2HPO_4 pH 8.0, 300 mM NaCl, 40 mM imidazole. The protein was eluted with 25 ml 50 mM Na_2HPO_4 pH 8.0, 300 mM NaCl, 250 mM imidazole. The fractions containing purified AAC(6′)-APH(2″) were pooled, concentrated over an Amicon PM30 membrane and dialyzed against 50 mM HEPES pH 7.5, 1 mM EDTA. Approximately 50 mg of His-tagged AAC(6′)-APH(2″) was purified from a 1 l cell culture.

Protein concentrations were determined using the Bradford method [29].

5.4. APH and AAC kinetic assays

The assays used to measure aminoglycoside phosphotransferase and acetyltransferase activities were previously described in [8]. However, most assays were scaled down from 1 ml to 250 μl volumes, so they could be conducted in 96 well microtiter plates using a Molecular Devices SpectraMax Plus microtiter plate reader.

For Michaelis–Menten kinetic determinations, initial rates were fit by non-linear least squares to Eq. 1, using Grafit version 4.0 [30].

$$v = (k_{cat}/E_t)[S]/(K_m + [S]) \quad (1)$$

5.5. Inactivation of AAC(6′)-APH(2″) by wortmannin

Wortmannin inactivation experiments were conducted in 50 mM HEPES-NaOH pH 7.5 at room temperature with various concentrations of wortmannin dissolved in dimethyl sulfoxide (DMSO; 5% v/v for all wortmannin concentrations including controls) in a total volume of 20 μl. Reactions were initiated by the addition of enzyme (0.3–0.5 nmol) and 5 μl aliquots were removed, added to 37°C temperature-equilibrated assay broth and assayed for kanamycin kinase or acetyltransferase activity.

5.6. Large scale affinity labeling of APH(2″)-Ia to determine the site(s) of modification

APH(2″)-Ia (100 nmol) was inactivated by the addition of several aliquots of wortmannin (1 μmol every 2 h for a total of 6 h) to the inactivation buffer consisting of 50 mM HEPES-NaOH pH 7.5 and 10 mM $MgCl_2$ in a total volume of 0.5 ml. The control incubation, without wortmannin but with an equivalent amount of DMSO, was carried out simultaneously. Inactivation was deemed complete when $< 2\%$ of the kanamycin phosphotransferase activity remained.

The volume of the reactions was increased to 1.5 ml, and the solutions were dialyzed against 2 l 25 mM NH_4HCO_3 pH 8.3 at 4°C. One volume of 200 mM Tris–HCl pH 8.3, 2 mM EDTA, 8 M urea and 4 mM dithiothreitol was then added and the solutions stirred for 1 h under nitrogen at room temperature. To cap cysteine residues, 1.1 equivalents iodoacetic acid per cysteine residue (550 nmol) were added to the reaction mixture and stirred for an additional 60 min in the dark. The reactions were subsequently dialyzed overnight against 20 mM NH_4HCO_3 pH 8.3 at 4°C. The solutions were then evaporated using a SpeedVac, before resuspension in 100 μl 100 mM NH_4HCO_3 pH 8.3, 8 M urea. The reactions were incubated at room temperature for 30 min prior to the addition of 45.5 μl 100 mM NH_4HCO_3 pH 8.3 and 5.5 μl of trypsin solution (2 mg trypsin in 50 ml 1.2 M HCl). The trypsin digestion was allowed to proceed for 24 h at room temperature in the dark before freezing and storing the reactions at −80°C.

Table 4
Oligonucleotide primers used in this study to generate appropriate APH(2″)-Ia mutants

Mutant	Mutagenic primer (5′ to 3′)
Lys13Ala	GCCACAAATGTTGCGGCAATGAAATATTTAATTGAGC
Lys52Ala	GGCATATTTAGTTAATAATGAATACATTTTTGCAACAAAATTTAG
Lys125Ala	GTCAGAAGAAGAACAAAATTTGTTAGCACGAGATATTGCC
Lys287Ala	GGAATTAAAAATATTGCACAGGAATTTATCGAAAATGGTAGAAAAG

5.7. Trypsin digest analysis

Multiple peptide sequences were determined in a single run by microcapillary reverse-phase chromatography directly coupled to a Finnigan LCQ quadrupole ion trap mass spectrometer equipped with a custom nanoelectrospray source. The column was packed in-house with 10 cm of POROS 10R2 into a New Objective one-piece 75 μm in diameter column terminating in an 8.5 μm tip. Flow rate was nominally 200 nl/min. The ion trap repetitively surveyed MS (395–1400 *m*/*z*), executing data-dependent scans on the three most abundant ions in the survey scan, allowing high resolution (zoom) scans to determine charge state and exact mass, and MS/MS spectra for peptide sequence information. MS/MS spectra were acquired with a relative collision energy of 30% and an isolation width of 2.5 Da. Recurring ions were dynamically excluded. After database correlation with SEQUEST, modified peptides were confirmed by manual interpretation of the MS/MS spectra using FuzzyIons [31,32].

5.8. Site-directed mutagenesis of selected lysine residues to alanine

Peptide mapping results identified four potential Lys residues that were being modified by wortmannin. To determine the role of each Lys in the activity of APH(2″)-Ia, they were individually mutated to Ala using the Quik-Change mutagenesis method (Stratagene, La Jolla, CA, USA). Briefly, the appropriate mutagenic oligonucleotides (Table 4) and their reverse complements were used in combination with 10 ng template DNA (pETB-FAPH) in *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA)-catalyzed PCR reactions. Parental DNA was digested with *Dpn*I, before the mutant plasmid DNA was transformed into $CaCl_2$-competent *E. coli* XL1-Blue. Positive clones were sequenced in their entirety before transformation into *E. coli* BL21(DE3) and subsequent protein purification.

5.9. Absorbance spectrum of HisAAC(6′)-APH(2″) inactivated with wortmannin

The course of the reaction between HisAAC(6′)-APH(2″) (17 nmol) and wortmannin (250 nmol) in 0.5 ml 50 mM HEPES-NaOH pH 7.5 at room temperature was followed by taking absorbance scans from 200 nm to 600 nm every 10 min using a Cary 3E UV/Vis spectrophotometer.

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