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Nucleoside triphosphate cosubstrates control the substrate profile and efficiency of aminoglycoside 3'-O-phosphotransferase type IIa†

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Aminoglycosides (AGs) are broad-spectrum antibiotics that play an important role in the control and treatment of bacterial infections. Despite the great antibacterial potency of AGs, resistance to these antibiotics has limited their clinical applications. The AG 3'-O-phosphotransferase of type IIa (APH(3')-IIa) encoded by the *neo^R* gene is a common bacterial AG resistance enzyme that inactivates AG antibiotics. This enzyme is used as a selection marker in molecular biology research. APH(3')-IIa catalyzes the transfer of the γ -phosphoryl group of ATP to an AG at its 3'-OH group. Although APH(3')-IIa has been reported to utilize exclusively ATP as a cosubstrate, we demonstrate that this enzyme can utilize a broad array of NTPs. By substrate profiling, TLC, and enzyme kinetics experiments, we probe AG phosphorylation by APH(3')-IIa with an extensive panel of substrates and cosubstrates (13 AGs and 10 NTPs) for the purpose of gaining a thorough understanding of this resistance enzyme. We find, for the first time, that the identity of the NTP cosubstrate dictates the set of AGs modified by APH(3')-IIa and the phosphorylation efficiency for different AGs.

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Introduction

The 2-deoxystreptamine aminoglycosides (2-DOS AGs, Fig. 1A) are broad-spectrum antibiotics that display excellent potency against Gram-positive and Gram-negative bacteria as well as mycobacteria.^{1–4} As one of the most effective weapons in the combat against bacterial infections, these aminocyclic sugars are classified as 4,5- or 4,6-disubstituted AGs based on the positions of substitution on the 2-DOS core.⁵ In the USA, amikacin (AMK), gentamicin (GEN), and tobramycin (TOB) are administered systemically.⁶ European countries also employ netilmicin (NET) and sisomicin (SIS) in their antibacterial regimens.⁷ Kanamycin (KAN) is used globally against drug-resistant *Mycobacterium tuberculosis* infections. In addition to their use as antibiotics, AGs have been widely explored as therapeutic options for premature termination codon (PTC) diseases for the ability of these drugs to promote

readthrough of a PTC to allow biosynthesis of functional full-length proteins.^{8–12}

Unfortunately, incorrect or non-compliant use of antibiotics or overuse of certain antibiotics has given rise to one of the greatest global health threats: antibiotic resistance. Bacteria can acquire resistance to AG antibiotics *via* three mechanisms: by changing the cell wall composition, modifying the antibiotic target (in this case the ribosome), and acquiring or upregulating AG-modifying enzymes (AMEs).¹³ AMEs represent the prevalent mechanism of AG resistance. AMEs are represented by three types of enzymes, each catalyzing a distinct chemistry: AG *N*-acetyltransferases (AACs), AG *O*-nucleotidyltransferases (ANTs), and AG *O*-phosphotransferases (APHs).¹⁴ APHs transfer a phosphoryl group from an NTP (Fig. 1B) onto a specific OH group of an AG yielding a phosphorylated AG and an NDP.^{15,16} This transfer occurs with the regiospecificity dictated by the specific class of the APH indicated in the enzyme name according to the AME nomenclature. Such phosphorylation abrogates AG binding to the ribosome, making the bacteria highly resistant to that AG.

Harnessing their AG resistance function, researchers have been using APH enzymes as antibiotic selection markers in recombinant DNA technology. A subject of the present study, APH(3')-IIa, encoded by the neomycin resistance (*neo^R*) gene *aph(3')-IIa*, has been used as an AG resistance marker in

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Fig. 1 Structures of A. AGs with their 2-DOS core highlighted in orange, and B. (d)NTPs used in this study.

molecular biology for decades, providing robust selection in AG-containing media.^{17–19}

A key issue to considering AGs as therapies or genetic selection tools is their modification by APHs. Therefore, a systematic investigation of the substrate–cosubstrate profile of APH enzymes is necessary. As indicated by the nomenclature, APH(3′)-IIa and other APH(3′) enzymes phosphorylate AGs at their 3′-OH group. A partial AG substrate profile of APH(3′)-IIa was previously reported.^{20,21} It was previously reported that APH(3′)-IIa used exclusively ATP, whereas another APH(3′) enzyme, APH(3′)-Ia, also transferred a phosphate from GTP.²² In fact, GTP was used in some *in vitro* studies of these enzymes.^{22–26} In contrast to APH(3′), some APH(2′) enzymes, such as APH(2′)-Ia,²⁷ APH(2′)-If,²⁸ and APH(2′)-IIIa,²⁷ showed a clear preference for GTP. The study of APH(3′)-IIa described herein addresses the enzyme activity for different substrate–cosubstrate pairs in order to help us better understand this resistance enzyme.

Results and discussion

Determination of AG profile of APH(3′)-IIa with various NTPs

To examine whether and, if so, how different NTPs may dictate the AG substrate profile of APH(3′)-IIa, we measured its AG phosphorylation activity by a well-established UV-vis assay. In this assay, we monitored the conversion of NADH to NAD⁺ by pyruvate kinase (PK), used here as a coupled NADH-dependent enzyme converting the NDP coproduct of the AG phosphorylation back to NTP (Fig. S1†).^{20,22,26,29} We tested 13 AGs (AMK, apramycin (APR), GEN, geneticin (G418), hygromycin (HYG), KAN, neamine (NEA), neomycin B (NEO), NET, paromomycin (PAR), ribostamycin (RIB), SIS, and TOB) along with 9 NTPs (ATP, dATP, GTP, dGTP, UTP, dUTP,

dCTP, TTP, and ITP). Due to the lack of a commercially available CTP of sufficient purity for this assay, we had to exclude CTP from our UV-vis assay. In addition to ATP, we found APH(3′)-IIa to use 7 out of the 9 (d)NTPs tested as cosubstrates. We discovered that purine-containing NTPs generally supported phosphorylation of a larger number of AGs than did pyrimidine-containing NTPs. When ATP was used as a cosubstrate, APH(3′)-IIa phosphorylated 7 AGs (AMK, G418, KAN, NEA, NEO, PAR, and RIB), amongst which PAR and RIB have not been reported previously (Table 1). Interestingly, when using GTP as a cosubstrate, we noticed that while KAN, NEA, NEO, PAR, and RIB could be readily phosphorylated by APH(3′)-IIa, the enzyme displayed a much lower activity for G418 and no activity for AMK. Among the purine-containing NTPs, ITP supported the narrowest AG profile, with only NEA, NEO, PAR, and RIB being efficiently phosphorylated. It was also notable that KAN, while being efficiently phosphorylated with ATP or GTP as cosubstrates, was a poor substrate when the enzyme was provided with ITP.

Even though UTP was a pyrimidine-containing NTP, potentially interacting suboptimally with the base binding site compared to a purine-containing NTP, such as ITP, the AG substrate profile for UTP was identical to that observed for ITP. These observations indicate that different perturbations of base structure and size can have similar effects of limiting the substrate profile.

To test the effect of the 2′-OH group on the ribose ring, we compared the AG profiles for NTP and dNTP as cosubstrates. We found that the AG profiles of the enzyme were similar for dATP and ATP, dGTP and GTP, and dUTP and UTP. As one minor exception, with dUTP the enzyme was slightly more active with KAN and slightly less active with RIB than with UTP. We found that the dNTPs that were cosubstrates for the

Table 1 AG phosphorylation by APH(3')-IIa with various (d)NTP cosubstrates by UV-vis assay and TLC

		AMK	APR	GEN	G418	HYG	KAN	NEA	NEO	NET	PAR	RIB	SIS	TOB
ATP	UV-vis	✓	✗	✗	✓	✗	✓	✓	✓	✗	✓	✓	✗	✗
	TLC	✓	✗	✗	✓	✗	✓	✓	✓	✗	✓	✓	✗	✗
GTP	UV-vis	✗	✗	✗	--	✗	✓	✓	✓	✗	✓	✓	✗	✗
	TLC	✓	✗	✗	✗	✗	✓	✓	✓	✗	✓	✓	✗	✗
CTP	UV-vis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	TLC	p	✗	✗	✗	✗	✓	✓	✓	✗	✓	✓	✗	✗
UTP	UV-vis	✗	✗	✗	✗	✗	--	✓	✓	✗	✓	✓	✗	✗
	TLC	✓	✗	✗	✗	✗	✓	✓	✓	✗	✓	✓	✗	✗
ITP	UV-vis	✗	✗	✗	✗	✗	--	✓	✓	✗	✓	✓	✗	✗
	TLC	✓	✗	✗	✗	✗	✓	✓	✓	✗	✓	✓	✗	✗
dATP	UV-vis	--	✗	✗	✓	✗	✓	✓	✓	✗	✓	✓	✗	✗
	TLC	✓	✗	✗	✓	✗	✓	✓	✓	✗	✓	✓	✗	✗
dGTP	UV-vis	--	--	✗	✓	--	✓	✓	✓	✗	✓	✓	✗	✗
	TLC	p	✗	✗	✓	✗	p	✓	✓	✗	✓	✓	✗	✗
dCTP	UV-vis	✗	✗	✗	✗	✗	✗	--	--	✗	--	--	✗	✗
	TLC	✗	✗	✗	✗	✗	✓	✓	✓	✗	✓	✓	✗	✗
TTP	UV-vis	✗	✗	✗	✗	✗	✗	--	--	✗	--	--	✗	✗
	TLC	✓	✗	✗	✓	✗	✓	✓	p	✗	✓	✓	✗	✗
dUTP	UV-vis	✗	✗	✗	✗	✗	✓	✓	✓	✗	✓	--	✗	✗
	TLC	✗	✗	✗	✓	✗	✓	✓	✓	✗	✓	✓	✗	✗

✓ = absorbance >0.1 (UV-vis) or phosphorylated (TLC), p = partially phosphorylated (TLC), -- = absorbance between 0.05–0.1 (UV-vis), ✗ = absorbance <0.05 (UV-vis) or not phosphorylated (TLC), N/A = not tested.

most limited sets of AGs were dCTP and TTP (note that TTP naturally contains a deoxyribose sugar). This was not surprising since both dNTPs contained a monocyclic base that may not engage optimally with the enzyme NTP binding site.

Thin-layer chromatography

In order to visualize directly the phosphoryl transfer activity of APH(3')-IIa, we performed TLC experiments (Fig. 2 and S2,† and Table 1). We first used ATP as a representative cosubstrate and carried out reactions with all 13 AGs. For the

AGs that were observed to be modified by the UV-vis assay, we made two time-point observations, at 2 h and at 24 h. For the other AGs, we quenched the reactions only after 24 h. For each AG, we also included an AG substrate standard as well as a full reaction that we quenched immediately after initiating it (time 0) to confirm that the reaction conditions did not interfere with AG migration on TLC plates.

Upon the enzymatic modification of the AGs that were previously found to be accepted by the enzyme as a substrate (AMK, G418, KAN, NEA, NEO, PAR, and RIB) we observed product species with significantly larger R_f values than those

Fig. 2 Pictures of TLC plates of APH(3')-IIa reacting with ATP and various AGs as well as the corresponding R_f values for each lane.

of the respective substrates. The AGs that were not observed to be substrates by the UV-vis assay (APR, GEN, HYG, NET, SIS, and TOB) did not display a shift in their R_f values after enzymatic reactions. These AGs were either highly structurally distinct (APR, HYG) or they did not contain a 3'-OH group (GEN, NET, SIS and TOB). These observations were in agreement with the AG phosphorylation results obtained by the UV-vis assay.

As the UV-vis assay relies on the activity of a coupled enzyme, PK, for conversion of the NDP product back to the respective NTP, the inability to observe activity by this assay for some nucleotides could be due to the NDP specificity of PK, and not APH(3')-IIa. Therefore, we performed TLC with the rest of NTP cosubstrates (24 h time point only, Table 1 and Fig. S2†). As expected, we found that for all the cosubstrates that were shown to support phosphorylation by the UV-vis assay, the reaction was also observed by TLC. In addition, we observed that APH(3')-IIa transferred a phosphoryl group from CTP (not tested in previous UV-vis substrate profile assays) as well as dCTP and TTP, indicating that the minimal UV-vis signal from the coupled enzyme assay was due to PK not accepting dCDP and TDP as substrates. Indeed, we did not observe the activity of PK when we tested this enzyme with dCDP and TDP in the absence of APH(3')-IIa and AG. Furthermore, we observed phosphorylation of AGs in some AG-NTP combinations where no phosphorylation was observed previously in substrate profiling experiment, including AMK with GTP, UTP, ITP, dATP, and dGTP (partial), G418 with dUTP, and KAN with UTP and ITP. This likely occurred due to the much higher reactant and enzyme concentrations as well as a longer reaction time used in the TLC assays.

Determination of enzyme kinetics by UV-vis assays

To characterize quantitatively the reaction kinetics for different substrates and cosubstrates, we determined the Michaelis-Menten kinetic parameters (K_m and k_{cat}) for APH(3')-IIa with respect to representative AGs using ATP, GTP, and UTP as cosubstrates at a fixed concentration (2 mM). We chose NEO and KAN as representative 4,5- and 4,6-disubstituted AGs, as they were shown to be good substrates for most NTP cosubstrates. We also selected the 4,6-disubstituted AGs AMK and G418, because APH(3')-IIa displayed the highest variation in its phosphorylation activity with these AGs among different NTPs as cosubstrates. Because APH enzymes characterized to date follow either an ordered sequential mechanism, where the NTP binds the enzyme prior to the AG, or a random sequential mechanism,¹⁵ K_m in these experiments can be interpreted in terms of the binding affinity of the AG to the enzyme-NTP complex.

We found that with ATP as a cosubstrate, APH(3')-IIa displayed the highest affinity for KAN with a K_m value of $3.1 \pm 0.9 \mu\text{M}$ (Table 2 and Fig. S3†). Of note, even though APH(3')-IIa is often referred to as the product of the *neo*^R gene, the K_m value for NEO is nearly 6-fold higher, 17 ± 4

Table 2 Kinetic parameters of APH(3')-IIa with respect to AGs

AG	NTP	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)
KAN	ATP	3.1 ± 0.9	34 ± 2	11 ± 3
	GTP	69 ± 9	6.3 ± 0.3	0.09 ± 0.01
	UTP	40 ± 6	2.8 ± 0.1	0.07 ± 0.01
NEO	ATP	17 ± 4	49 ± 3	2.9 ± 0.7
	GTP	23 ± 5	43 ± 2	1.9 ± 0.4
	UTP	5.4 ± 1.1	10.0 ± 0.4	1.9 ± 0.4
G418	ATP	113 ± 12	89 ± 3	0.79 ± 0.09
	GTP	2172 ± 732	12 ± 2	0.006 ± 0.002
AMK	ATP	1040 ± 260	51 ± 7	0.05 ± 0.01

μM . APH(3')-IIa displayed similar maximum turnover rates of phosphorylation of NEO and KAN ($k_{cat} = 49 \pm 3 \text{ min}^{-1}$ and $34 \pm 2 \text{ min}^{-1}$, respectively). As a result, when using ATP as a cosubstrate, the enzyme was found to be more efficient with KAN ($k_{cat}/K_m = 11 \pm 3 \text{ min}^{-1} \mu\text{M}^{-1}$) than with NEO ($k_{cat}/K_m = 2.9 \pm 0.7 \text{ min}^{-1} \mu\text{M}^{-1}$). APH(3')-IIa showed much lower binding affinities for G418 ($K_m = 113 \pm 12 \mu\text{M}$) and AMK ($K_m = 1040 \pm 260 \mu\text{M}$) than for NEO and KAN. A likely structural explanation for the lack of affinity for AMK and G418 is that the bulky (S)-4-amino-2-hydroxybutyryl (AHB) group at the 1-position of AMK or the additional methyl group at the 6'-position of G418 might not fit well into the substrate binding pocket (Fig. 3A). The 6'-amino group of KAN and presumably NEO is bound in a negatively-charged environment of the C-terminal carboxyl group, Asp154 and Asp157. G418 does not contain a 6'-amino group, lacking a positive charge at this position. In addition, the 3"-amino group of G418 is methylated and does not bear a positive charge, unlike its non-methylated counterpart in KAN and NEO. This amino group in KAN is in a negatively-charged environment of Glu230 (Fig. 3A). The absence of positive charges at 6'- and 3"-positions in G418 is predicted to disfavor its binding. Despite the drastically higher K_m values, the maximum turnover rates for G418 ($k_{cat} = 89 \pm 3 \text{ min}^{-1}$) and AMK ($k_{cat} = 51 \pm 7 \text{ min}^{-1}$) were found to be comparable to those for NEO and KAN, indicating that the geometry of the phosphoryl transfer reaction was unchanged. Consequently, the catalytic efficiencies of the enzyme with AMK ($k_{cat}/K_m = 0.049 \pm 0.014 \text{ min}^{-1} \mu\text{M}^{-1}$) and G418 ($k_{cat}/K_m = 0.79 \pm 0.09 \text{ min}^{-1} \mu\text{M}^{-1}$) were lower than with NEO and KAN.

Interestingly, when we switched the cosubstrate from ATP to GTP, we observed a change in the AG binding preference. With GTP, APH(3')-IIa had the highest affinity for NEO ($K_m = 23 \pm 5 \mu\text{M}$), which was similar to that observed with ATP. Furthermore, the enzyme turnover and overall efficiency ($k_{cat} = 43 \pm 2 \text{ min}^{-1}$ and $k_{cat}/K_m = 1.9 \pm 0.4 \text{ min}^{-1} \mu\text{M}^{-1}$) were similar to those observed with ATP as the cosubstrate. These observations indicate that GTP binds the enzyme and acts as a phosphoryl donor similarly to ATP in the presence of NEO. With GTP, the enzyme had a much weaker, 20-fold lower affinity for KAN ($K_m = 69 \pm 9 \mu\text{M}$) than it did with ATP (see next section for structural rationale). GTP also had a deleterious (6-fold) effect on the maximum turnover rate of phosphoryl transfer from GTP to KAN: $k_{cat} = 6.3 \pm 0.3 \text{ min}^{-1}$. It is possible that the

Fig. 3 Computational superimposition of the protein structures of A. APH(3')-IIa (in pale yellow) with KAN (in bright yellow) bound (PDB ID: 1ND4)²⁵ and APH(3')-IIIa (in pale blue) with KAN (in teal) bound (PDB ID: 1L8T).³² Asp154, Asp157, Asp227, Glu230, and Phe264 in APH(3')-IIa are shown in orange. Ser227 in APH(3')-IIIa as well as the two residues forming the salt bridge, Glu160 and Arg229, are shown in dark teal. B. APH(3')-IIa (in pale yellow) with KAN (in bright yellow) bound (PDB ID: 1ND4)²⁵ and APH(3')-IIIa (in pale green) with ADP (in dark green) and NEO (in bright green) bound (PDB ID: 2B0Q).³² Leu103, Cys192, Pro194, and Glu231 of APH(3')-IIa are shown in orange. The Na⁺ ion coordinating Cys192 and the 2'- and 3'-OH of KAN is shown in purple.

dissociation of GDP, if rate limiting, as observed for APH(3')-IIIa,^{30,31} was slower than that of ADP. The different k_{cat} values for the same AG and different NTP cosubstrates can be rationalized as a consequence of minor shifts in the positioning and orientation of the γ -phosphate of different NTPs; the differences in K_{m} values suggest an allosteric effect of these small changes on interactions of the AG with the enzyme.

When GTP was used as a cosubstrate with G418, the K_{m} value for G418 was 20-fold higher ($K_{\text{m}} = 2172 \pm 732 \mu\text{M}$) than that measured with ATP as a cosubstrate, indicating that G418 was a poor substrate for this enzyme. With the much larger K_{m} and smaller k_{cat} ($12 \pm 2 \text{ min}^{-1}$) values, the catalytic efficiency for G418 with GTP became more than 100-fold smaller than that with ATP as a cosubstrate ($k_{\text{cat}}/K_{\text{m}} = 0.006 \pm 0.002 \text{ min}^{-1} \mu\text{M}^{-1}$). Phosphoryl transfer from GTP to AMK was below the detection limit. This again indicated that the minor chemical differences in NTP transmit to both the catalytic center and the AG binding pocket. Overall, these kinetic measurements were consistent with those observed during the substrate profile determination (Table 1).

When we explored the enzyme kinetics for UTP as a cosubstrate, we found that APH(3')-IIa displayed higher binding affinity and lower turnover rates than with GTP for both KAN and NEO, which resulted in similar overall catalytic efficiencies to those observed with GTP. It is possible that the pyrimidine base of UTP, while having a favorable allosteric effect on the AG binding site, resulted in a slower dissociation of UDP. Alternatively, the change from a purine to a pyrimidine base may have resulted in slight mispositioning of the γ -phosphate group, slowing the phosphoryl transfer step. APH(3')-IIa did not accept G418 and AMK as substrates when UTP was used.

The underlying assumption of these kinetic measurements is that the observed coupled reaction is not rate-limited by a

coupled enzyme, PK, acting on the NDP product of the reaction of interest. We tested this directly by carrying out representative kinetic assays for all relevant nucleotides with 2-fold higher concentrations of the coupled enzymes than those used in the above measurements (Fig. S4†). The progress curves with the original and with the doubled concentrations of the coupled enzymes were the same within experimental uncertainty, indicating that, indeed, the above kinetics were not affected by potential variations in activity of PK with different NDP substrates.

Structural insight into the NTP effect of the AG profile

We examined available crystal structures of APH(3')-IIa in complex with KAN (PDB ID: 1ND4)²⁵ as well as two crystal structures of APH(3')-IIIa, a thoroughly characterized homologous APH, in complexes with ADP and KAN (PDB ID: 1L8T)³² and with ADP and NEO (PDB ID: 2B0Q)³² to help us understand the effects of NTP on the AG profile.

The structures of APH(3')-IIa in complex with KAN and that of APH(3')-IIIa in complex with ADP and KAN are highly superimposable (Fig. 3A). Nevertheless, differences can be observed between the two superimposed structures, specifically at the different accommodation of KAN ring III of the two proteins (Fig. 3A). For instance, APH(3')-IIa contains a salt bridge formed by Glu160 and Arg229 that is absent in APH(3')-IIIa. The salt bridge could help stabilize the flexible loop (Asp150-Phe165 in APH(3')-IIIa)³² at the AG-binding site in a closed form upon KAN binding. Another difference in binding of KAN ring III between the two enzymes is in that the larger Asp227 in APH(3')-IIa than Ser227 in APH(3')-IIIa positions ring III of KAN further away from the protein core, consequently forcing the AG-gating flexible loop outward into a seemingly looser conformation in APH(3')-IIa.

Next, we superimposed the crystal structures of APH(3')-IIa in complex with KAN and APH(3')-IIIa in complex with ADP and NEO (Fig. 3B). Please note that NEO is a 4,5-disubstituted AG with rings III and IV positioned in a different orientation from that of ring III of the 4,6-disubstituted KAN. APH(3')-IIIa was previously reported to be the most substrate promiscuous amongst all APH(3') enzymes.^{32,33} For APH(3')-IIa, the 2'- and 3'-OH of KAN are coordinated, through a Na⁺ ion, to the side chain of Cys192. This highly restrained binding mode, not observed for APH(3')-IIIa, could be an AG-selection mechanism for APH(3')-IIa. The AGs that do not satisfy this coordination, such as NEO, which bears an NH₂ group instead of OH at its 2'-position, may be less favored than KAN. In addition, we can see that Leu103, Pro194, and Cys192 in APH(3')-IIa form a hydrophobic wall that may prevent binding of NEO, which contains an extra ring (IV). Specifically, Leu103, Pro194, and Glu231 sterically clash with ring IV of NEO bound to APH(3')-IIIa in the superimposition. This steric hindrance may contribute to the reason why NEO is a less favored substrate than KAN for APH(3')-IIa when using ATP as a cosubstrate. This surface, especially Pro194, is located close to the ATP binding site, apparently to bind AGs in a U-shaped conformation. Due to this proximity, the binding of a different NTP may induce structural shifts propagated *via* this surface to the AG-binding cavity to affect AG selection.

In addition, we compared APH(3')-IIa to another APH enzyme known for its dual cosubstrate specificity, APH(2'')-IVa.³⁴ We superimposed the crystal structure of APH(3')-IIa (PDB ID: 1ND4)²⁵ with the two structures of APH(2'')-IVa bound to adenosine (PDB ID: 4DT8) and guanosine (PDB ID: 4DT9) (Fig. 4 and S5†).³⁴ The dual nucleotide specificity in

APH(2'')-IVa was attributed to a shift in the hydrogen bonding network when adenine was replaced by guanine. Specifically, the N6 of adenine donates a hydrogen to the carbonyl oxygen of Thr96 of APH(2'')-IVa and the N1 of adenine accepts the hydrogen from the amide nitrogen of Ile96 of APH(2'')-IVa (Fig. S5A†). The O6 and N1 of guanine form hydrogen bonds with the amide nitrogen and carbonyl oxygen Ile98, respectively (Fig. S5B†). We asked if a similar shift could occur with APH(3')-IIa. We found potential hydrogen bond donors and acceptors for both adenine and guanine in this case as well. The N6 and N1 of adenine could serve as hydrogen bond donor and acceptor, respectively, to interact with the backbone oxygen of Gly95 and the backbone nitrogen of Val97 in APH(3')-IIa (Fig. S5C†). On the other hand, guanine could interact with APH(3')-IIa by forming hydrogen bonds between O6, N1, and N2 and the backbone nitrogen and oxygen of Val97, as well as the backbone oxygen of Gly99 (hydrogen bond acceptor) of APH(3')-IIa, respectively (Fig. S5D†). This structural model provides a potential explanation for the broad cosubstrate promiscuity of APH(3')-IIa. These structural proposals are to be tested by detailed structural studies.

Conclusions

In this study, we investigated for the first time how cosubstrate NTPs define the AG profile for APH(3')-IIa. A complex dependence of the AG profile on the identity of the base of the NTP strongly suggests allosteric communication between the cosubstrate and the substrate binding sites. Even though ATP is the most abundant NTP *in vivo*, this study indicates other NTPs should not be disregarded as potential cosubstrates in the context of AG antibiotic overuse.

Experimental

Materials and instruments

The enzymes and buffers used for the molecular cloning of APH(3')-IIa, were purchased from New England BioLabs (NEB; Ipswich, MA), whereas primers for PCR were purchased from Sigma-Aldrich (Milwaukee, WI). All AGs used in this study were purchased from AK Scientific (Union City, CA). ATP, dATP, dCTP, GTP, UTP, TTP, and ITP were purchased from Sigma-Aldrich. dGTP and dUTP were purchased from Promega (Madison, WI). TLC plates (silica gel 60 F254 coated) were purchased from VWR (Radnor, PA). UV-vis assays were performed on a SpectraMax M5 plate reader. Kinetics parameters were determined using SigmaPlot 13.0 (SysStat Software, San Jose, CA).

Cloning, expression and purification of APH(3')-IIa

Cloning of *aph(3')-IIa*-pET28a was performed in *E. coli* TOP10. The *aph(3')-IIa* gene was PCR amplified by using the forward primer 5'-GCGTTTCATATGATTGAACAAGATGG-3' and reverse primer 5'-CCAGAGCTCGAGTCAGAAGAACTCGTC-3' using the pcDNA3.1 vector from Invitrogen. After PCR, the amplified *aph(3')-IIa* gene (817 bp) was purified, digested, with *Nde*I

Fig. 4 Superimposition of APH(3')-IIa (in pale yellow) with KAN (in bright yellow) bound (PDB ID: 1ND4)²⁵ and two crystal structures of APH(2'')-IVa with adenosine (APH(2'')-IVa in light blue, adenosine in dark green, PDB ID: 4DT8) and guanosine (APH(2'')-IVa in light purple, guanosine in purple, PDB ID: 4DT9) bound.³⁴ Residues of APH(3')-IIa are colored in orange, whereas the residues in APH(2'')-IVa are colored in teal and dark purple. The specific interactions between each enzyme and nucleoside are shown in four separate panels in Fig. S5†

and *XhoI* (restriction sites are underlined above) and ligated into an *NdeI/XhoI* digested pET28a vector. The DNA sequence was confirmed (sequencing performed by Eurofins Genomics, Louisville, KY). The *aph(3')-IIa*-pET28a vector was transformed into *E. coli* BL21 (DE3). An overnight culture was grown at 37 °C from a fresh transformant colony. The protein was overexpressed starting with a 1% inoculum in Luria-Bertani (LB) broth (2 L) containing KAN (50 µg mL⁻¹) at 37 °C with shaking at 200 rpm until the attenuation of 0.6 at 600 nm. The protein expression was then induced with IPTG (1 mM) and the cells were grown overnight at 25 °C with shaking at 200 rpm. The cell pellet was resuspended in 50 mL of lysis buffer (25 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 10% glycerol) and the cells were lysed by intermittent sonication. The protein was purified using Ni²⁺-NTA affinity column chromatography in lysis buffer with a gradient of imidazole (10 mL of 5 mM, 3 × 5 mL of 20 mM, 40 mM, and 250 mM). Pure fractions were then dialyzed in 3 × 2 L of Tris-HCl (50 mM, pH 8.0), NaCl (300 mM) and 10% glycerol before being concentrated to 100 µM using a 10 kDa molecular weight cut-off Millipore Amicon Ultra centrifugal device. The pure 32.1 kDa NHis₆-tagged protein was flash-frozen in liquid nitrogen and stored at -80 °C. Protein yield was 8 mg per L of culture.

Determination of AG profile of APH(3')-IIa with various NTPs

To determine the substrate profiles of APH(3')-IIa for different NTP cosubstrates, we used a protocol previously described.^{20,22,26,29} In a 96-well plate, each 200 µL reaction contained AG (100 µM), Tris-HCl (50 mM, pH 8.0), MgCl₂ (10 mM), KCl (40 mM), NADH (0.5 mg mL⁻¹ freshly prepared), PEP (2.5 mM), NTP (2 mM), pyruvate kinase-lactate dehydrogenase (PK-LDH, 4 µL of 10 mg mL⁻¹ solution) (P0294, Sigma Aldrich), and APH(3')-IIa (1 µM), which was added last to the plate to initiate the reaction. The assay was carried out at 37 °C and absorbance at 340 nm was recorded every 30 s for 20 min to monitor the disappearance of NADH. After subtraction from the final absorbance of each reaction that measured for a control reaction in the absence of AG, the absorbance values greater than 0.1 were considered significant and indicated accordingly in Table 1. The AGs for which the corrected absorbance values were in the range of 0.05–0.1 were considered poor substrates for a given NTP. The AGs for which the absorbance values were below 0.05 were considered not to be substrates for APH(3')-IIa.

Thin-layer chromatography

The TLC assays with stains that detected AGs and their phosphorylated products were performed in order to confirm directly that the reactions we observed from the UV-vis assay resulted from phosphorylation of AGs. We first used ATP as a representative NTP. Each reaction contained Tris-HCl (50 mM, pH 8.0), AG (1 mM), NTP (2 mM), MgCl₂ (10 mM), KCl (40 mM), and APH(3')-IIa (5 µM). The eluent system for each AG consisted of different ratios of MeOH and NH₄OH as follows: 3:2/MeOH:NH₄OH for AMK and NEO, 5:2 for APR,

HYG, and SIS, 15:2 for GEN, G418, NET, and TOB, 7:3 for KAN, and 3:1 for NEA, PAR, and RIB. TLC plates for most AGs were stained with ninhydrin, with the exception of G418 and RIB, which were stained with 10% sulfuric acid in MeOH. The stained plates were heated for visualization (Fig. 2). We then performed TLC for the rest of (d)NTPs in the same reaction condition (24 h reactions). The results were summarized in Fig. S2.†

Determination of enzyme kinetics by UV-vis assays with respect to AGs

The reaction conditions were the same as those described in the AG profile by UV-vis assay section and the data collected were processed as previous published.⁵ The first 3 min were used to determine the initial rate of reaction. The best-fit kinetic parameters with respect to each AG are summarized in Table 2 with the Michaelis-Menten curves shown in Fig. S3.†

Validation of PK efficiency in kinetics UV-vis assays

To confirm that kinetic parameters determined for APH(3')-IIa in the UV-vis assay with PK-LDH were not affected by the activity of PK, we used NEO as a representative AG and performed time course reactions containing 1× and 2× (compared to the concentration of the coupled enzymes used in the kinetic assays above) with each relevant nucleotide cosubstrate. Specifically, each reaction contained NEO (250 µM) and the rest of the ingredients at concentrations described in the kinetics assays. We used either 1× (as in the kinetics analysis) or 2× PK-LDH to test whether the concentration of PK-LDH in our kinetics analysis was limiting the rate of APH(3')-IIa enzymes (Fig. S4†).

Conflicts of interest

The authors declare no conflicts of interest.

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