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An Aminoglycoside Microarray Platform for Directly Monitoring and Studying Antibiotic Resistance†

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ABSTRACT: Antibiotic resistance is a major threat to human health. Since resistance to the aminoglycoside class of antibiotics is most commonly caused by enzymatic modification, we developed a high-throughput microarray platform for directly assaying resistance enzyme activity on aminoglycosides. After modification, the array can be hybridized with the therapeutic target, a bacterial rRNA A-site mimic, to study the effect that modification has on binding. Such studies will help identify important factors that contribute to high-affinity recognition of therapeutic targets and low-affinity recognition of and modification by resistance enzymes. This platform may also be useful for screening chemical libraries to discover new antibiotics that evade resistance.

Many antibiotics target the bacterial ribosome by forming direct contacts with ribosomal RNA (*1*−*3*). These observations validate both the ribosome and RNA as important drug targets. Aminoglycosides make up one class of antibiotics that bind rRNA.[1] Most aminoglycosides form specific contacts to the bacterial rRNA A-site, a region of the ribosome that has a central 1 × 2 all-adenine internal loop (*4*, *5*). A myriad of brilliant studies have elucidated a mechanism for aminoglycoside activity that centers on altering the dynamics of nucleotides in the A-site upon mRNA decoding (*6*, *7*). Clinically, aminoglycosides are used to treat severe infections, including those caused by *Mycobacterium tuberculosis* and Gram-negative bacteria (*8*).

Bacterial resistance to aminoglycosides is most commonly caused by O-phosphorylation, O-nucleotidylation (Figure 1), and N-acetylation of the substrate by resistance enzymes. It has been shown for O-phosphorylation and N-acetylation that these modifications weaken the affinity of the drug for the rRNA A-site (*9*). Ideal antibacterials, therefore, would be poor substrates for resistance enzymes and high-affinity binders to the rRNA A-site. New antibiotics are often identified by synthesizing and screening libraries of designer antibiotics (*10*−*12*). The compounds are then screened *in vivo* to test their efficacy or *in vitro* to identify compounds that are not enzymatically modified. Both screening methods, however, have disadvantages. Cell culture studies may not

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[1] Abbreviations: A-site, aminoacyl site; ANT(2″), aminoglycoside nucleotidyltransferase(2″); APH(3′), aminoglycoside phosphotransferase(3′); Boc, *tert*-butylcarbamate; bs, broad singlet; d, doublet; dd, doublet of doublets; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DCM, dichloromethane; HPLC, high-performance liquid chromatography; HR MS, high-resolution mass spectrometry; LR MS, low-resolution mass spectrometry; m, multiplet; MS, mass spectrometry; MWCO, molecular weight cutoff; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; rRNA, ribosomal RNA; s, singlet; TFA, trifluoroacetic acid.

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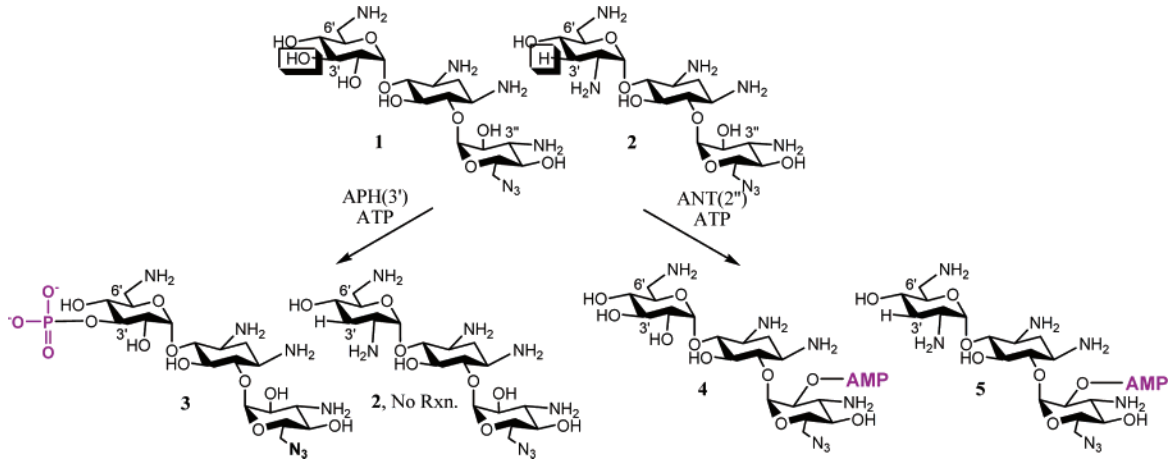


FIGURE 1: Structures of the 6″-azido derivatives of kanamycin A (**1**) and tobramycin (**2**) and the products after reaction with an aminoglycoside phosphotransferase [APH(3′)] and an aminoglycoside nucleotidyltransferase [ANT(2″)]. The boxed atoms represent differences in the structures that affect modification by APH(3′); compound **1** is phosphorylated by APH(3′) while **2** is not.

provide direct information about how structural features of a library member affect binding to the therapeutic target or modification by resistance enzymes. Such studies can be biased by factors such as differential drug uptake. Library members with potent activities but poor uptake are eliminated from the screen, removing the possibility for derivatization to improve uptake. *In vitro* methods, such as filter binding (*13*) and spectrophotometric assays (*14*), can accurately characterize the kinetics of resistance enzymes for a given aminoglycoside substrate; however, they provide no information about the effect that modification has on the affinity for the A-site. Such experiments must be completed subsequently, usually by fluorescence or surface plasmon resonance assays (*9*, *12*, *15*, *16*). Though these methods are useful, they are generally not high-throughput and do not involve examination of modification and the effects that modification has on binding RNA in parallel. More effective *in vitro* screening methods could determine the structural features of the compound that lead to high-affinity recognition of therapeutic targets and low-affinity recognition of and modification by resistance enzymes using a single platform. One such platform amenable to these studies is an aminoglycoside microarray.

Microarrays have several advantages as screening tools. The most significant one is their high-throughput nature since several thousand compounds can be probed on a single array (*17*−*19*). Previous studies have used microarrays to study binding of aminoglycosides to A-site mimics and to resistance enzymes (*20*, *21*). In these studies, however, compounds were immobilized through their amines onto succinimide-functionalized surfaces. This is a significant deficiency because the amine groups used for immobilization form important contacts with A-site rRNA (*5*, *9*, *22*). Immobilization through an amine that makes critical hydrogen bonding interactions with the target RNA will significantly affect RNA recognition (*4*, *5*, *9*). Array-based studies of resistance enzymes were used only to interrogate binding of antibiotics (*20*). The main cause of antibiotic resistance with aminoglycosides, however, is enzymatic modification by resistance enzymes. Therefore, we have developed an aminoglycoside microarray platform that allows for defined immobilization of aminoglycoside substrates, and direct monitoring of both resistance enzyme activity and the effect that modification has on RNA binding.

MATERIALS AND METHODS

General Synthetic Methods. All NMR spectra were recorded on a Varian NMR spectrometer operating at 500, 400, or 300 MHz on proton. Chemical shifts were referenced to residual solvent and are reported in parts per million. Low-resolution mass spectra were recorded on a LCQ Advantage IonTrap LC−MS and Surveyor HPLC system. All chemicals were purchased from Sigma Aldrich and used without further purification unless otherwise noted. Tobramycin was purchased in the free base form, and kanamycin A was purchased as a sulfate salt. Kanamycin A was treated with Dowex resin (−OH form) to convert the sulfate salt to the free base. The free base form was used for all chemical syntheses.

TLCs for all Boc-protected samples were visualized by staining with ninhydrin after removal of the Boc groups. This was accomplished by dipping a plate in a 10% TFA/DCM solution and then drying the plate with a heat gun; this step was repeated. The plate was stained with a ninhydrin solution (0.1 g of ninhydrin, 40 mL of 2-butanol, and 9.2 mL of glacial acetic acid) and developed by heating with a heat gun.

1,3,6′,3″-Tetra-N-(tert-butoxycarbonyl)kanamycin A. A solution of kanamycin A free base (2.57 g, 5.7 mmol) was dissolved in 125 mL of a 4:1 DMSO/H_2O mixture. To this solution was added di-*tert*-butyldicarbonate (12.2 g, 57 mmol, 12 equiv). The reaction mixture was equipped with a reflux condenser and heated at 70 °C overnight, after which a white precipitate formed. The reaction was cooled to room temperature, and 20 mL of NH_4OH was added to further precipitate the product. Filtration afforded a white solid that was washed with H_2O (2 × 300 mL) and dried under vacuum (yield 4.09 g, 81%). The crude sample was not further purified but was suitable for use in subsequent steps: ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.85 (s, 1H), 6.55 (s, 1H), 6.46 (d, 1H, J = 9.5 Hz), 6.3 (s, 1H), 5.32 (d, 1H, J = 4.5 Hz), 5.2 (s, 1H), 4.65 (d, 1H, J = 6.0 Hz), 4.16 (t, 1H, J = 5.0, 5.5 Hz), 3.77 (d, 1H, J = 9.5 Hz), 3.55−3.15 (m, 15H), 3.03 (s, 1H), 1.76 (d, 1H, J = 12.0 Hz), 1.33 (s, 36H); ^{13}C

NMR (75 MHz, DMSO-d_6) δ 157.07, 156.81, 156.03, 155.59, 101.83, 98.53, 84.69, 81.05, 78.46, 77.93, 75.69, 73.60, 73.38, 72.85, 71.02, 68.15, 61.03, 56.62, 50.75, 49.78, 42.12, 35.39, 28.91; LR MS calcd *m*/*z* 907.4 ($M + Na^+$), found *m*/*z* 907.4.

*6″-(2,4,6-Triisopropylbenzenesulfonyl)-1,3,6′,3″-tetra-N-(tert-butoxycarbonyl)kanamycin A (**8**)*. A sample of 1,3,6′,3″-tetra-*N*-(*tert*-butoxycarbonyl)kanamycin A (538 mg, 597 μmol) was dissolved in 8.8 mL of pyridine. To this solution was added 2,4,6-triisopropylbenzenesulfonyl chloride (1.2 g, 3.5 mmol, 6 equiv) in 1 mL of pyridine, and the reaction mixture was stirred overnight. To quench the reaction, 10 mL of MeOH was added and the reaction mixture was stirred for 30 min. Concentrated HCl (7.9 mL) in 100 mL of ice-cold water was added to the reaction mixture, and the mixture was then extracted with EtOAc (3 × 150 mL). The organic layers were combined and extracted with brine, dried over Na_2SO_4, and concentrated to a solid. Crude product was purified by silica gel chromatography (3% MeOH/DCM): yield 320 mg, 270 μmol, 46%; R_f = 0.69 (15% MeOH/DCM); ^{1}H NMR (400 MHz, CD_3OD) δ 7.29 (s, 2H), 5.06 (s, 2H), 4.40 (m, 2H), 4.16 (m, 3H), 3.79−3.34 (m, 13H), 3.20 (t, 1H), 2.96 (q, 1H), 2.02 (d, 1H, J = 10.0 Hz), 1.46−1.48 (m, 37H), 1.29−1.26 (m, 18H); ^{13}C (75 MHz, CD_3OD) δ 159.39, 159.17, 157.86, 157.62, 155.24, 152.24, 130.65, 124.90, 102.76, 99.74, 85.74, 80.96, 80.59, 80.35, 80.18, 76.76, 74.48, 73.86, 72.28, 71.81, 71.57, 69.14, 57.34, 56.03, 52.10, 50.75, 41.85, 35.44, 30.75, 28.85, 28.82, 28.78, 25.16, 25.06, 23.94, 23.94; LR MS calcd *m*/*z* 1173.6 ($M + Na^+$), found *m*/*z* 1174.1; HR MS calcd *m*/*z* 1173.5710 ($M + Na^+$), found *m*/*z* 1173.5720.

*6″-Azido-1,3,6′,3″-tetra-N-(tert-butoxycarbonyl)kanamycin A (**9**)*. A sample of 6″-(2,4,6-triisopropylbenzenesulfonyl)-1,3,6′,3″-tetra-*N*-(*tert*-butoxycarbonyl)kanamycin A (**8**) (150 mg, 130 μmol) was dissolved in 5 mL of DMF to which lithium azide (120 mg, 2.4 mmol, 20 equiv) was added, and the reaction mixture was heated at 60 °C overnight. After completion, the mixture was added to 150 mL of H_2O and extracted with EtOAc (3 × 150 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4, and concentrated to a solid. The sample was purified by silica gel chromatography (3% MeOH in DCM) to yield 110 mg of product (93%): R_f = 0.4 (5% MeOH DCM); ^{1}H NMR (400 MHz, CD_3OD) δ 5.08 (s, 2H), 4.32 (s, 1H), 3.71−3.30 (m, 15H), 3.18 (t, 1H, J = 9.2, 8.4 Hz), 2.01 (d, 1H, J = 3.2 Hz), 1.44 (s, 36H); ^{13}C (75 MHz, CD_3OD) δ 158.43, 158.22, 156.97, 156.67, 101.89, 98.80, 84.80, 80.54, 79.42, 79.22, 75.80, 73.57, 72.97, 72.24, 71.38, 71.05, 70.81, 69.41, 56.28, 51.49, 51.07, 49.86, 40.94, 35.08, 27.88; LR MS calcd *m*/*z* 932.4 ($M + Na^+$), found *m*/*z* 932.4; HR MS calcd *m*/*z* 932.4423 ($M + Na^+$), found *m*/*z* 932.4423.

*6″-Azidokanamycin A (**1**)*. A sample of 6″-azido-1,3,6′,3″-tetra-*N*-(*tert*-butoxycarbonyl)kanamycin A **(9)** (70 mg, 77 μmol) was dissolved in 2 mL of a 1:1 DCM/TFA mixture and the reaction mixture stirred at room temperature for 20 min. The sample was evaporated to dryness, co-evaporated several times with toluene, and then stirred in 2 mL of diethyl ether. The solid was filtered away from the ether and purified by silica gel chromatography using a 7:2:1 2-propanol/NH_4OH/H_2O mixture to yield 30 mg, 59 μmol, 77%: R_f = 0.2 (7:2:1 2-propanol/NH_4OH/H_2O); ^{1}H NMR (300 MHz, D_2O) δ 5.47 (d, 1H, J = 3.9 Hz), 5.04 (d, 1H, J = 3.6 Hz), 4.06 (bs, 1H), 3.95 (t, 1H, J = 7.5, 7.2 Hz), 3.76−3.28 (m, 12H), 3.04 (m, 4H), 2.01 (m, 1H), 1.30 (q, 1H, J = 12.3 Hz); ^{13}C (75 MHz, D_2O) δ 100.16, 98.41, 87.01, 84.82, 73.99, 72.54, 71.51, 71.21, 71.09, 70.96, 69.81, 69.69, 54.25, 51.11, 50.63, 48.80, 40.79, 34.27; HR MS calcd *m*/*z* 510.2524 ($M + H^+$), found *m*/*z* 510.2539.

1,3,2′,6′,3″-Penta-N-(tert-butoxycarbonyl)tobramycin. This was synthesized as previously described by Michael et al. (*23*).

6″-(2,4,6-Triisopropylbenzenesulfonyl)-1,3,2′,6′,3″-penta-N-(tert-butoxycarbonyl)tobramycin. The 6″-hydroxyl group of 1,3,2′,6′,3″-penta-*N*-(*tert*-butoxycarbonyl)tobramycin was activated with 2,4,6-triisopropylbenzenesulfonyl chloride as described by Michael et al. (*23*).

6″-Azido-1,3,2′,6′,3″-penta-N-(tert-butoxycarbonyl)tobramycin. To a sample of 6″-(2,4,6-triisopropylbenzenesulfonyl)-1,3,2′,6′,3″-penta-*N*-(*tert*-butoxycarbonyl)tobramycin (210 mg, 171 μmol) in 5 mL of DMF was added lithium azide (100 mg, 2 mmol, 12 equiv). The reaction was heated at 60 °C overnight. The crude reaction mixture was diluted with 200 mL of H_2O and extracted with EtOAc (3 × 150 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4, and concentrated to a solid. The sample was purified by silica gel chromatography (4% MeOH in DCM) to yield 170 mg, 170 μmol, quantitative: R_f = 0.3 (4% MeOH in DCM); ^{1}H NMR (500 MHz, CD_3OD) δ 5.12 (d, 2H, J = 9.5 Hz), 4.18 (d, 1H, J = 9.0 Hz), 3.73−3.33 (m, 16H), 2.13 (d, 1H, J = 12.0 Hz), 2.04 (bs, 1H), 1.65 (q, 1H, J = 11.5), 1.42 (s, 46H); ^{13}C NMR (125 MHz, CD_3OD) δ 157.94, 156.53, 156.40, 98.20, 81.52, 79.31, 79.07, 78.97, 78.79, 75.31, 72.10, 70.53, 68.76, 65.01, 55.65, 51.06, 49.78, 40.55, 32.81, 27.46; HR MS calcd *m*/*z* 1015.5170 ($M + Na^+$), found *m*/*z* 1015.5171.

*6″-Azidotobramycin (**2**)*. A sample of 6″-azido-1,3,2′,6′,3″-penta-*N*-(*tert*-butoxycarbonyl)tobramycin (42 mg, 41 μmol) was dissolved in 2 mL of a 1:1 TFA/DCM mixture, and the reaction mixture was stirred at room temperature for 30 min. A 10 mL aliquot of toluene was added to the reaction, and the mixture was evaporated to yield a tan oil. The oil was stirred in 10 mL of diethyl ether to yield a white solid that was dissolved in water and lyophilized. The product was then purified via silica gel chromatography (7:2:1 2-propanol/NH_4OH/H_2O) to yield 16.2 mg, 328 μmol, 80%: R_f = 0.30 (7:2:1 2-propanol/NH_4OH/H_2O); ^{1}H NMR (500 MHz, D_2O) δ 5.59 (d, 1H, J = 3.5 Hz), 5.07 (d, 1H, J = 3.5 Hz), 4.04 (bs, 1H), 3.9, (m, 1H), 3.78−3.08 (m, 15H), 2.20 (m, 2H), 1.88 (q, 1H, J = 12 Hz), 1.54 (q, 1H, J = 12.5 Hz); ^{13}C NMR (75 MHz, D_2O) δ 100.32, 95.41, 85.87, 81.83, 74.48, 71.21, 69.69, 69.63, 68.06, 65.40, 54.56, 50.89, 50.51, 48.97, 48.35, 40.40, 32.41, 31.49; LR MS calcd *m*/*z* 493.3 ($M + H^+$) found *m*/*z* 493.2; HR MS calcd *m*/*z* 493.2729 ($M + H^+$), found *m*/*z* 493.2748.

Expression of APH(3′)-IIIa and Harvesting of Cell Lysate. APH(3′) is an aminoglycoside phosphotransferase, transferring the γ-phosphoryl group from ATP to the 3′-OH position of an aminoglycoside. For APH(3′) expression [described by McKay et al. (*24*)], *Escherichia coli* [JM109(DE3)] was transformed with a pETSACG1 plasmid carrying the APH(3′) resistance gene. Cultures were grown at 37 °C in 1 L of LB medium containing 100 mg/L ampicillin. Cultures were induced at an OD_{600} of ~0.5 by addition of 1 mM IPTG, and the culture was incubated for an additional 3 h at

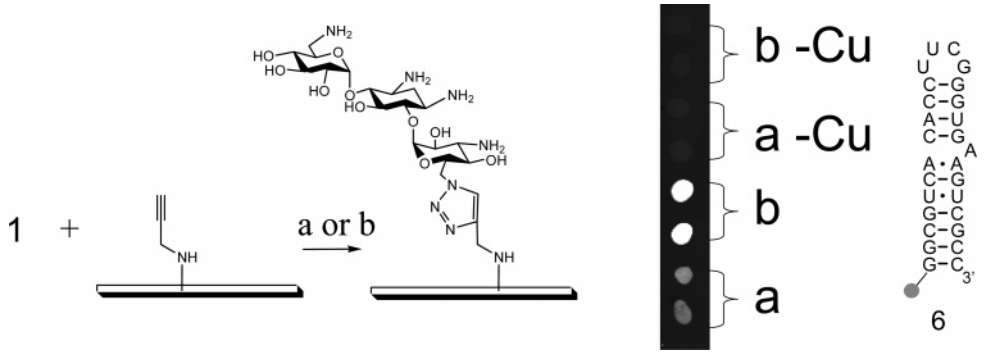


FIGURE 2: Schematic of the Huisgen dipolar cycloaddition reaction to anchor **1** and **2** onto alkyne-displaying agarose slides. Conditions used for reaction a: 10 mM phosphate buffer (pH 8.0), 100 μM tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), 100 μM ascorbic acid, and 10% glycerol in the presence or absence of 1 mM $CuSO_4$. Conditions used for reaction b: 10 mM Tris-HCl (pH 8.5), 100 μM TBTA, 100 μM ascorbic acid, and 10% glycerol in the presence or absence of 1 mM $CuSO_4$. Tris buffer gives a 5-fold higher signal than phosphate buffer; the conditions in b were used in all subsequent experiments. After reaction with modifying enzymes, arrays were probed with 5′-end-labeled DY547 A-site mimic (**6**), an oligonucleotide mimic of the bacterial rRNA A-site (*5*, *15*, *21*).

37 °C. Cells were pelleted by centrifugation, washed in ice-cold 0.85% (w/v) NaCl, resuspended in a minimal volume of ice-cold lysis buffer [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.1 mM DTT, and 1 mM PMSF], and lysed by sonication. Cellular debris was pelleted via centrifugation. The supernatant was placed into 3500 MWCO dialysis tubing and dialyzed against a buffer containing 50 mM Tris-HCl (pH 7.5), 40 mM KCl, and 10 mM $MgCl_2$ at 4 °C. The protein was concentrated to 500 μL using a Sartorius (Goettingen, Germany) Vivaspin concentration device (10 000 MWCO, polyethersulfone membrane).

Expression of ANT(2″)-Ia and Harvesting of Cell Lysate. ANT(2″) is an aminoglycoside nucleotidyltransferase and adenylates the 2″-OH position of aminoglycosides. For ANT-(2″) expression [described by Wright et al. (*25*)], *E. coli* [JM109(DE3)] cells were transformed with a pET 22b(+) plasmid carrying the ANT(2″) resistance gene. Cultures were grown at 37 °C in 1 L of LB medium containing 50 mg/L ampicillin and 10 mg/L kanamycin A. Expression was induced at an OD_{600} of ~0.5 by addition of 0.5 mM IPTG and the culture incubated for an additional 8 h at 37 °C. Cells were pelleted as described above, resuspended, and washed in ice-cold buffer [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA]. Cells were resuspended in a minimal volume of cold lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 5 mM β-mercaptoethanol] and sonicated. The cellular debris was pelleted by centrifugation and the supernatant dialyzed against 60 mM Tris-HCl (pH 7.1), 40 mM $MgCl_2$, 0.4 M NH_4Cl, and 0.16 mM DTT (*26*) at 4 °C. The crude protein mixture was concentrated to 500 μL as described above.

Functionalization of Slides. A 1% agarose solution was prepared by microwaving an aqueous solution of agarose until it dissolved. To the solution were added $NaIO_4$ and propargylamine at final concentrations of 10 mM. A 1.5 mL aliquot of the solution was then applied to the surface of silane-prep glass slides (*27*−*29*). The agarose was allowed to solidify, and then the slides were submerged in a 0.01 M $NaHCO_3$ solution for 1 h followed by water for 1 h. The slides were then left to dry to a thin film overnight at room temperature. Arrays were submerged in an aqueous solution of 20% ethylene glycol for 45 min and rinsed with water. This was followed by submersion in 100 mM Tris-HCl (pH 7.5) for 30 min and then in a solution containing 80 mL of 1× PBS, 20 mL of ethanol, and 300 mg of $NaCNBH_3$. Slides were finally rinsed with 0.2% SDS and then water and were dried prior to use.

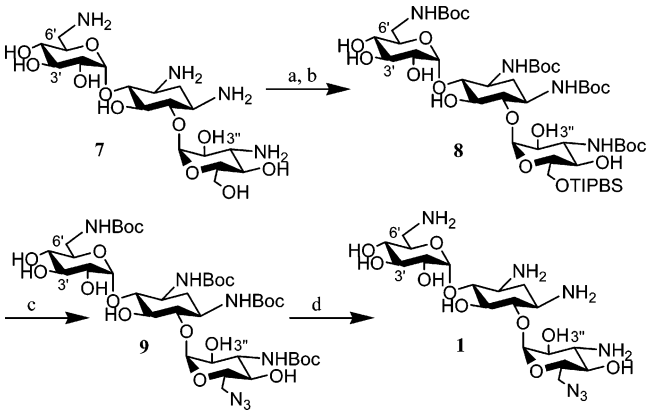
Immobilization of 6″-Azido-Aminoglycosides onto the Slide Surface. All stock solutions were prepared with nanopure water unless otherwise noted. Spotting solutions consisted of 10 mM Tris-HCl (pH 8.5), 100 μM tris(benzyltriazolylmethyl)amine (TBTA) (*30*) (dissolved in 4:1 butanol/DMSO), 1 mM $CuSO_4$, 100 μM ascorbic acid, 10% glycerol, and serially diluted concentrations of the 6″-azido-aminoglycoside (**1** and **2**). These conditions provided higher loadings than ones using phosphate buffer.

Slides were equipped with a multiwell silicon gasket to display 50 microwells on the surface of the slide (Grace Bio Labs, Bend, OR) (*28*), and 0.4 μL of spotting solution was delivered to each well. Slides were incubated at room temperature for 2 h and rinsed with 10 μL of 1× assay buffer [200 mM HEPES (pH 7.5), 11 mM $MgCl_2$, and 22 mM KCl] (*9*) in each well. The solution was incubated for 5 min at room temperature and then pipetted out of the wells; this step was repeated four times.

*Enzymatic Modification of Immobilized **1** and **2**.* Microarrays in which serially diluted concentrations of compound **1** or **2** were immobilized were washed and air-dried. Reactions were completed in a total volume of 12 μL of 1× assay buffer, 2.3 nmol of phosphoenolpyruvate, 0.018 unit of pyruvate kinase, 11.4 nmol of ATP, and either 0.2 OD_{260} of APH and 500 000 cpm of [γ-^{32}P]ATP or 0.8 OD_{260} of ANT and 500 000 cpm of [α-^{32}P]ATP. Control experiments were completed as described above, except the lysate containing APH or ANT was replaced with a lysate from the same bacterial strain that did not harbor the plasmid encoding the resistance enzyme. Reactions were carried out for 12 h at 37 °C and 100% humidity to prevent evaporation. After reaction, the silicon gasket was removed from the surface and the slides were submerged in 0.2% SDS (2 × 15 min) and then in water for 30 min at 37 °C. Slides were air-dried, exposed to a phosphorimager cassette, and scanned using a Bio-Rad phosphorimager. Data were quantified with Bio-Rad's QuantityOne software.

Probing Modified Slides with the Bacterial rRNA A-Site Mimic. A 600 μL solution of 1 μM 5′-DY543-labeled *E. coli* ribosomal A-site (**6**, purchased from Dharmacon) in 1× hybridization buffer [8 mM Na_2HPO_4 (pH 7.1), 1 mM

Scheme 1: Synthesis of the 6″-Azido-Aminoglycoside Derivative of Kanamycin A (**1**)[a]



[a] Synthesis of the tobraymcin (**2**) derivative was completed in the same manner: (a) Boc_2O, 4:1 DMSO/H_2O, 70 °C, 12 h, 81% crude; (b) triisopropylbenzenesulfonyl chloride (TIPBS-Cl), pyridine, room temperature, 46%; (c) LiN_3, *N*,*N*-dimethylformamide, 60 °C, 12 h, 93%; (d) methylene chloride/trifluoroacetic acid (1:1), room temperature, 20 min, 77%.

EDTA, and 0.185 M NaCl] was refolded by incubation at 95 °C for 3 min and then cooled slowly to room temperature over ∼25 min. After the solution had cooled, 6 μL of 10 mg/mL bovine serum albumin (BSA) was added. Prior to hybridization of the A-site, slides were prewet with 600 mL of 1× hybridization buffer containing 200 μg/mL BSA, and the solution was evenly spread over the surface with a piece of Parafilm. After incubation for 5 min, the Parafilm and excess buffer were removed. The 600 μL solution containing refolded A-site was pipetted onto the slide and covered with a sheet of Parafilm. The RNA solution was incubated for 30 min at room temperature in the dark, and unbound RNA was removed from the surface by rinsing with ten 1 mL aliquots of hybridization buffer containing 200 μg/mL BSA. The slides were then rinsed with water, air-dried, and scanned with an Axon GenePix 4000A microarray scanner at 532 nm. Data were quantified using Bio-Rad's QuantityOne.

RESULTS AND DISCUSSION

To develop an effective microarray-based assay for these studies, derivatives of aminoglycosides with a chemical handle for defined surface immobilization were required. By studying structures of aminoglycoside−A-site mimic complexes (*22*, *31*), we found that no contacts are formed between an oligonucleotide mimic of the A-site (**6**, Figure 2) and the 6″-OH of tobramycin. Therefore, aminoglycosides with an azide chemical handle at the 6″-position were synthesized to anchor the compounds onto alkyne-functionalized slides via a Huisgen 1,3 dipolar cycloaddition reaction (*30*, *32*).

We synthesized target compounds **1** and **2** (Figure 1 and Scheme 1), derivatives of kanamycin A and tobramycin, respectively, by modifying a synthetic scheme reported by the Tor group for the synthesis of "amino-aminoglycosides" (*33*). Their hydrogenation-labile benzylcarbamate amine protecting group was replaced with the acid-labile *tert*-butylcarbamate because hydrogenation conditions could also reduce the azido group. Acidic conditions, however, would remove the *tert*-butylcarbamate group while leaving the azido group intact (Scheme 1).

The syntheses of both **1** and **2** were completed in the same manner starting from the parent aminoglycosides in their free

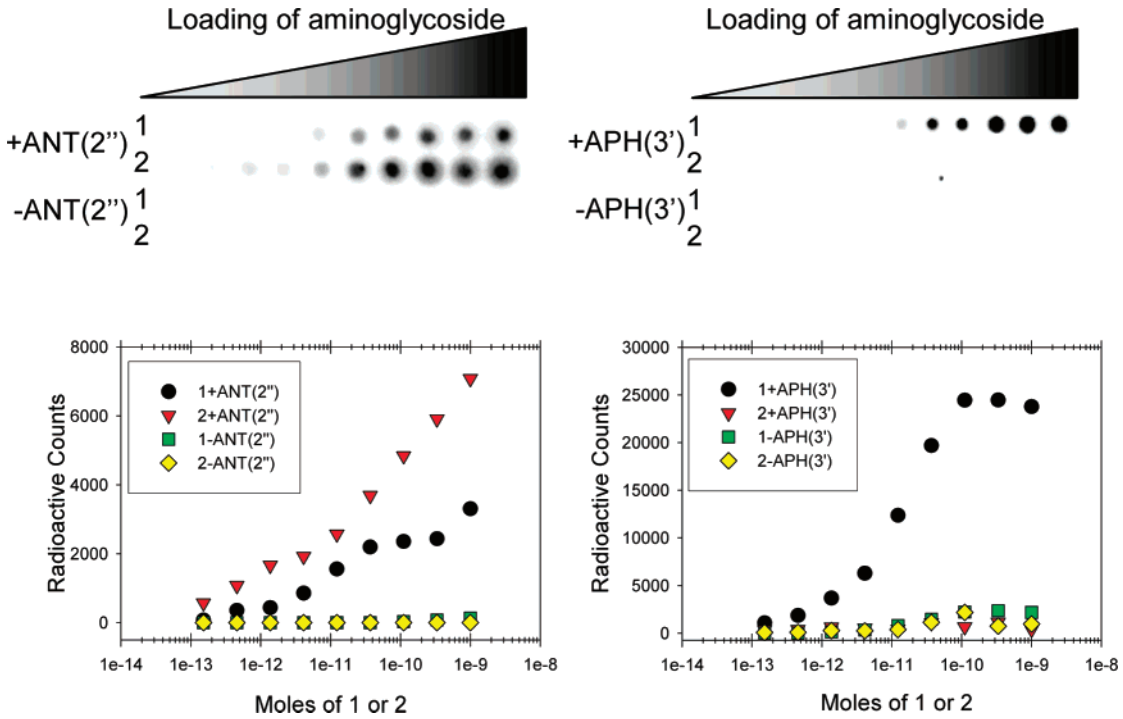


FIGURE 3: Modification of array-immobilized **1** and **2** by ANT(2″) (left) and APH(3′) (right). +ANT indicates samples incubated with a lysate containing ANT(2″), while −ANT indicates samples incubated with a lysate without ANT(2″). +APH indicates samples incubated with a lysate containing APH(3′), while −APH indicates samples incubated with a lysate without APH(3′). The top panels are autoradiograms of arrays after incubation with ^{32}P-labeled ATP and the appropriate lysate. The bottom panels are plots of data from the autoradiograms. Moles refer to the moles of aminoglycoside delivered to the microarray surface. Measurements were completed in triplicate, and errors are ±10%.

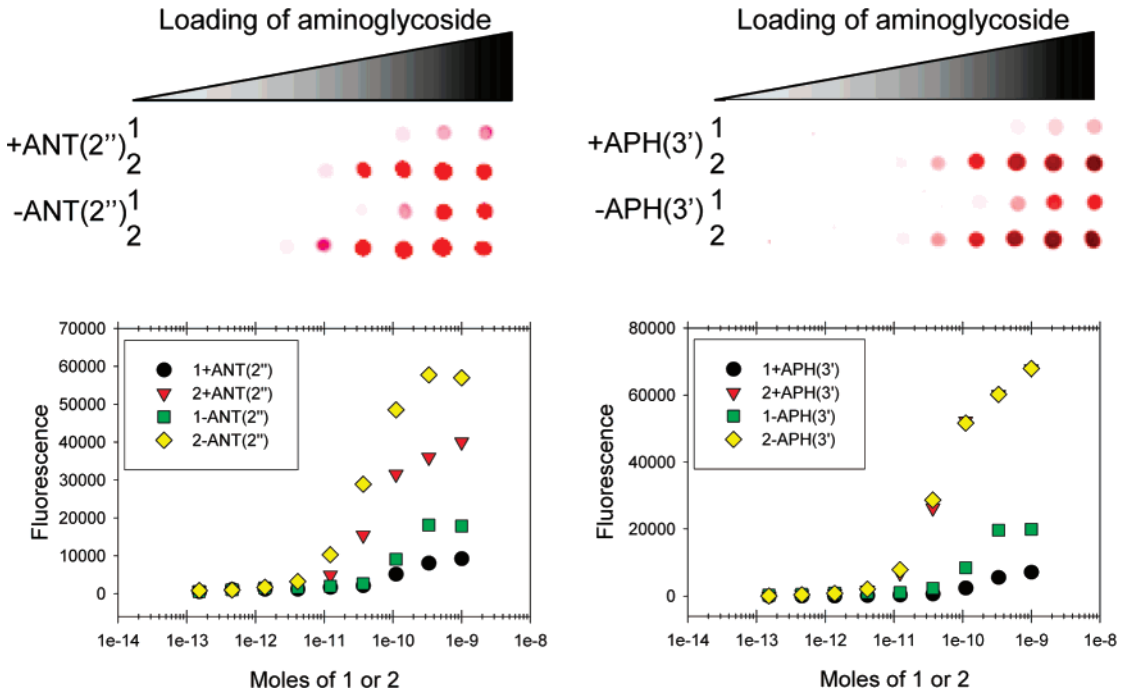


FIGURE 4: Ability of array-immobilized azido-aminoglycosides to bind to **6** after modification by ANT(2″) or APH(3′). +ANT indicates samples incubated with a lysate containing ANT(2″), while −ANT indicates samples incubated with a lysate without ANT. +APH indicates samples incubated with a lysate containing APH(3′), while −APH indicates samples incubated with a lysate without APH(3′). The left panels show results for ANT(2″) and the right panels results for APH(3′). The top panels are images of arrays hybridized with **6** after incubation with the appropriate lysates, and the bottom panels are plots of data for binding to **6** after incubation with the appropriate lysates. Moles refer to the moles of aminoglycoside that were delivered to the microarray surface. Measurements were completed in triplicate, and errors are ±10%.

base form (Scheme 1). The amino groups were protected as the *tert*-butylcarbamates via reaction with di-*tert*-butyl dicarbonate at 70 °C in a DMSO/water mixture (*23*). The products of this reaction were treated with triisopropyl benzyl sulfonyl chloride in pyridine to activate the 6″-hydroxyl group. The activating group was then displaced via reaction with LiN_3 overnight at 60 °C in *N*,*N*-dimethylformamide to afford the Boc-protected azido-aminoglycosides. Global Boc deprotection was achieved by treating the sample in a 1:1 methylene chloride/trifluoroacetic acid mixture.

Next, immobilization conditions for deposition of compounds **1** and **2** onto alkyne-functionalized agarose slides via a Huisgen dipolar cycloaddition reaction were optimized (Figure 2). We prefer agarose-coated surfaces because of their three-dimensional nature and high compound loading (*27*−*29*). The three-dimensional, hydrophilic surface of these arrays suggested that they would support enzymatic modification of immobilized compounds (*34*). We monitored immobilization by capturing of a fluorescently labeled rRNA A-site mimic (**6**) (*5*, *21*). We found that 10 mM Tris (pH 8.5) is superior to phosphate buffer for immobilization, in agreement with a previous report that used a Huisgen dipolar cycloaddition reaction to label a polyvalent viral surface (*35*). It therefore appears that Tris buffer may be generally superior to phosphate buffer and could prove to be useful in a variety of other biolabeling experiments. In all cases, no reaction occurs in the absence of $CuSO_4$, as expected (Figure 2).

Two classes of aminoglycoside resistance enzymes, nucleotidyl transferase (ANT) and phosphotransferase (APH), were used to determine if the array was suitable for direct monitoring of resistance enzyme activity. The 2″-position is a hydroxyl group in both **1** and **2**; therefore, both compounds are substrates for ANT(2″). Compound **1** has a hydroxyl group at the 3′-position and is therefore a substrate for APH(3′). This position in compound **2**, however, is a hydrogen; thus, it is not a substrate for APH(3′).

Serial dilutions of **1** and **2** were spotted into microwells created by a silicon gasket (*28*) affixed to alkyne-agarose arrays. The enzymatic activity of ANT(2″) was probed by incubating cell lysate containing ANT(2″) and [α-^{32}P]ATP in assay buffer (*36*) in microwells displaying **1** or **2**. Lysate from the same bacterial strain that does not express ANT(2″) was used as a negative control. As expected, ANT(2″) transfers radioactively labeled AMP to both **1** and **2**; however, the magnitude of the signal is greater for **2** than for **1** (Figures 1 and 3). No label is transferred when a lysate that does not contain ANT(2″) is used. Previous kinetic experiments with ANT(2″) have shown that the k_{cat} values are similar (~2 s^{-1}) for tobramycin (underivatized **2**) and kanamycin A (underivatized **1**), while the K_M is 22 μM for tobramycin and 50 μM for kanamycin (*36*). Inspection of the array data shows that tobramycin is labeled to a greater extent that kanamycin A, which can be explained, at least in part, by the differences in the k_{cat}/K_M and K_M values between the two substrates.

Enzymatic activity of APH(3′) (*37*) was assayed analogously except [γ-^{32}P]ATP was used to monitor transfer of the ^{32}P-labeled phosphoryl group (Figures 1 and 3). As expected, the phosphoryl group was transferred to only **1**, since **2** has a hydrogen instead of a hydroxyl group at the 3′-position (Figure 1). No label is transferred when a lysate that does not contain APH(3′) is used. Since only **1** is modified by APH(3′) while **1** and **2** are modified by ANT-

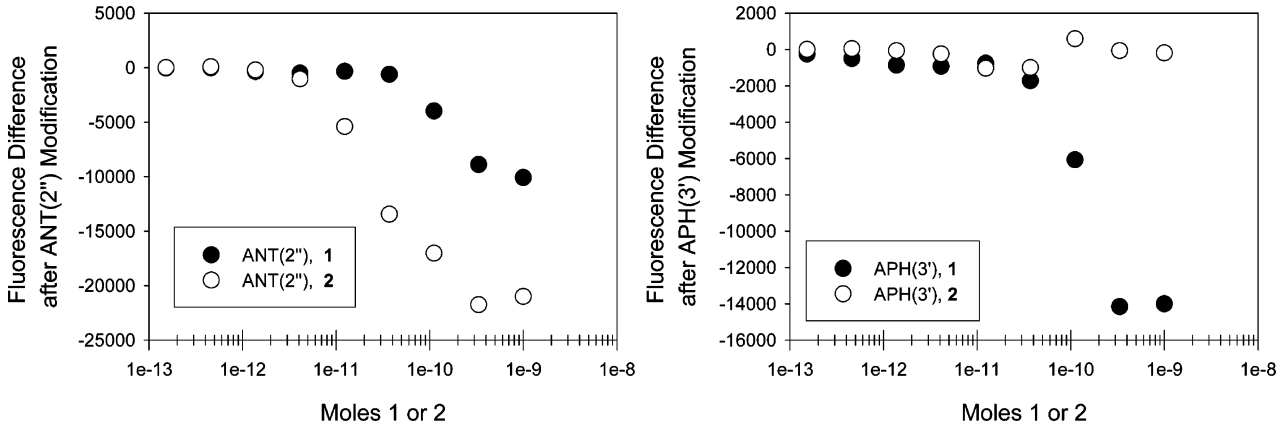


FIGURE 5: Difference in binding to **6** after modification of **1** and **2** by ANT(2″) and APH(3′). Data were taken from Figure 4. The fluorescent signal from binding to **6** from the −ANT or −APH samples was subtracted from the fluorescent signal from the +ANT or +APH samples. Negative values reflect the reduction in binding to **6** after incubation with the lysate containing the resistance enzyme. Reduced binding is observed only when the aminoglycoside is modified by the resistance enzyme. Moles refer to the moles of aminoglycoside that were delivered to the microarray surface. Measurements were completed in triplicate, and errors are ±10%.

(2″), the array-immobilized compounds retain their expected specificities.

Experiments were completed to determine the extent of modification of array-immobilized **1** by APH(3′) and ANT(2″). Results show that **1** is on average ~3-fold more modified by APH(3′) than by ANT(2″). This could be because the 6″-position used for immobilization is closer to the 2″-OH group modified by ANT(2″) than the 3′-OH group modified by APH(3′) (for details, see the Supporting Information). It is interesting to note that the k_{cat}/K_M values of APH(3′) and ANT(2″) for kanamycin are 1.43×10^5 M^{-1} s^{-1} (*24*) and 3.93×10^4 M^{-1} s^{-1} (*36*), respectively.

The relative binding affinities of **1** and **2** for the rRNA A-site mimic (**6**) were compared before and after modification. The arrays shown in Figure 3 were washed with 0.2% SDS to remove bound protein and were probed for binding to **6**. We observed that unmodified **2** has a higher affinity for A-site mimic **6** than unmodified **1** does, which is expected because tobramycin binds the A-site 12-fold more tightly than kanamycin A (*15*). This trend is also observed when the binding of **6** to enzymatically modified **2** is compared to the binding to enzymatically modified **1**.

From the results shown in Figure 3, both **1** and **2** are adenylated by ANT(2″). Therefore, reduced binding of **6** is expected after the compounds are incubated with a lysate containing the resistance enzyme (+ANT rows, Figure 4). As shown in Figure 4, enzymatically modified **1** and **2** (+ANT) bind more weakly to **6** than the unmodified aminoglycosides (−ANT). The reduction in binding as a function of immobilized aminoglycoside concentration is shown in Figure 5. In contrast, only **1** is a substrate for APH(3′) (+APH **1**, Figure 3). Thus, a reduced level of binding of **6** to modified **1** (+APH) compared to unmodified **1** (−APH) should be observed. Indeed, binding of **6** to **1** incubated with a lysate containing APH(3′) is reduced compared to that with unmodified **1** (Figures 4 and 5). Since **2** is not a substrate for APH(3′) (Figure 3), no difference in the binding of **2** in the presence and absence of APH(3′) is observed, as expected (Figures 4 and 5). Though a decrease in binding of **6** to modified aminoglycosides is observed, it is smaller than the decrease expected from previously reported solution measurements. This is due to the failure to modify all of the aminoglycoside displayed on the surface (for details, see the Supporting Information).

Our results show that modification of aminoglycosides by both ANT(2″) and APH(3′) reduces the affinity of the aminoglycosides for their rRNA target. Previously, this was known for only the aminoglycosides modified by acetyltransferases (AAC) and APHs (*9*). Therefore, a general mode of aminoglycoside resistance via enzymatic modification is a reduction in the affinity for the therapeutic target.

SUMMARY

We developed a microarray platform that can directly monitor aminoglycoside resistance enzyme activity from the APH and ANT families. The platform also allows studies of the effects that modification has on binding to the rRNA A-site on a single array. Results of modification experiments are in good agreement with the results of solution studies. It is likely that this platform is general for monitoring enzymatic activity and studying the effects that modification has on binding to therapeutic targets and can be applied to other classes of antibiotics.

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SUPPORTING INFORMATION AVAILABLE

^1H and ^{13}C NMR spectra and mass spectra for all newly reported compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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