Inhibition of Aminoglycoside Antibiotic Resistance Enzymes by Protein Kinase Inhibitors*

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Bacterial resistance to the aminoglycoside antibiotics is manifested primarily through the expression of enzymes which covalently modify these drugs. One important mechanism of aminoglycoside modification is through ATP-dependent O-phosphorylation, catalyzed by a family of aminoglycoside kinases. The structure of one of these kinases, APH(3′)-IIIA has recently been determined by x-ray crystallography, and the general fold is strikingly similar to eukaryotic protein kinases (Hon, W. C., McKay, G. A., Thompson, P. R., Sweet, R. M., Yang, D. S. C., Wright, G. D., and Berghuis, A. M. (1997) Cell 89, 887–895). Based on this similarity, we have examined the effect of known inhibitors of eukaryotic protein kinases on two aminoglycoside kinases, APH(3′)-IIIA and the enzyme AAC(6′)-APH(2′) which also exhibits acetyl-CoA-dependent aminoglycoside modification activity. We report that several known protein kinase inhibitors are also good inhibitors of aminoglycoside kinases. Compounds belonging to the isoquinolinesulfonamide group are especially effective in this regard, giving competitive inhibition in the micromolar range with respect to ATP and noncompetitive inhibition versus the aminoglycoside substrate. This study provides the basis for future aminoglycoside kinase inhibitor design and for the development of compounds which could reverse antibiotic resistance in the clinic.

Bacterial resistance to antibiotics continues to escalate and is widely recognized as a serious health threat (1, 2). Aminoglycoside antibiotics are an important group of antimicrobials which are used primarily to treat infections in the clinic due to the fact that parenteral rather than oral administration is required to obtain sufficient serum concentrations for effective control of bacterial infections (3). A major advantage to these cationic antibiotics is the fact that, despite the generally accepted view that these drugs target the bacterial ribosome, they have a bactericidal effect. This is thought to be the result of membrane damage effects which occur after impairment of ribosome function (4). Aminoglycoside therapy is especially important for the treatment of serious enterococcal infections where it is required to achieve synergistic killing when co-administered with a penicillin (5).

Clinical resistance to the aminoglycosides is generally the result of the synthesis of modifying enzymes, which can use ATP to either phosphorylate or adenylate the drugs, or acetyl-CoA to acylate them (6, 7). These modified aminoglycosides are no longer effective antibiotics and thus the cells manifest a resistance phenotype. There are well over 50 aminoglycoside-modifying enzymes that have been characterized at the gene level (6, 7). In the aminoglycoside kinase family (APH), the deduced amino acid sequences show relatively low overall similarity (10–50%), but they do share conserved sequences, primarily in the C-terminal region. Of particular importance is the sequence H/G/NIDX_{3–4}N, a homologue of which is also found in eukaryotic Ser/Thr and Tyr protein kinases: (H/ N/ X/ N/ X/ N). The significance of this short peptide lies in the fact that the conserved Asp is required for catalysis in protein kinases and most likely acts as an active site base deprotonating the substrate hydroxyl group to permit effective phosphoryl transfer (8, 9). We have recently determined the three-dimensional structure of the enterococcal aminoglycoside kinase, APH(3′)-IIIA and found that the overall structure of the enzyme is highly similar to eukaryotic protein kinases (Fig. 1) (10). Site-directed mutagenesis of the conserved Asp-190 resulted in a dramatic reduction of enzyme activity, consistent with an important role of this residue in catalysis as is the case with the corresponding Asp residues in eukaryotic protein kinases (10). Our previous work had demonstrated as well that APH(3′)-IIIA, like protein kinases, operates by an associative phosphoryl transfer mechanism by direct attack of the nucleophilic hydroxyl group on the γ-phosphate of ATP (11). Thus, this aminoglycoside kinase shares a similar three-dimensional fold and chemical mechanism with eukaryotic protein kinases.

In view of the fact that all aminoglycoside kinases have the H/G/NIDX_{3–4}N catalytic sequence, it is not unreasonable to predict that they will also share a similar kinase fold. Of particular interest to us is AAC(6′)-APH(2′), an enzyme with both aminoglycoside acetyltransferase and kinase activities harbored by many aminoglycoside-resistant staphylococci and enterococci (12, 13). This enzyme confers high level resistance (minimal inhibitory concentration >1,000 μg/ml) to a broad range of clinically important aminoglycosides including gentamicin, tobramycin, and amikacin. The close mechanistic and three-dimensional structural relationship between APH(3′)-IIIA and protein kinases have led us to explore the sensitivity of aminoglycoside kinases to known inhibitors of protein kinases. Kinase inhibitors of three classes, the indole carbazoles, the flavanoids, and the isoquinolinesulfonamides (Fig. 2) were examined. The latter were found to be good inhibitors of two aminoglycoside kinases, APH(3′)-IIIA and AAC(6′)-APH(2′). This study thus provides the basis for future inhibitor designs, which could lead to compounds that reverse antibiotic resistance.

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1 The abbreviations used are: APH, aminoglycoside phosphotransferase; AAC, aminoglycoside acetyltransferase; CKI-7, N-(2-aminoethyl)-5-chloroisouquinoline-8-sulfonamide; CKI-8, 1-(5-chloroisouquinoline-8-sulfonyl)piperazine; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H-9, N-(2-aminoethyl)-5-isoquinolinesulfonamide; HA-1004, N-(2-guanidinoethyl)-5-isoquinolinesulfonamide.
**Inhibition of Aminoglycoside Antibiotic Kinases**

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Genistein, quercetin, and staurosporine were from Sigma. N-(2-Aminoethyl)-5-chloroisouquinoline-8-sulfonamide (CKI-7) and 1-(5-chloroisouquinoline-8-sulfonamido)piperazine (CKI-8) were from Seikagaku America (Rockville, MD). 1-(5-Isouquinolinesulfonyl)-2-methylpiperazine (H-7), N-(2-aminoethyl)-5-isouquinolinesulfonamide (H-9), and N-(2-guanidinoethyl)-5-isouquinolinesulfonamide (HA-1004) were from Research Biochemicals International (Natick, MA). APH(3')-IIIa and AAC(6')-APH(2') were purified as described previously (14).

**Enzyme Assays and Data Analysis**—Aminoglycoside kinase activity was monitored by coupling of ADP release to NADH oxidation by pyruvate kinase and lactate dehydrogenase in the presence of excess phosphoenolpyruvate (14).

Assays were performed at 37 °C and contained 1 mM ATP, 1.28 mM NADH, 2.5 mM phosphoenolpyruvate, 5 units of lactate dehydrogenase, 3.5 units of pyruvate kinase, 50 mM HEPES, pH 7.5, and inhibitor dissolved in water or dimethyl sulfoxide in the case of the isoflavonoid compounds. The amount of dimethyl sulfoxide in the reaction mixture never exceeded 5% (v/v) and did not affect the APH(3')-IIIa activity but did diminish the activity of AAC(6')-APH(2'). Reactions were initiated by the addition of 0.5 nmol of purified APH(3')-IIIa or 0.21 nmol of pure AAC(6')-APH(2'). For experiments with varying kanamycin A, ATP was held at 1 mM. Inhibition constants were determined by fitting data determined in duplicate at least four different inhibitor concentrations to Equation 1 for competitive inhibition or Equations 2 or 3 for non-competitive inhibition using Grafit version 3.0 software (16).

\[
\frac{v}{V_{\max}} = \frac{[S]}{K_m(1 + I/K_i) + [S]}, \quad \text{(Eq. 1)}
\]

\[
\frac{v}{V_{\max}} = \frac{[S]}{K_m(1 + I/K_i) + [S]} \cdot \frac{1}{1 + I/K_i} \quad \text{(Eq. 2)}
\]

\[
\frac{v}{V_{\max}} = \frac{[S]}{K_m(1 + I/K_i) + [S]} \cdot \frac{1}{1 + I/K_s + [S]/K_i} \quad \text{(Eq. 3)}
\]

where \(K_m\) is the Michaelis constant, \(V_{\max}\) is the maximal velocity, \(K_i\) is the slope inhibition constant, \(K_i\) is the intercept inhibition constant, and \(K_s\) is the substrate inhibition constant (APH(3')-IIIa is substrate inhibited by kanamycin A (14)).

**RESULTS AND DISCUSSION**

The indole carbazole containing alkaloid staurosporine did not inhibit either APH(3')-IIIa or AAC(6')-APH(2') at 0.7–0.1 µM concentration (Table I). This compound is a potent inhibitor of protein kinase C and many eukaryotic protein kinases with \(K_i\) values generally below 10 nM (17). The current evidence supports binding of staurosporine at the ATP site of protein kinases (17). Despite the fact that the general structure of the ATP site is shared between APH(3')-IIIa and protein kinases, this is not translated in similar affinity for staurosporine.

The isoflavonoids genistein and quercetin have been shown to be inhibitors of the epidermal growth factor receptor kinase and other Tyr kinases and are competitive with ATP (18).

Genistein did not inhibit either APH(3')-IIIa or AAC(6')-APH(2'), but the related compound quercetin showed modest inhibition of APH(3')-IIIa which was competitive with ATP (Table I).

The isouquinolinesulfonamide inhibitors are well known competitive inhibitors of ATP for many protein kinases (19–21). Remarkable target specificity has been reported to be achieved by varying the side chain and the placement of the isouquinoline ring nitrogen (reviewed in Ref. 19). Thus H-9 is an inhibitor of protein kinase C, whereas CKI-7, which differs only by the presence of a chlorine atom linked to C-5 (Fig. 2), is a potent inhibitor of casein kinases. Recent x-ray crystallographic structures of CKI-7 bound to casein kinase (22) and H-7, N-(2-(methylamino)ethyl)-5-isouquinolinesulfonamide(H-8), and N-(2-(p-bromocinnamylamino)ethyl)-5-isouquinolinesulfonamide (H-89) complexed with cAMP-dependent protein kinase (23) have demonstrated the structural basis for inhibition of protein kinases by this family of compounds.

A key element which is common to both CKI-7 and the H-series inhibitor protein complexes is a common hydrogen bond between the isouquinoline ring nitrogen and a main chain amide hydrogen. An equivalent hydrogen bond is also made between N-1 of ATP in the

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*2 D. M. Daigle and G. D. Wright, submitted for publication.*
Inhibition of Aminoglycoside Antibiotic Kinases

TABLE I

Effects of protein kinase inhibitors on APH(3')-IIIa and AAC(6')-APH(2')

| Inhibitor | Variable substrate | Patterna | $K_a$ (μM) | $K_i$ (μM) | Equationb |
|-----------|--------------------|----------|------------|------------|-----------|
| APH(3')-IIIa | ATP | C | 66.1 ± 7.5 | 1 | |
| CIKI-7     | ATP | C | 290 ± 89 | 1 | |
| CIKI-8     | ATP | C | 730 ± 130 | 1 | |
| H-7        | ATP | C | 138 ± 40 | 1 | |
| H-9        | Kanamycin A | NC | 260 ± 19 | 3 | |
| HA-1004    | ATP | C | 48.9 ± 12.3 | 1 | |
| Genistein  | ATP | — | 126 ± 22 | 1 | |
| Quercetin  | ATP | C | 161 ± 42 | 1 | |
| Staurosporine | ATP | — | — | — | |
| AAC(6')-APH(2') | ATP | C | 87.1 ± 17.8 | 1 | |
| CIKI-7     | ATP | C | 137 ± 29 | 1 | |
| CIKI-8     | ATP | C | 63 ± 19 | 1 | |
| H-7        | ATP | C | 260 ± 19 | 3 | |
| H-9        | Kanamycin A | NC | 998 ± 307 | 2 | |
| HA-1004    | ATP | C | 151 ± 42 | 1 | |
| Genistein  | ATP | — | 125 ± 49 | 2 | |
| Quercetin  | ATP | C | 151 ± 42 | 1 | |
| Staurosporine | ATP | — | — | — | |

a C, competitive; NC, noncompetitive.
b Data fit to equations under “Experimental Procedures.”
c No inhibition at 250 μM.
d No inhibition at 0.7 μM.
e No inhibition at 500 μM.
f No inhibition at 500 μM; dimethyl sulfoxide required to solubilize these compounds dramatically reduced enzyme activity (approximately 50% at 5% dimethyl sulfoxide); thus these compounds may bind to the enzyme, but nonetheless with poor affinity.
g No inhibition at 1 μM.

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The bifunctional enzyme lacks an overlapping substrate enzyme complex and the same amide hydrogen, thus the isoquinoline and the purine rings occupy the same binding pocket and employ identical positioning elements when bound to protein kinases. The structure of the APH(3')-IIIa-ADP complex also reveals an analogous hydrogen bond between N-1 of ADP and the amide hydrogen of Ala-93 (10), and thus we predicted that the isoquinolinesulfonamides could indeed bind to APH(3') in a similar fashion (Fig. 3). The compounds H-7, H-9, HA-1004, CIKI-7, and CIKI-8 were competitive inhibitors of ATP binding to APH(3')-IIIa (Table I), and additional studies determined that H-9 was a non-competitive inhibitor of the second substrate kanamycin. Thus, as predicted by the protein fold and adenine nucleotide binding strategy which is common to the aminoglycoside kinase APH(3')-IIIa and protein kinases, isoquinolinesulfonamides bind to the active site of the drug resistance kinase in the ATP binding site, presumably through a hydrogen bond between the isoquinoline ring nitrogen and the amide hydrogen of Ala-93.

Among the implications of this conclusion was the prediction that other aminoglycoside kinases, while low in overall amino acid sequence homology, would also be inhibited by the isoquinolinesulfonamides. The enzyme AAC(6')-APH(2') confers resistance to virtually all aminoglycoside antibiotics with the exception of streptomycin and spectinomycin. The 57-kDa enzyme has both aminoglycoside kinase and acetyltransferase activities which, respectively, reside in the C-terminal and N-terminal regions of the intact enzyme (12). AAC(6')-APH(2') shows only 16% overall primary sequence similarity to APH(3')-IIIa. The bifunctional enzyme lacks an overlapping lysyl-tRNA synthetase homologue (APH(3')-IIIa numbering) which is used to position the ATP by bridging the $\alpha$- and $\beta$-phosphates in APH(3')-IIIa and protein kinases, yet the C-terminal APH(2') portion of AAC(6')-APH(2') does have the sequence H188GDL-GDNSN, similar to the catalytically important H188GDL-GDNSN sequence of APH(3')-IIIa which incorporates Asp-190, the proposed catalytic base. AAC(6')-APH(2') was sensitive to the isoquinolinesulfonamide inhibitors tested (Table I). General inhibition behavior was identical to that found with APH(3')-IIIa, that is competitive versus ATP and non-competitive versus aminoglycoside substrate. There were significant differences in sensitivity toward specific compounds between the kinases however. Whereas CIKI-7 was a comparable inhibitor of both enzymes, APH(3')-IIIa was more sensitive to the guanidinoethyl-containing HA-1004 and AAC(6')-APH(2') was more sensitive to the methylpiperazine compound H-7. The latter result was all the more surprising given the lack of inhibition by the homologue CIKI-8. The AAC(6') activity was not impaired by the inhibitors tested, thus these compounds are limited to kinase inhibition. Based on these results, we predict that the three-dimensional structure of the AAC(6')-APH(2') C terminus will have the general protein kinase fold despite the low sequence similarity with APH(3')-IIIa or protein kinases.

Whereas the isoquinolinesulfonamides tested are significant inhibitors of the aminoglycoside kinases in vitro, with the best compounds tested thus far yielding inhibition constants in <100 μM concentration (corresponding to 20–30 μM/ml), they did not reverse antibiotic resistance in cultures of Enterococcus faecalis harboring aph(3')-IIIa or aac(6')-aph(2') in either liquid or solid media (not shown). Nonetheless, these studies demonstrate the potential effectiveness of protein kinase inhibitors as inhibitors of antibiotic resistance, an important target for drug design (15). Compounds which incorporate the isoquinoline nucleus but exploit the unique features of the aminoglycoside kinases could show selective inhibition of these...
enzymes and thus would find use as therapeutic agents for the reversal of antibiotic resistance.

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