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Inhibitors of the Aminoglycoside 6'-N-Acetyltransferase type Ib [AAC(6')-Ib] Identified by In-Silico Molecular Docking

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Abstract

AAC(6)-Ib is an important aminoglycoside resistance enzyme to target with enzymatic inhibitors. An in-silico screening approach was used to identify potential inhibitors from the ChemBridge library. Several compounds were identified, of which two of them, 4-[(2-{[1-(3-methylphenyl)-4,6-dioxo-2-thioxotetrahydro-5(2H)-pyrimidinylidene]methyl}phenoxy)methyl]benzoic acid and 2-{5-[(4,6-dioxo-1,3-diphenyl-2-thioxotetrahydro-5(2H)-pyrimidinylidene)methyl]-2-furyl}benzoic acid, showed micromolar activity in inhibiting acetylation of kanamycin A. These compounds are predicted to bind the aminoglycoside binding site of AAC(6)-Ib and exhibited competitive inhibition against kanamycin A.

Keywords

aminoglycoside resistance; acetyltransferase; aminoglycoside modifying enzymes; enterobacterial; *Klebsiella*

Aminoglycosides are mainly used to treat infections caused by gram-negative aerobic bacilli like *Enterobacteriaceae* and *Pseudomonas aeruginosa*¹ and, in combination with other classes of antibiotics such as β -lactamases or vancomycin, to treat infections caused by some gram-positives¹. Other life-threatening infections that can be treated with aminoglycosides are tularemia, brucellosis, enterococci-caused endocarditis, streptococci infections, and plague¹. Aminoglycosides have also been very useful in the treatment of nosocomial infections caused by enterobacteria such as *Klebsiella pneumoniae*, which presents a high rate of morbidity and mortality^{2,3}. However, the rise in multidrug resistant strains is

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Supplementary data

Supplementary information associated with this work can be found at <http://>

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threatening the efficacy of treatments. In the case of aminoglycosides, one of the most important mechanisms of resistance is enzymatic modification⁴. Numerous enzymes have been found that inactivate aminoglycosides by transfer of acetyl, phosphoryl, or nucleotidyl groups⁴. Aminoglycoside acetyltransferases are an important class of resistance enzymes and catalyze the inactivation of aminoglycosides through transfer of an acetyl residue to an –NH₂ group of the 2-deoxystreptamine nucleus or the sugar moieties⁴. The aminoglycoside 6-*N*-acetyltransferase type Ib [AAC(6)-Ib] confers resistance to kanamycin and other aminoglycosides and has been detected in about 70% of gram-negative clinical isolates with an AAC(6)-I resistance profile^{4–6}. The *aac(6)-Ib* gene has been found within numerous plasmids, integrons, and transposons^{4–8}. Several variants of this gene have been recently identified that extend the spectrum to include other aminoglycosides such as gentamicin or antibiotics of a different family like quinolones^{9, 10}.

One possible solution to the problem of AAC(6)-Ib-mediated resistance to aminoglycosides is to inhibit the expression of the *aac(6)-Ib* gene. Efforts in this direction have been partially successful but are far from practical application^{11–13}. Another alternative, as shown by the inhibitors of β -lactamases¹⁴, could be the development of compounds that interfere with the activity of AAC(6)-Ib. Although inhibitors of aminoglycoside acetyltransferases have been described, none potent and efficient enough to be used in the clinics is already available^{15–17}. Computational methods have been used to identify or design compounds that bind the active sites of enzymes and inhibit their activity^{18–20}. Here we describe inhibitors of AAC(6)-Ib that were selected by testing compounds selected with in-silico molecular docking.

AAC(6)-Ib was overexpressed and partially purified by ionic exchange chromatography from a recombinant clone where *aac(6)-Ib* was placed under the control of the BAD promoter in the cloning vehicle pBAD102 as recommended by the supplier to obtain a His-Patch containing thioredoxin fused protein. Briefly, *Escherichia coli* harboring the recombinant clone was cultured to OD₆₀₀=0.6, at this moment 0.2% arabinose was added and the culture was allowed to incubate for 6 hours at 30°C. Cells were harvested by centrifugation, resuspended in 50 mM Tris pH 7.5 buffer, and lysed by sonication. Cell debris was removed by centrifugation and the soluble lysate was subjected to DEAE Sepharose ionic exchange chromatography eluting with a 0 to 1 M NaCl gradient. Multiple purification runs were pooled to obtain enough partially purified protein to carry out kinetic analysis. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the sample of this preparation is shown in Fig. S1. All chemical compounds tested as potential inhibitors were purchased from ChemBridge Corp (San Diego, CA) and dissolved in 100% dimethyl sulfoxide (DMSO). Nuclear magnetic resonance data for biologically active compounds is shown in Fig. S2.

The computational search for inhibitors was done by in-silico molecular docking using the X-ray crystal structure of AAC(6)-Ib complexed with kanamycin C and coenzyme A retrieved from the Protein Databank (code: 1V0C)²¹ and the molecular docking program Autodock Vina 1.1.2²⁰ with commercially available compounds from the ChemBridge chemical library (obtained from the ZINC database)²². Using AutoDock 4.0, the macromolecule AAC(6)-Ib was prepared for virtual screening by removing both Kanamycin C and Acetyl CoA ligands from the active site, removing all water molecules, and applying the partial charges as assigned by AutoDock²³. Virtual screening was performed using PyRx as a platform for AutoDock Vina²⁴. The Chembridge chemical library subset of ligands downloaded from the ZINC database were prepared using Open Babel 2.3.0 within the PyRx platform²⁵. The gridbox for docking was designed to include the entire aminoglycoside binding site. The docking gridbox had dimensions of 15 Å × 13 Å × 13 Å, and was centered on the aminoglycoside binding site as reported by Vetting et al.²¹.

In vitro activity was assessed by monitoring the increase in absorbance at 412 nm that occurs when 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reacts with the CoA-SH released from acetyl CoA after acetylation of the antibiotic substrate²⁶. The standard assay mixture contained 20 mM Tris pH 7.5 buffer, 0.2 mM DTNB, 50 μ M acetyl CoA, and 18 μ M kanamycin A, and 100 μ M of compound in 10% DMSO. Mixtures were incubated for 10 minutes at room temperature prior to the addition of enzyme. After the addition of partially purified AAC(6)-Ib, progress of the reaction was followed using a BioTek Synergy 2 plate reader monitoring absorbance at 412 nm. Initial velocities (V_i) were calculated using the Gen 5 software, version 2.01.13. Percent inhibition was determined by comparing the initial velocities of reactions taking place in the presence or absence of each tested compound. Those compounds that exhibited a level of inhibition higher than 20% were also assayed in the presence of 0.01% Triton X-100 to rule out inhibition by non-specific protein aggregation. All results are reported as a mean of three separate experiments.

To characterize the mode of inhibition, a range of inhibitor concentrations was tested while one substrate was held constant in excess and the other was varied. The standard conditions defined above were modified using kanamycin A concentrations ranging from 3 to 18 μ M while acetyl CoA was held constant in excess at 100 μ M or acetyl CoA concentrations ranging from 5 to 72 μ M while kanamycin A was held constant in excess at 25 μ M. All other conditions were those of the standard reaction described above. All experiments were performed in triplicate. K_i values were determined by nonlinear regression analysis using GraphPad Prism 6 software²⁷.

We first screened a subset of 1,000 compounds from the ZINC database using a computational molecular docking approach as described above. We selected the 20 compounds that were predicted to have the highest binding affinity to AAC(6)-Ib. Enzymatic reactions in the presence of each one of these compounds showed that only one produced modest inhibition of the acetylation of kanamycin A (Tables 1 and S1, these compounds are identified as "screen 1"). Compound **1**, 4-[(2-([1-(3-methylphenyl)-4,6-dioxo-2-thioxotetrahydro-5(2H)-pyrimidinylidene]methyl)phenoxy)methyl]benzoic acid (ChemBridge library compound 6443779, Tables 1 and S1), exhibited competitive inhibition with respect to kanamycin A and noncompetitive inhibition with respect to acetyl CoA. The K_i values calculated using the nonlinear regression method were 267 and 141 μ M for acetyl CoA and kanamycin A, respectively (Table 1). We then identified analogs of this compound using the "Show me analogs" function of the Chembridge website and determined their predicted binding affinity using Autodock Vina. The 10 compounds with highest affinity were then analyzed to assess their inhibition properties, 5 of them showed inhibition characteristics similar to compound **1** (Tables 1 and S1, these compounds are identified as "screen 1-analogs"). Although these were encouraging preliminary results, the K_i values were still considered too high. Therefore, we carried out a second docking analysis using a larger subset (12,000 compounds) from the ZINC database from which we selected 50 compounds (listed in Table S2, identified as "screen 2"). Only one of them, compound **2**, 2-{5-[(4,6-dioxo-1,3-diphenyl-2-thioxotetrahydro-5(2H)-pyrimidinylidene)methyl]-2-furyl}benzoic acid (ChemBridge library compound 5646906, Figure 1 and Tables 1 and S2), showed significant inhibition of the acetylation of kanamycin A. However, in this case the K_i values calculated using the nonlinear regression method were significantly lower than the K_i values of compounds found in the first round of analysis. The K_i values for compound **2** were 35 and 12 μ M for acetyl CoA and kanamycin A, respectively (Table 1). Lineweaver-Burk plots used to characterize the mode of inhibition of AAC(6)-Ib showed that compound **2** inhibits the reaction in competitive and noncompetitive manners with respect to kanamycin A and acetyl CoA, respectively (Figure 1). As we did in our first round of analyses, we identified analogs to compound **2** and on the basis of docking analysis and visual inspection we selected eight of them to test (Table S2, identified as "screen 2-

analogs”). None of them were significantly stronger inhibitors than compound **2**, but two of them, compounds **2a** and **2b**, 5-(2-furylmethylene)-1-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione and 1-(4-chlorophenyl)-5-(2-furylmethylene)-2,4,6(1H,3H,5H)-pyrimidinetrione, respectively (ChemBridge library compounds 5174640 and 5174745, respectively, Table S2), exhibited K_i values comparable to compound **2** (Table 1).

It has been described before that in some instances the effect of potential enzymatic inhibitors may be due to protein aggregation rather than enzymatic inhibition²⁸. To rule out this possibility, all compounds that showed inhibition were also tested in the presence of 0.01% Triton X-100. Of all these assays only compound 5309471 did not show the same level of inhibitory activity when tested with 0.01% Triton X-100 (Table S2) indicating that nonspecific aggregation may play a role in inhibition. This compound was not further considered.

All compounds with observable activity that were characterized in this study inhibited the acetylation reaction in a competitive manner with respect to the aminoglycoside substrate. These results are in agreement with the docking-predicted interaction between the substrate molecule and the protein. A representative model of the interactions between one inhibitor and AAC(6)-Ib is shown in Figure 2. In this predicted model a short distance between an amino acid and the inhibitor is an indicator of interaction strength. As it can be seen in Figure 2, amino acids D115 and D152 are near the inhibitor molecule, which agrees with results obtained by X-ray crystallography of AAC(6)-Ib complexed to kanamycin C and acetyl CoA²¹ and by molecular dynamics simulations²⁹.

Interestingly, the two separate docking assessments yielded inhibitors with similar core scaffold structures. In both screenings, the inhibitors identified consisted of substituted pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione or 2-thioxodihydro-4,6(1*H*,5*H*)-pyrimidinedione. All active compounds had a phenyl group, in some cases with one or two substitutions, bound to one of the Ns. The most potent inhibitors, compounds **2** and **2a**, also had a furan-2-ylmethylidene substitution at position 5. Compound **2** possessed another phenyl substitution on the 2-thioxodihydro-4,6(1*H*,5*H*)-pyrimidinedione and a benzoic acid substitution on the furan-2-ylmethylidene group. These substitutions did not result in substantial changes to efficiency of inhibition. A chloride substitution on the phenyl group (compound **2b**) resulted in weaker inhibition. The important role that substitutions may play in the inhibitory characteristics of these compounds is illustrated by the lack of activity observed when compounds **2c** and **2d** were tested (Table S2). These compounds resemble compound **2**, without the benzoic acid residue suggesting that the benzoic acid group compensates a negative effect produced by the second phenyl substitution. Our ability to predict the effect of adding functional groups to the pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione or 2-thioxodihydro-4,6(1*H*,5*H*)-pyrimidinedione scaffolds awaits further data.

In conclusion, the results presented here demonstrate that computer docking is a good initial step to identify compounds that inhibit the acetylating activity of AAC(6)-Ib thereby inhibiting the ability of bacteria to resist the toxic effect of aminoglycosides. Further work including the use of additional docking tools and testing a higher number of compounds could lead to the identification of stronger inhibitors active against groups of aminoglycoside modifying enzymes making them prospective candidates for their utilization in the clinics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AAC(6)-Ib	aminoglycoside 6 -N-Acetyltransferase type Ib
acetyl CoA	Acetyl coenzyme A
DMSO	dimethyl sulfoxide
DTNB	5,5 -dithiobis(2-nitrobenzoic acid)
V_i	initial velocity

References

1. Yao, J.; Moellering, R. Manual of Clinical Microbiology; Murray, P.; Baron, E.; Jorgensen, J.; Landry, M.; Tenover, M., editors. Vol. 1. American Society for Microbiology Press; Washington, DC: 2007. p. 1077
2. Keynan Y, Rubinstein E. Int J Antimicrob Agents. 2007; 30:385. [PubMed: 17716872]
3. Abbott, S. Manual of Clinical Microbiology. Murray, P.; Baron, E.; Jorgensen, J.; Landry, M.; Tenover, M., editors. Vol. 1. ASM Press; Washington, DC: 2007. p. 698
4. Ramirez MS, Tolmasky ME. Drug Resist Updat. 2010; 13:151. [PubMed: 20833577]
5. Ramirez MS, Nikolaidis N, Tolmasky M. Front Microbiol. 2013; 4:121. [PubMed: 23730301]
6. Vakulenko SB, Mobashery S. Clin Microbiol Rev. 2003; 16:430. [PubMed: 12857776]
7. Tolmasky, M. Enzyme-mediated resistance to antibiotics: mechanisms, dissemination, and prospects for inhibition. Bonomo, R.; Tolmasky, M., editors. ASM Press; Washington, DC: 2007. p. 35
8. Sarno R, McGillivray G, Sherratt DJ, Actis LA, Tolmasky ME. Antimicrob Agents Chemother. 2002; 46:3422. [PubMed: 12384346]
9. Casin I, Hanau-Bercot B, Podglajen I, Vahaboglu H, Collatz E. Antimicrob Agents Chemother. 2003; 47:697. [PubMed: 12543680]
10. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, Bush K, Hooper DC. Nat Med. 2006; 12:83. [PubMed: 16369542]
11. Soler Bistue AJ, Martin FA, Vozza N, Ha H, Joaquin JC, Zorreguieta A, Tolmasky ME. Proc Natl Acad Sci U S A. 2009; 106:13230. [PubMed: 19666539]
12. Soler Bistue AJ, Ha H, Sarno R, Don M, Zorreguieta A, Tolmasky ME. Antimicrob Agents Chemother. 2007; 51:1918. [PubMed: 17387154]
13. Sarno R, Ha H, Weinschel N, Tolmasky ME. Antimicrob Agents Chemother. 2003; 47:3296. [PubMed: 14506044]
14. Drawz SM, Bonomo RA. Clin Microbiol Rev. 2010; 23:160. [PubMed: 20065329]
15. Boehr DD, Draker KA, Koteva K, Bains M, Hancock RE, Wright GD. Chem Biol. 2003; 10:189. [PubMed: 12618191]
16. Chen W, Green KD, Tsodikov OV, Garneau-Tsodikova S. Biochemistry. 2012; 51:4959. [PubMed: 22646013]
17. Lombes T, Begis G, Maurice F, Turcaud S, Lecourt T, Dardel F, Micouin L. Chembiochem. 2008; 9:1368. [PubMed: 18464231]
18. Morris GM, Green LG, Radic Z, Taylor P, Sharpless KB, Olson AJ, Grynszpan F. J Chem Inf Model. 2013
19. Yelekci K, Karahan O, Toprakci M. J Neural Transm. 2007; 114:725. [PubMed: 17401533]
20. Trott O, Olson AJ. J Comput Chem. 2010; 31:455. [PubMed: 19499576]

21. Vetting MW, Park CH, Hegde SS, Jacoby GA, Hooper DC, Blanchard JS. *Biochemistry*. 2008; 47:9825. [PubMed: 18710261]
22. Irwin JJ, Sterling T, Mysinger MM, Bolstad ES, Coleman RG. *J Chem Inf Model*. 2012
23. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. *J Comput Chem*. 2009; 30:2785. [PubMed: 19399780]
24. Wolf LK. *Chemical & Engineering News*. 2009; 87:48.
25. O'Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. *J Cheminform*. 2011; 3:33. [PubMed: 21982300]
26. Green KD, Chen W, Garneau-Tsodikova S. *ChemMedChem*. 2012; 7:73. [PubMed: 21898832]
27. Motulsky, H.; Christopoulos, A. *A Practical Guide to Curve Fitting*. Oxford University Press; New York, NY: 2004. *Fitting Models to Biological Data using Linear and Nonlinear Regression*.
28. Feng BY, Shelat A, Doman TN, Guy RK, Shoichet BK. *Nat Chem Biol*. 2005; 1:146. [PubMed: 16408018]
29. Romanowska J, Reuter N, Trylska J. *Proteins*. 2013; 81:63. [PubMed: 22907688]

Figure 1.

Inhibition of AAC(6)-Ib catalyzed acetylation of kanamycin A. A and D. Chemical structures of compounds **2** and **2a**. B and E. Lineweaver-Burk plots at variable concentrations of kanamycin A. C and F. Lineweaver-Burk plots at variable concentrations of acetyl CoA. The numbers on the curves indicate the concentration of inhibitor.

Figure 2. Interactions of kanamycin A and compound **2a** with AAC(6)-Ib. In silico model of kanamycin A (panel A) or ChemBridge compound **2a** (panel B) with AAC(6)-Ib. Important amino acids in the interaction substrate-enzyme²¹ are shown.

Table 1

Inhibition parameters

Compound	ChemBridge Compound ID	K_i (μM) ^a		Structure
		Kanamycin A	Acetyl CoA	
Screen 2				
2	5646906	12 ± 1	35 ± 3
2a	5174640	15 ± 2	16 ± 1
2b	5174745	33 ± 7	74 ± 12
Screen 1				
1	6443779	141 ± 29	267 ± 33
1a	6199444	84 ± 12	97 ± 11

Compound	ChemBridge Compound ID	K_i (μM) ^a		Structure
		Kanamycin A	Acetyl CoA	
1b	7280272	129 $\pm$ 16	292 $\pm$ 25
1c	6078578	157 $\pm$ 20	258 $\pm$ 30
1d	6343976	155 $\pm$ 19	297 $\pm$ 26
1e	6881166	139 $\pm$ 27	181 $\pm$ 14

^aCalculated by nonlinear regression