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Kinetic characterization and molecular docking of novel allosteric inhibitors of aminoglycoside phosphotransferases

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Abbreviations: AME, aminoglycoside-modifying enzyme; AAC, aminoglycoside *N*-acetyltransferase; ANT, aminoglycoside *O*-nucleotidyltransferase; APH, aminoglycoside *O*-phosphotransferase; GOLD, Genetic Optimization for Ligand Docking.

Keywords: allosteric inhibitors, antibiotic resistance, docking, inhibition modes, protein dynamics, steady state kinetics.

ABSTRACT

Background: Bacterial antibiotic resistance often leads to treatment failure which may have serious consequences, especially in critically sick patients. Resistance to aminoglycosides is mainly due to the expression of antibiotic-modifying enzymes. One important mechanism of aminoglycoside modification is the ATP/GTP-dependent O-phosphorylation catalyzed by aminoglycoside phosphotransferases, APHs. The aim of this study is to identify specific inhibitors of APHs that could restore bacterial susceptibility to aminoglycosides.

Methods: We focused on the search for allosteric inhibitors that bind to small cavities of the protein and block the enzyme function by perturbing its dynamics.

Results: From normal mode analysis, a cavity of variable volume belonging to a large groove which splits the protein into two parts was chosen as target. By molecular docking, we screened a large library of commercially available compounds. Seventeen of the highest ranked compounds were tested by *in vitro* kinetic experiments in order to evaluate their ability to inhibit APHs. Site-directed mutagenesis was carried out with the aim of confirming the inhibition mechanism determined kinetically and the interactions with the protein predicted by *in silico* studies. These interactions were also confirmed by the use of structurally-related molecules.

Conclusions: Two compounds showed interesting inhibition properties, and one was able to block two different classes of APH.

General significance: This study gives new insights into the inhibition of APHs by such allosteric inhibitors, and provides the basis for the future development of combined therapies, antibiotic plus APH inhibitor, which may reverse the resistance to aminoglycosides in a clinical context.

1. Introduction

Aminoglycosides constitute a large family of water soluble, polycationic amino sugars of considerable structural diversity. They are broad spectrum antibacterial agents that are products of bacterial or fungal metabolism. Bacterial resistance to aminoglycosides manifests itself by one of the following mechanisms: (i) the presence of aminoglycoside-modifying enzymes [1]; (ii) the decrease of bacteria membrane permeability towards aminoglycoside uptake into the bacteria or extrusion of the aminoglycosides from the cell by efflux pumps [2]; or (iii) the modification of the drug target which can be either mutations that result in structural alterations of the ribosome [3] or methylations by 16S rRNA methyltransferases which interferes with the aminoglycoside binding [4]. Among these mechanisms, inactivation by aminoglycoside-modifying enzymes (AMEs) is the most important both in terms of level and frequency of resistance conferred to the bacteria [5]. There are three types of AMEs, each of which transfers a functional group onto the aminoglycoside structure thereby inactivating the antibiotic: aminoglycoside O-nucleotidyltransferases (ANTs) transfer a nucleoside monophosphate from a nucleoside triphosphate on a hydroxyl group of the antibiotic; aminoglycoside N-acetyltransferases (AACs) transfer an acetyl group from acetyl-CoA on an amine group; and aminoglycoside O-phosphotransferases (APHs) transfer the γ -phosphate of a nucleoside triphosphate on a hydroxyl group [6]. Each family of enzymes consists of different isoenzymes that differ in substrate specificity and selectivity. At least 50 different genes for AMEs have been identified in bacteria [1,7]. A number of bacteria also harbor a bifunctional enzyme that catalyzes both acetylation and phosphorylation [8,9].

In bacterial resistant isolates that possess antibiotic-inactivating enzymes, drugs are enzymatically modified when they penetrate into the bacteria. Therefore, the altered drug is unable to interact efficiently with its target, the ribosome. Moreover, in the absence of active aminoglycosides that normally perturb bacterial protein synthesis, the next step of high-rate accumulation of antibiotic does not take place [10,11]. Since the discovery of the antibiotic-inactivating enzymes, many efforts have been carried out in order to chemically modify the

81 existing antibiotics and produce new drugs that retain their antibacterial activity without being
82 inactivated. This strategy has led to considerable success, especially in the fight against
83 penicillin resistance. For instance, the use of methicillin, a derivative of the early penicillins,
84 overcame enzyme-mediated inactivation in most penicillin-resistant, gram-positive organisms
85 [12]. Another strategy to overcome enzyme-associated resistance consists in combining the
86 antibiotics with an inhibitor of the corresponding modifying enzyme. For example beta-
87 lactamase inhibitors, such as clavulanic acid, associated with amoxicillin inhibit most strains
88 of penicillinase-producing *S. aureus*, *H. influenza*, *E. coli*, *Klebsiella* spp,... [13]. Based on
89 this strategy, several teams have searched for AMEs inhibitors. The new discovery of AAC
90 inhibition by metal salts (mechanism still unknown) may offer a promising therapeutic issue
91 [14,15]. Shi *et al.* reviewed recent progresses in development of APHs inhibitors [16]. The
92 success in discovering specific inhibitors for AMEs depends on our ability to understand the
93 basic properties of their mode of action and of their substrate specificity. So far, main studies
94 focused on the development of competitive inhibitors that target either the nucleotide binding
95 site, such as protein kinase inhibitors [17,18], or the aminoglycoside binding site, with
96 aminoglycoside analogues [19,20] or non-carbohydrate inhibitors [21]. Using *in silico*
97 screening of chemical libraries in the aminoglycoside binding site of AAC(6')-Ib, competitive
98 inhibitors were recently identified [22].

99 However, therapeutic enzyme inhibitors are not restricted to active site competitors.
100 Identification of small molecules that stabilize inactive conformations of the protein may
101 represent a powerful starting point for rational drug design. The underlying idea is to complex
102 the enzyme with a molecule that binds to a cavity other than the active site, and stabilizes the
103 enzyme in a non-catalytically competent conformation. For example, the inhibition of
104 APH(3')-IIIa by AR_3a, an ankyrin repeat protein, has been shown to be due to a significant
105 change of the APH conformation upon AR_3a allosteric binding [23]. However, the large size
106 of this modulator makes it hardly druggable.

107 Here, using normal mode analysis on two different APH crystal structures, we
108 identified a common cavity of variable volume and used it as target for screening a library of

109 small commercially available compounds. By molecular docking, several potential allosteric
110 inhibitors against two aminoglycoside-modifying enzymes, aminoglycoside 3'-
111 phosphotransferase type IIIa, APH(3')-IIIa, and aminoglycoside 2"-phosphotransferase type
112 IVa, APH(2")-IVa, were identified. We performed kinetic and molecular modelling studies to
113 assess their mechanism of inhibition and to spotlight their binding site in the protein.
114

2. Materials and methods

2.1. Normal mode analysis and molecular docking

Two starting crystal structures of APH(3')-IIIa (PDB ID: 1L8T, including kanamycin A and ADP, from [24]) and APH(2'')-IVa (PDB ID: 3SG8, including tobramycin, from [25]) were selected for normal modes computation and cavity search. In these structures, the aminoglycosides were removed to favour the binding of inhibitors near the target cavity. ADP was maintained in 1L8T, except otherwise stated in the text. The first two hundreds normal modes were calculated (all-atoms parameter set 27) using the VIBRAN module of CHARMM program [26,27] allowing to define fifty conformations for each enzyme. Briefly, after addition of hydrogen using the H-build routine, the potential energy of each system was minimized for 5,000 steps of conjugate gradient algorithm (tolerance gradient of 0.01 kcal/mol/Å) followed by 50,000 steps of Adopted Basis Newton-Raphson to reach a mean energy gradient of less than 10^{-5} kcal/mol/Å. Diagonalization of the Hessian matrix and normal modes were computed from these energy-minimized structures. Trajectories of superimposed modes (modes 7 to 200, after removal of the six first modes corresponding to intrinsic translational or rotational motions) were computed with CHARMM to produce fifty frames corresponding to protein conformers (hereafter called "trajectory" allowing to visualize the displacement of the whole coordinates on the superposed modes 7 to 200). Atomic fluctuations averaged by residues were computed from normal modes 7-200 using the fluctuation subroutine implemented in the Vibran module of Charmm. Structure and normal modes trajectories were analyzed with VMD software [28]. From these conformers, all cavities were identified and characterized using MDpocket software [29], and a small cavity fulfilling the criteria for allosteric inhibition (location and variable volume) located behind the kanamycin binding site was selected for subsequent screening.

All docking was performed using GOLD (Genetic Optimization for Ligand Docking) program v5.2 [30,31]. Zinc database (<http://zinc.docking.org>) [32] was queried to find drug-

like molecules according to Lipinski's rule-of-five [33]. Briefly, 100,000 compounds from the Zinc database were selected after applying these drug-like filtering criteria, except that logP was defined below or equal to 2 in order to increase the selection of aqueous-soluble compounds. All these molecules were screened by ensemble docking on both APHs using 20 genetic algorithm runs within a 15 Å radius of the sphere centered on the targeted site and using Goldscore as scoring function. The docking poses were analyzed by the clustering method (complete linkage) from the rmsd matrix of ranking solutions. A second round of molecular docking (50 genetic algorithm runs) was performed on each APH separately, using the highest ranked compounds (the first hundred) from the ensemble screening. Twenty one compounds corresponding to the highest scores were selected, but finally, only seventeen compounds were commercially available and purchased.

Structural analysis and visualization of docking poses were carried out using the PyMOL Molecular Graphics System (version 1.3, Schrödinger, LLC).

2.2. Kinetic studies

In all *in vitro* kinetic experiments, equimolar concentrations of MgCl_2 were added to ATP. Thus, in the text, ATP and ADP refer to MgATP and MgADP, respectively. Experimental buffer was 50 mM Tris-HCl pH 7.5, 1 mM free MgCl_2 , 40 mM KCl, and the temperature was 25°C. The APH steady-state activity was evaluated using purified recombinant proteins, produced as previously reported [34], by measuring the time courses of ADP production by two different methods.

For a rapid screening of the compounds, the steady-state rate constants, k_{ss} , of enzyme activity were monitored in 96-well plates by coupling the release of ADP to a pyruvate kinase/lactate dehydrogenase reaction, as described by McKay *et al.* [35]. Reaction mixtures contained 0.5 μM APH, 4 mM phosphoenol pyruvate, 280 μM NADH, 20 U/ml pyruvate kinase, 25 U/ml lactate dehydrogenase, 25 or 350 μM ATP and 50 or 100 μM kanamycin A, with APH(3')-IIIa or APH(2'')-IVa, respectively. Experiments were carried out in

the absence or in the presence of potential inhibitors at 500 μ M. Reactions were initiated by the addition of ATP and monitored by reading the absorbance at 340 nm every 11 seconds. Values of k_{ss} were determined from the slope of linear phase of the reaction time courses using GraFit 7.0.2 software. The experiments were carried out in triplicate and the averaged inhibition was computed.

For the best compounds identified by the previously described enzymatic coupled system, the inhibition constants and inhibition modes were determined by the quench-flow method and HPLC analysis as previously described [36]. Briefly, quench-flow method consists in interrupting enzymatic reactions at different times with a quencher, which is usually a strong acid [37]. Here, 10% perchloric acid was used to stop the reaction every 3–7 s, depending on the enzyme. The reaction was triggered by addition of ATP in a thermostatically-controlled beaker (25°C) containing the enzyme and the aminoglycoside, with or without inhibitor. After quenching, the separation of ADP and ATP was carried out using HPLC (Alliance, Waters) and a partisphere SAX column (AIT France). The mobile phase was 200 mM ammonium phosphate buffer pH 5.5 and 10% acetonitrile. Samples from quench-flow were first spin at 19,000 g for 20 min at 4°C to remove precipitated protein. A volume of 100 μ l of supernatant quenched sample was diluted into 900 μ l of mobile phase supplemented with KOH to readjust the pH to 5.5. Volumes of 50 or 100 μ l of these samples were injected on the HPLC column. Quantification of ADP and ATP was obtained by integrating absorption peaks at 259 nm from the chromatograms. The inhibition mode and inhibition constants were determined by repeating the experiment at different concentrations of ATP (keeping kanamycin A concentration constant, see figure legends) or at different concentrations of kanamycin A (ATP concentration constant). APH concentrations were 0.1–0.5 μ M. Fittings were performed using GraFit 7.0.2 software using four different inhibition modes: competitive, non-competitive, uncompetitive and mixed. Only the most appropriate fit (which describes the inhibition with the lowest χ^2) is shown on the figures with the corresponding Lineweaver-Burk representation.

2.3. Site-directed mutagenesis

R211A mutant of APH(3')-IIIa was constructed by PCR (QuickChange Lightning Multi Site-Directed Mutagenesis Kit) using the two primers :
5'-TTTATTGATCTTGGGGCAAGCGGCAGGGCGGAC-3,' and
5'-GTCCGCCCTGCCGCTTGCCCCAAGATCAATAAA-3'.

Each 50 µl PCR reaction contained 2.5 µl of 10× QuickChange Lightning Multi reaction buffer, 0.75 µl of QuickSolution, 100 ng of DNA template (wild type *aph(3')-IIIa* in a pET15b plasmid), 1 µl of each mutagenic primer (100 ng), 10 mM dNTP and 2.5 units of QuickChange Lightning Multi enzyme blend. The PCR product was digested by DpnI restriction endonuclease for 1 h at 37°C. Mutant plasmids were recovered after transformation in *E. coli* BL21 (DE3) chemo-competent cells. Successful introduction of the desired mutations was controlled by sequencing the plasmid DNA (Fig. S1).

The mutant APH(3')-IIIa was produced in *E. coli* and purified as the wild type protein.

3. Results

3.1. Target cavity identification by normal mode analysis

Here, we focused on the search for APH allosteric inhibitors that bind to small cavities of the protein and block the enzyme function by perturbing its dynamics. Thus, a specific cavity was defined according to the following criteria: the cavity must be present in different APH subfamilies, it must be outside of the substrate binding sites, and lastly, it must undergo obvious volume change during the dynamics of the enzyme, required for its function. Subfamilies APH(3') and APH(2'') were chosen because of their prevalence [1].

From the normal mode analysis, one cavity behind the aminoglycoside binding site was selected because it was fulfilling all the predefined criteria (Fig. 1, Fig. S2 also showing videos). It belongs to a large groove which splits the proteins into two parts. Moreover, the region defining this cavity was found to be flexible according to the fluctuations computed from the superposed normal modes (Fig. 1). It must be emphasized that the observed atomic fluctuations were relatively small and therefore were not considered as the main criteria to select the cavity (commonly, fluctuations of residues located at the interior of the protein are less important compared to external flexible loops). Typically, APH proteins do not require large collective motions like other kinases for their biological function and this feature renders more difficult the search for internal dynamic cavities. Nevertheless and taking into account to these small fluctuations, the target binding pocket was also selected in respect to the volume changes observed along the normal modes. The target cavities were surrounded by residues Gly192 and Arg226 in 1L8T structure and by residues Ser199 and Ser232 in 3SG8 (yellow balls in Fig. S2). They are situated at a similar place within the two APHs and their compositions in amino acids were similar but not identical (at least one Phe, Leu, Ile, Ser and Asp found in each case).

Figure 1

3.2. Molecular docking

Using the targeted cavity defined above in APH(3')-IIIa and APH(2'')-IVa structures and the ZINC chemicals database [32], a virtual screening was carried out in order to predict and evaluate the potential affinity of these enzymes for drug-like molecules using Gold software. The Goldscore scoring function was used to rank the screened molecules – the highest scores predict highest affinities. In order to favor the binding of inhibitors near the target cavity which is located behind the aminoglycoside binding site, kanamycin A was removed from 1L8T structure (but not ADP) and tobramycin from 3SG8 prior docking.

In a first case study using an ensemble docking after overlaying both APH (same center of coordinates defining the target cavity at the same location for the two APHs), the first 100 out of the 100,000 compounds from the drug-like ZINC subset were selected based on their docking scores. Then, a second thorough docking run (50 GA runs) was performed on each APH separately with these selected compounds. We finally selected 21 best ranking ligands ($63 < \text{score} < 81$) after named NL1 to NL21 (Table 1). NL4, NL7, NL10 and NL15 were not commercially available, and therefore only 17 NL compounds were purchased for the further experimental assays.

3.3. Rapid *in vitro* screening of APH inhibitors using a coupled enzyme kinetic assay.

To evaluate the potential APH inhibition promoted by the selected molecules, *in vitro* inhibition studies were performed. The 17 molecules were rapidly screened using an enzymatic coupled-system, as previously described [35]. We used kanamycin A for these studies because both APHs can phosphorylate this aminoglycoside with a good efficiency through a Michaelis-Menten kinetics. Steady-state rate constants (k_{ss}) were measured at fixed concentrations of ATP and kanamycin A, in the absence or in the presence of NL compounds (500 μM). Percentages of inhibition by NL compounds were determined and normalized in respect to the activity without compound. The results are summarized in Table

1. In these conditions, a 40% or higher inhibition of APH(3')-IIIa activity was observed with compounds NL6 and NL8, and for APH(2'')-IVa with compounds NL6, NL9 and NL16. Two compounds seemed particularly interesting: NL8 because it inhibits APH(3')-IIIa by almost 80%, and NL6 for its ability to inhibit both APHs.

In order to determine the inhibition mode of these two compounds, steady-state kinetic experiments were carried out using a direct quenched-flow method. Results are described below and their mechanism is discussed on the basis of docking experiments.

3.3.1. Inhibition by NL8 compound.

NL8 was the most potent inhibitor amongst the 17 molecules tested here by the coupled system, showing a 79% inhibition of APH(3')-IIIa activity at 500 μ M. However, it showed little inhibition of APH(2'')-IVa activity. The steady-state rate constants, k_{ss} , of APH(3')-IIIa were determined at different concentrations of kanamycin A or ATP, and at 0, 50 or 100 μ M concentrations of NL8 by the quenched-flow method. Double reciprocal plots of $1/k_{ss}$ versus $1/[ATP]$ or $1/[kanamycin\ A]$ are shown in Fig. 2.

Figure 2

The NL8 compound was found to be a competitive inhibitor of APH(3')-IIIa towards ATP with a K_i of 9 ± 2 μ M, and a non-competitive inhibitor towards kanamycin A with a K_i of 55 ± 2 μ M.

It should be noted that NL8 was initially identified as potential allosteric inhibitor on the basis of a docking on APH(3')-IIIa-ADP complex. To explain the unexpected ATP competitive inhibition mode found here, we carried out another docking of NL8 but in the absence of ADP (Fig. S3). In these conditions, NL8 was found to overlap with the nucleotide binding site, especially with the phosphate groups of the nucleotide. Unfortunately, it was difficult to predict with accuracy the binding of NL8 as the molecule presents a high degree of variability, even within repetitions of dockings (several rounds using identical parameters).

Similar variabilities were obtained when NL8 was docked in the apo, in the kanamycin-bound or in the ADP-bound structures.

NL8 was identified on the basis of an *in silico* screening in which the cavity of the targeted protein was located behind the aminoglycoside binding pocket (Fig. 1). However, the competition between ATP and NL8 upon binding to APH(3')-IIIa suggests that the inhibitor may bind to a different location, presumably closer to the nucleotide binding pocket without interfering with the aminoglycoside site.

3.3.2. Inhibition by NL6 compound.

The coupled enzyme system method showed that NL6 inhibited efficiently both enzymes with 43% and 63% inhibition for APH(3')-IIIa and APH(2'')-IVa, respectively.

We determined the mode of inhibition of each protein by NL6. Fig. 3A and B show that NL6 is a non-competitive inhibitor of APH(2'')-IVa towards both ATP and kanamycin A with K_i of $85 \pm 10 \mu\text{M}$ and $74 \pm 4 \mu\text{M}$, respectively. Similarly, kinetics with APH(3')-IIIa showed NL6 acts again as a non-competitive inhibitor towards ATP and kanamycin A with K_i of $75 \pm 3 \mu\text{M}$ and $76 \pm 6 \mu\text{M}$, respectively (Fig. 3C and D).

Figure 3

To further investigate the binding of NL6 to APH(3')-IIIa, molecular docking of NL6 was performed in the apo protein, in the ADP-bound structure and in the ternary complex (PDB ID: 1L8T). Although NL6 is acting on both APHs' activity, it was more difficult to predict the binding position of NL6 in APH(2'')-IVa. Therefore, we focused on APH(3')-IIIa here. A cluster of the best docking poses is shown in Fig. 4A, B, C for each simulation. Interestingly, if one part of the molecule presents an important degree of flexibility in the apo and in the ADP-bound structures, the dihydro-benzopyran ring of NL6 shows a rigorously identical location in all docking predictions, suggesting the importance of this group for NL6 binding. The interactions occurring between this moiety and the protein are detailed in Fig. 4D.

Figure 4

All molecular dockings predicted that the dihydro-benzopyran ring is stabilized by the backbone atoms of Leu209 and Gly210 and by a hydrogen bond between the carbonyl near the benzopyran ring and the side chain of Arg211 (Fig. 4D). This latter interaction seems to be of a crucial importance to stabilize the molecule as NL6 presents a completely different predicted location when Arg211 is replaced by an alanine (Fig. 4E). To assess the importance of this interaction for the *in vitro* binding of NL6, we engineered the R211A mutant of APH(3')-IIIa and performed kinetic studies in the absence or in the presence of NL6. The activity of this mutant was significantly affected: k_{cat} was decreased by 62%. Interestingly, the molecule was completely unable to inhibit the APH mutant (Fig. 4F). This result confirms the binding site of NL6 predicted by *in silico* studies and highlights the importance of Arg211 for its binding to APH.

To further investigate the importance of the two other interactions occurring with the benzopyran ring, we tested the activity of structurally related compounds. Indeed, the benzopyran moiety is stabilized by two hydrogen bonds with the backbones of Leu209 and Gly210, it was thus impossible to modify these interactions by mutations. Consequently, we evaluated the activity of two structurally related molecules. One of them, named NL6-1 (Zinc14108604), contains the same dihydro-benzopyran ring than NL6 but with a slightly shorter structure, while the other compound, NL6-2 (Zinc13235262), has a naphthyl group instead. According to ZINC chemical database, the two analogues show more than 70% of structural similarity with NL6 (Fig. 5A).

Dockings were achieved in the APH(3')-IIIa with NL6 and its two analogues using the same parameters. According to these *in silico* studies, the two molecules bind to the same position to that observed for NL6 in the substrate-bound structure of APH(3')-IIIa (Fig. 5C and D). However, the naphthyl group of NL6-2 does not interact with the protein in contrast to the dihydro-benzopyran group NL6-1 which has similar interactions to those of NL6. Similarly, when docked in the apo protein, NL6 and NL6-1 show a strict overlay of their dihydro-benzopyran group, while NL6-2 presents a completely different binding mode (Fig. 5E and F). The naphthyl group of NL6-2 is indeed located at the opposite side from ADP in contrast

to the dihydro-benzopyran moiety of NL6 and NL6-1, located near the nucleotide binding site. This suggests that the benzopyran ring is also important for the stabilization of NL6.

Figure 5

Based on this prediction, these molecules were tested *in vitro* to estimate their ability to inhibit the APH(3')-IIIa activity. As shown in Fig. 5B, NL6-1 inhibits the activity of the APH. Similarly to NL6, NL6-1 was confirmed to act as a non-competitive inhibitor towards ATP with a K_i of $185 \pm 12 \mu\text{M}$ (data not shown). The 2-fold higher K_i of NL6-1 compared to NL6 can be explained by its shorter structure (20 carbons) compared to NL6 (23 carbons), resulting in a smaller number of interactions with the protein and consequently in a weaker inhibition. In contrast, NL6-2 was completely unable to inhibit the APH activity, confirming the hypothesis that the dihydro-benzopyran group is of major importance for APH inhibition.

According to kinetic and molecular docking studies, NL6 is likely to bind between both substrates, without affecting their binding. The presence of NL6 should greatly perturb the phosphotransfer of the γ -phosphate of ATP on the 3'-OH of kanamycin A which could explain the enzyme inhibition.

To conclude, the dihydro-benzopyran ring is of crucial importance for the binding of NL6 to APH enzymes. In addition, the two interactions with side chain of Arg211 and backbones of Leu209 and Gly210 were shown to play an essential role in the inhibition of APH.

4. Discussion

The problem of bacterial resistance to antibiotic due to aminoglycoside phosphotransferases APH(2'')-IVa and APH(3')-IIIa enzymes could be overcome by the development of novel drugs that target APHs, as suggested by Shi and co-authors in a recent review [16]. For example, because of the structural similarity of the ATP binding domain with protein kinases, APHs can be inhibited by eukaryotic protein kinase inhibitors. In this way, protein kinase inhibitors of the isoquinoline sulfonamide family have been shown to be competitive inhibitors of APH(3')-IIIa towards ATP, although they were unable to restore aminoglycoside susceptibility of strains harboring *aph(3')-IIIa* gene [38]. In the same manner, Shakya *et al.* screened a library of known kinase inhibitors and identified pyrido-pyrimidines as selective inhibitors of APH(3')s and flavonoids as general inhibitors of APHs [39]. A crystal structure of the APH(2'')-IVa-kanamycin-quercetin complex allowed to confirm that the inhibitor binds to the ATP binding site of the enzyme.

The problem with such kinase inhibitors is their intrinsic poor selectivity because they target the ATP binding site of APHs which is similar to that of many eukaryotic important enzymes. However, the recent structure-guided optimization of protein kinase inhibitors constitutes a promising strategy to enhance their specificity for APHs [18]. The originality of our inhibitors, compared to these protein kinase inhibitors, is that they do not target the substrate binding site, hence avoiding or reducing off-target effects. Therefore, they are neither going to compete with the antibiotic itself for the binding to APHs or to the rRNA target, nor with ATP which has a central role in many physiological processes. Instead, they have been selected for their ability to interfere with important dynamic properties of the targeted enzyme.

A similar strategy was successfully applied to APH with the development of allosteric inhibitors based on engineered ankyrin repeat motives. These compounds inhibited the enzyme activity, both *in vitro* and *in vivo*, and were able to restore the bacteria sensitivity to kanamycin and amikacin to a level comparable to that of the corresponding knockout strains

[40]. Later, the resolution of the crystal structure of APH(3')-IIIa complexed with one of the most potent ankyrin repeat protein inhibitor has confirmed the allosteric inhibition mechanism [23]. Indeed, the binding of the inhibitor stabilizes the APH in a non-catalytically competent conformation. However, the designed ankyrin repeat protein is a large molecule that is hardly druggable.

Here, on the basis of *in silico* screening of a chemical library, our idea was to find small molecules that are more druggable than large proteins and that inhibit APHs in a similar manner to that of ankyrin repeat protein. Allosteric small-molecule kinase inhibitors are indeed promising alternatives to active site binders, as recently reviewed [41].

We have identified twenty one small molecules that are potential allosteric inhibitors of APHs. Out of the seventeen that were evaluated, four molecules were able to inhibit significantly APH activity *in vitro*. We determined the mode of inhibition for two of them and showed that they are non-competitive towards kanamycin A. One of them, NL8, was a competitive inhibitor of APH(3')-IIIa towards ATP, suggesting that it should bind near the nucleotide binding position, but its precise binding position remains elusive. The fact that it did not inhibit APH(2'')-IVa suggests that it does not bind to the nucleotide binding site which is conserved between the two APHs. The most promising molecule, NL6, is able to inhibit non-competitively the two APHs tested. Its binding site was confirmed both by mutating one interacting residue of APH and by the use of NL6 analogues with one missing the dihydro-benzopyran ring required for an efficient binding. As shown in Fig. 6, NL6 binds to a different location in APH(3')-IIIa that of the protein kinase inhibitor CKI, and of the ankyrin-repeat protein inhibitor AR-3A.

Figure 6

This confirms that non-allosterically modulated proteins such as APHs can be inhibited by small druggable allosteric compounds, which brings new insights for the rational drug discovery of APHs inhibitors.

The seventeen compounds will be further tested for their effect on antibiotic susceptibility of different Gram-positive and Gram-negative bacteria strains as well as on

430 their potential cell toxicity. The integration of dynamic motions by using normal mode
431 analysis in combination with molecular docking, enzyme kinetics and antibacterial activity
432 studies, provided important insights into the molecular basis underlying ligand binding and
433 enzyme inhibition. Co-crystallization assays of APHs with these molecules are currently in
434 progress and rational lead optimization processes are considered in order to design new
435 derivatives with higher inhibitory properties. Efforts should be made to increase the affinity of
436 APH for the inhibitor by adding chemical substitutions that would interact with additional
437 residues of the cavity. This should avoid that a single mutation of the protein is enough to
438 prevent the inhibition.
439

5. Conclusion

Optimization of the lead compounds identified in this study should help in the design of efficient APHs inhibitors that may overcome APH-dependent bacterial resistance to aminoglycosides. Emergence and frequency of potential resistance mutants will be evaluated *in vitro*. Moreover, this strategy may be applied to other multi-resistant strains for which the major AMEs have been identified.

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References

- [1] Ramirez MS & Tolmasky ME (2010) Aminoglycoside modifying enzymes. *Drug Resist. Updat.* **13**, 151–171.
- [2] Kumar A & Schweizer HP (2005) Bacterial resistance to antibiotics: active efflux and reduced uptake. *Adv. Drug Deliv. Rev.* **57**, 1486–1513.
- [3] Pfister P, Hobbie S, Vicens Q, Böttger EC & Westhof E (2003) The molecular basis for A-site mutations conferring aminoglycoside resistance: relationship between ribosomal susceptibility and X-ray crystal structures. *Chembiochem* **4**, 1078–1088.
- [4] Doi Y & Arakawa Y (2007) 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. *Clin. Infect. Dis.* **45**, 88–94.
- [5] Vakulenko SB & Mobashery S (2003) Versatility of aminoglycosides and prospects for their future. *Clin. Microbiol. Rev.* **16**, 430–450.
- [6] Wright GD, Berghuis AM & Mobashery S (1998) Aminoglycoside antibiotics. Structures, functions, and resistance. *Adv. Exp. Med. Biol.* **456**, 27–69.
- [7] Shaw KJ, Rather PN, Hare RS & Miller GH (1993) Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev.* **57**, 138–163.
- [8] Ferretti JJ, Gilmore KS & Courvalin P (1986) Nucleotide sequence analysis of the gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2"-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. *J. Bacteriol.* **167**, 631–638.
- [9] Azucena E, Grapsas I & Mobashery S (1997) Properties of a bifunctional bacterial antibiotic resistance enzyme that catalyzes ATP-dependent 2"-phosphorylation and acetyl-CoA-dependent 6'-acetylation of aminoglycosides. *J. Am. Chem. Soc.* **119**, 2317–2318.
- [10] Dickie P, Bryan LE & Pickard MA (1978) Effect of enzymatic adenylation on dihydrostreptomycin accumulation in *Escherichia coli* carrying an R-factor: model

- explaining aminoglycoside resistance by inactivating mechanisms. *Antimicrob. Agents Chemother.* **14**, 569–580.
- [11] Höltje JV (1979) Induction of streptomycin uptake in resistant strains of *Escherichia coli*. *Antimicrob. Agents Chemother.* **15**, 177–181.
- [12] Bush K & Mobashery S (1998) How beta-lactamases have driven pharmaceutical drug discovery. From mechanistic knowledge to clinical circumvention. *Adv. Exp. Med. Biol.* **456**, 71–98.
- [13] Drawz SM & Bonomo RA (2010) Three decades of beta-lactamase inhibitors. *Clin. Microbiol. Rev.* **23**, 160–201.
- [14] Chiem K, Fuentes BA, Lin DL, Tran T, Jackson A, Ramirez MS & Tolmasky ME (2015) Inhibition of aminoglycoside 6'-N-acetyltransferase type Ib-mediated amikacin resistance in *Klebsiella pneumoniae* by zinc and copper pyrithione. *Antimicrob. Agents Chemother.* **59**, 5851–5853.
- [15] Li Y, Green KD, Johnson BR & Garneau-Tsodikova S (2015) Inhibition of aminoglycoside acetyltransferase resistance enzymes by metal salts. *Antimicrob. Agents Chemother.* **59**, 4148–4156.
- [16] Shi K, Caldwell SJ, Fong DH & Berghuis AM (2013) Prospects for circumventing aminoglycoside kinase mediated antibiotic resistance. *Front. Cell. Infect. Microbiol.* **3**, 22.
- [17] Fong DH, Xiong B, Hwang J & Berghuis AM (2011) Crystal structures of two aminoglycoside kinases bound with a eukaryotic protein kinase inhibitor. *PLoS One* **6**, e19589.
- [18] Stogios PJ, Spanogiannopoulos P, Evdokimova E, Egorova O, Shakya T, Todorovic N, Capretta A, Wright GD & Savchenko A (2013) Structure-guided optimization of protein kinase inhibitors reverses aminoglycoside antibiotic resistance. *Biochem. J.* **454**, 191–200.

- 516 [19] Szychowski J, Kondo J, Zahr O, Auclair K, Westhof E, Hanessian S & Keillor JW
517 (2011) Inhibition of aminoglycoside-deactivating enzymes APH(3')-IIIa and AAC(6')-II
518 by amphiphilic paromomycin O²-ether analogues. *ChemMedChem* **6**, 1961–1966.
- 519 [20] Vong K, Tam IS, Yan X & Auclair K (2012) Inhibitors of aminoglycoside resistance
520 activated in cells. *ACS Chem. Biol.* **7**, 470–475.
- 521 [21] Welch KT, Virga KG, Whittemore NA, Özen C, Wright E, Brown CL, Lee RE &
522 Serpersu EH (2005) Discovery of non-carbohydrate inhibitors of aminoglycoside-
523 modifying enzymes. *Bioorg. Med. Chem.* **13**, 6252–6263.
- 524 [22] Lin DL, Tran T, Adams C, Alam JY, Herron SR & Tolmasky ME (2013) Inhibitors of the
525 aminoglycoside 6'-N-acetyltransferase type Ib [AAC(6')-Ib] identified by *in silico*
526 molecular docking. *Bioorg. Med. Chem. Lett.* **23**, 5694–5698.
- 527 [23] Kohl A, Amstutz P, Parizek P, Binz HK, Briand C, Capitani G, Forrer P, Plückthun A &
528 Grütter MG (2005) Allosteric inhibition of aminoglycoside phosphotransferase by a
529 designed ankyrin repeat protein. *Structure* **13**, 1131–1141.
- 530 [24] Fong DH & Berghuis AM (2002) Substrate promiscuity of an aminoglycoside antibiotic
531 resistance enzyme via target mimicry. *EMBO J.* **21**, 2323–2331.
- 532 [25] Shi K, Houston DR & Berghuis AM (2011) Crystal structures of antibiotic-bound
533 complexes of aminoglycoside 2"-phosphotransferase IVa highlight the diversity in
534 substrate binding modes among aminoglycoside kinases. *Biochemistry* **50**, 6237–6244.
- 535 [26] Brooks BR, Brooks CL, Mackerell AD, Nilsson L, Petrella RJ, Roux B, Won Y,
536 Archontis G, Bartels C, Boresch S, Caflisch A, Caves L, Cui Q, Dinner AR, Feig M,
537 Fischer S, Gao J, Hodoscek M, Im W, Kuczera K, Lazaridis T, Ma J, Ovchinnikov V,
538 Paci E, Pastor RW, Post CB, Pu JZ, Schaefer M, Tidor B, Venable RM, Woodcock HL,
539 Wu X, Yang W, York DM & Karplus M (2009) CHARMM: the biomolecular simulation
540 program. *J. Comput. Chem.* **30**, 1545–1614.
- 541 [27] Zhu X, Lopes PEM & Mackerell AD (2012) Recent Developments and Applications of
542 the CHARMM force fields. *Wiley Interdiscip. Rev. Comput. Mol. Sci.* **2**, 167–185.

- 543 [28] Humphrey W, Dalke A & Schulten K (1996) VMD: visual molecular dynamics. *J. Mol.*
544 *Graph.* **14**, 33–38, 27–28.
- 545 [29] Schmidtke P, Bidon-Chanal A, Luque FJ & Barril X (2011) MDpocket: open-source
546 cavity detection and characterization on molecular dynamics trajectories.
547 *Bioinformatics* **27**, 3276–3285.
- 548 [30] Jones G, Willett P & Glen RC (1995) Molecular recognition of receptor sites using a
549 genetic algorithm with a description of desolvation. *J. Mol. Biol.* **245**, 43–53.
- 550 [31] Jones G, Willett P, Glen RC, Leach AR & Taylor R (1997) Development and validation
551 of a genetic algorithm for flexible docking. *J. Mol. Biol.* **267**, 727–748.
- 552 [32] Irwin JJ, Sterling T, Mysinger MM, Bolstad ES & Coleman RG (2012) ZINC: a free tool
553 to discover chemistry for biology. *J. Chem. Inf. Model.* **52**, 1757–1768.
- 554 [33] Lipinski CA (2004) Lead- and drug-like compounds: the rule-of-five revolution. *Drug*
555 *Discov. Today Technol.* **1**, 337–341.
- 556 [34] Lallemand P, Leban N, Kunzelmann S, Chaloin L, Serpersu EH, Webb MR, Barman T
557 & Lionne C (2012) Transient kinetics of aminoglycoside phosphotransferase(3')-IIla
558 reveals a potential drug target in the antibiotic resistance mechanism. *FEBS Lett.* **586**,
559 4223–4227.
- 560 [35] McKay GA, Thompson PR & Wright GD (1994) Broad spectrum aminoglycoside
561 phosphotransferase type III from *Enterococcus*: overexpression, purification, and
562 substrate specificity. *Biochemistry* **33**, 6936–6944.
- 563 [36] Kaplan E, Guichou J-F, Chaloin L, Kunzelmann S, Leban N, Serpersu EH & Lionne C
564 (2016) Aminoglycoside binding and catalysis specificity of aminoglycoside 2"-
565 phosphotransferase IVa: A thermodynamic, structural and kinetic study. *Biochim.*
566 *Biophys. Acta* **1860**, 802–813.
- 567 [37] Barman TE, Bellamy SRW, Gutfreund H, Halford SE & Lionne C (2006) The
568 identification of chemical intermediates in enzyme catalysis by the rapid quench-flow
569 technique. *Cell. Mol. Life Sci.* **63**, 2571–2583.

- 570 [38] Daigle DM, McKay GA & Wright GD (1997) Inhibition of aminoglycoside antibiotic
571 resistance enzymes by protein kinase inhibitors. *J. Biol. Chem.* **272**, 24755–24758.
- 572 [39] Shakya T, Stogios PJ, Waglechner N, Evdokimova E, Ejim L, Blanchard JE, McArthur
573 AG, Savchenko A & Wright GD (2011) A small molecule discrimination map of the
574 antibiotic resistance kinome. *Chem. Biol.* **18**, 1591–1601.
- 575 [40] Amstutz P, Binz HK, Parizek P, Stumpp MT, Kohl A, Grütter MG, Forrer P & Plückthun
576 A (2005) Intracellular kinase inhibitors selected from combinatorial libraries of designed
577 ankyrin repeat proteins. *J. Biol. Chem.* **280**, 24715–24722.
- 578 [41] Wu P, Clausen MH & Nielsen TE (2015) Allosteric small-molecule kinase inhibitors.
579 *Pharmacol. Ther.* **156**, 59–68.
- 580

Figure captions

Fig. 1. Identification of the target cavities using normal mode analysis. (A) Crystal structure of APH(3')-IIIa shown as blue cartoon, complexed with ADP and kanamycin A in stick representation (PDB ID: 1L8T). (B) Detection of all the dynamic cavities, shown in mesh representation, from the fifty frames of the trajectory of the 200 first normal modes of 1L8T structure depleted of ligands. (C) Selected cavity used for virtual screening. (D) Crystal structure of APH(2'')-IVa shown as pink cartoon, complexed with tobramycin in stick representation (PDB ID: 3SG8). (E) Detection of dynamic cavities of 3SG8 structure without tobramycin. (F) Selected cavity used for virtual screening. (G) Volume of the target cavities (shown in C and F) showing variation along the normal modes trajectory of APH(3')-IIIa (in blue) and APH(2'')-IVa (in red). (H and I) Atomic fluctuations averaged by residue computed from normal mode analysis for APH(3')-IIIa (1L8T) and APH(2'')-IVa, respectively. The numbers highlight residues showing significant fluctuations (for more details, see Fig. S2).

Fig. 2. Determination of APH(3')-IIIa inhibition mode by NL8. (A) Competitive inhibition profile by NL8 towards ATP. Final concentrations were 0.1 μ M APH(3')-IIIa, 50 μ M kanamycin A and 5–500 μ M ATP. (B) Non-competitive inhibition profile by NL8 towards kanamycin A. Final concentrations were 0.1 μ M APH(3')-IIIa, 5–200 μ M kanamycin A and 25 μ M ATP. Lineweaver-Burk representations are shown but the fits were carried out on raw data. NL8 concentrations were 0 μ M (circles), 50 μ M (squares) or 100 μ M (triangles).

Fig. 3. Determination of the inhibition mode of APH(2'')-IVa (A, B) or APH(3')-IIIa (C, D) by NL6. Final concentrations were (A) 0.5 μ M APH(2'')-IVa, 100 μ M kanamycin A and 50–500 μ M ATP; (B) 0.5 μ M APH(2'')-IVa, 5–150 μ M kanamycin A and 400 μ M ATP; (C) 0.1 μ M APH(3')-IIIa, 50 μ M kanamycin A and 5–250 μ M ATP; (D) 0.1 μ M APH(3')-IIIa, 5–100 μ M kanamycin A and 25 μ M ATP. NL6 concentrations were 0 μ M (circles), 20 μ M (triangles) or 50 μ M (squares).

609

610 **Fig. 4.** (A, B, C) Clusters of docking poses of NL6 in the apo, in the ADP-bound and in the
611 ternary structures of APH(3')-IIIa, respectively. The stable dihydro-benzopyran group of NL6
612 is shown surrounded by a dashed line. (D) Details of interactions of the dihydro-benzopyran
613 ring of NL6 with APH(3')-IIIa. (E) Docking of NL6 in the substrate-bound structure of APH(3')-
614 IIIa R211A mutant. (F) Steady state activity of APH(3')-IIIa R211A mutant as a function of
615 ATP concentration in the absence (circles) or in the presence of NL6 (squares). Final
616 concentrations were 0.2 μ M R211A APH(3')-IIIa, 50 μ M kanamycin A, 5-100 μ M ATP, and 0
617 or 50 μ M NL6.

618

619 **Fig. 5.** (A) Structure of NL6 and the two selected analogues, NL6-1 and NL6-2. (B)
620 Comparison of APH(3')-IIIa steady state activity as a function of ATP concentration in the
621 absence of NL compound (circles) or in the presence of NL6 (squares), NL6-1 (triangles) or
622 NL6-2 (stars). Final concentrations were 0.1 μ M APH(3')-IIIa, 50 μ M kanamycin A, 5–100 μ M
623 ATP, and 0 or 50 μ M NL compounds. (C, D) Overlay of the best docking poses of NL6 (grey)
624 and NL6-1 (pink) or NL6-2 (dark cyan) respectively in the APH(3')-IIIa-ADP-kanamycin A
625 complex. (E, F) Overlay of the best docking poses of NL6 and NL6-1 or NL6-2 respectively in
626 the apo structure of APH(3')-IIIa.

627

628 **Fig. 6.** (A, B) Crystal structures of the APH(3')-IIIa bound to CKI-inhibitor (yellow sticks, PDB
629 ID: 3Q2J) or to AR_3A inhibitor (green cartoon, PDB ID: 2BKK), respectively. (C) Predicted
630 binding position of NL6 (grey sticks) in APH(3')-IIIa.

631

Table 1.

Docking scores and averaged *in vitro* APH inhibitions of selected NL compounds. Compounds that were not commercially available are indicated by an asterisk. Zinc numbers are indicated in brackets. Inhibitions of APH activity by 500 μ M of NL compound are expressed as percentage relative to the activity without compound. Inhibitions by 40% or more are highlighted in bold.

Compound (ZINC number)	Structure	APH(3')-IIIa		APH(2'')-IVa		Compound (ZINC number)	Structure	APH(3')-IIIa		APH(2'')-IVa	
		Score	Inhibition	Score	Inhibition			Score	Inhibition	Score	Inhibition
NL1 (08981682)		71	7	81	10	NL12 (40455179)		75	0	66	17
NL2 (20785546)		73	0	80	26	NL13 (1792615)		75	8	69	27
NL3 (13001091)		77	7	79	9	NL14 (6751595)		61	0	68	0
NL4* (13001089)		78	nd	78	nd	NL15* (36046879)		76	nd	69	nd
NL5 (22406103)		80	20	77	19	NL16 (13572054)		81	37	63	68
NL6 (13236811)		83	43	77	63	NL17 (14746000)		80	0	63	12
NL7* (52627502)		80	nd	77	nd	NL18 (36589946)		79	0	60	12
NL8 (8790387)		81	79	74	7	NL19 (8981627)		75	3	63	0
NL9 (1143187)		80	12	71	45	NL20 (22357542)		73	0	71	14
NL10* (05277506)		71	nd	68	nd	NL21 (12636347)		64	37	63	24
NL11 (20737652)		67	2	64	14						

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B

C

Fig. S1. Sequencing result showing the representative part of the *aph(3')-IIIa* gene where the R211A mutation has been introduced.

Fig. S2. Identification of the target cavities using normal mode analysis. (A, B) Crystal structures of APH(3')-IIIa and APH(2'')-IVa shown as blue or pink cartoon, respectively. The fluctuated residues involved in the cavity are shown as red balls: H188, K217 and I247 in APH(3')-IIIa and D237, E238 and K255 in APH(2'')-IVa. Yellow balls indicate CA atoms of the residues surrounding each cavity (G192 and R226 in APH(3')-IIIa and S199 and S232 in APH(2'')-IVa). (C, D) Movies showing the motions of APH(3')-IIIa and APH(2'')-IVa from superposed normal modes. Same representations as in A and B, respectively.

Fig. S3. Predicted binding poses obtained for NL8 by molecular docking using identical parameters either in the APH(3')-IIIa apo protein (A, B) or in the APH(3')-IIIa-kanamycin A complex (C, D). Best docking poses of NL8 in the ADP-bound structure (E) or in the ternary complex (F). NL8 was docked into the 1L8T structure in which none, one or both ligands were conserved for docking. Removed ligands are shown in transparent sticks.

Video Fig S2C

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Video Fig S2D

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