

Cellular studies of an aminoglycoside potentiator reveal a new inhibitor of aminoglycoside resistance

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Abstract: Aminoglycosides are a group of broad-spectrum antibiotics that have been used in the clinic for almost a century. The rapid spread of bacterial genes coding for aminoglycoside-modifying enzymes has however dramatically decreased the utility of aminoglycosides. We have previously reported several aminoglycoside potentiators which work by inhibiting aminoglycoside *N*-6'-acetyltransferase, one of the most common determinants of aminoglycoside resistance. Among those, pro-drugs that combine the structure of an aminoglycoside with that of pantothenate into one molecule are especially promising. We report here, a series of cellular studies to further investigate the activity and mechanism of action of these prodrugs. Our results reveal a new aminoglycoside resistance inhibitor, and the possibility that these prodrugs are transformed into more than one inhibitor in bacteria. We also report that the onset of the potentiators is rapid. Their low cell cytotoxicity, good stability and potentiation of various aminoglycosides, against both Gram-positive and Gram-negative bacteria, make them interesting compounds for the development of new drugs.

Introduction

Aminoglycosides are a group of highly potent, broad-spectrum antibiotics currently used to treat serious infections in a hospital setting.^{[1]-[2]} Their lethal effect on bacteria is mostly attributed to their binding to bacterial ribosomes, and causing infidelity during protein synthesis.^{[1]-[2]} As with all antibiotics, resistance to aminoglycosides has spread widely.^[3] The most common mechanism of aminoglycoside resistance involves the production of aminoglycoside-modifying enzymes such as aminoglycoside *N*-acetyltransferases (AACs), aminoglycoside *O*-phosphotransferases (APHs) and aminoglycoside *O*-nucleotidyltransferases (ANTs).^[4] The AAC family of enzymes is the largest one of the three, with over fifty AACs identified in various organisms.^{[3],[5]} They use acetyl-coenzyme A (AcCoA) to acetylate free amines at the 1, 3, 2' or 6' position of aminoglycosides (Figure 1), and are thus classified as AAC(1), AAC(3), AAC(2') or AAC(6'), respectively.^[1] AACs are further divided between subtypes I or II, based on the resistance profile that they cause (*i.e.* substrate scope), and a lower case letter at the end of the name defines the specific isoform.^[1] AAC(6') is by far the most prevalent in clinical strains.^[3] Interestingly, some bacteria, such as the important human pathogen *Enterococcus faecium*, harbor an ACC(6')-coding gene on a chromosome, which renders them intrinsically resistant to aminoglycosides.^[6]

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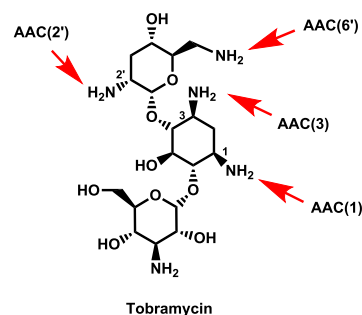


Figure 1. Susceptible sites for modifications by different AACs, shown using tobramycin as an example.^[1]

To overcome aminoglycoside resistance, two strategies have been investigated by different labs: 1) modifying aminoglycosides to make them refractory to transformations by resistance-causing enzymes while maintaining their potency; and 2) blocking aminoglycoside resistance.^[5] Our lab has had interest in the latter,^{[7]-[15]} with neamine derivatives **1a-e** being promising inhibitors of the Enterococci isoform AAC(6')-II (Figure 2).^{[7],[15]} While derivatives of other aminoglycosides have also been reported, they show slightly lower activity (*e.g.* the K_i of the neamine derivative **1a** is 76 nM, compared to 119 nM and 111 nM for the corresponding kanamycin A and ribostamycin derivatives respectively).^[7] Unfortunately, **1a-e** cannot penetrate bacterial cells and have no activity *in cellulo*. To address the permeability issue, a series of prodrugs, **P-1a-e**, designed to be bioactivated into **1a-e** by enzymes of the bacterial CoA biosynthetic pathway, were next reported.^[15] **P-1a-e** have no antibacterial activity of their own, do not inhibit AAC(6')-II significantly, yet, except for **P-1a**, potentiate the activity of kanamycin A against aminoglycoside-resistant *E. faecium* (harboring a chromosomally-encoded *aac(6')*).^[15] The difference between **P-1a** and **P-1b-e** was rationalized via *in vitro* studies, which showed that compounds **P-1b-e** were transformed into inhibitors **1b-e** by three of the *E. coli* CoA biosynthetic enzymes (Figure 2): pantothenate kinase (PanK, EC 2.7.1.33), phosphopantetheine adenylyltransferase (PPAT, EC 2.7.7.3) and dephospho-CoA kinase (DPCK, EC 2.7.1.24), whereas **P-1a** was not converted to inhibitor **1a**. Since **1a** was not produced, AAC(6')-II may not be inhibited, which may explain the absence of kanamycin A potentiation by **P-1a**.

Compounds **P-1a-e** are not inhibiting bacterial growth on their own, consequently exerting much less pressure on bacterial growth, and since the unnatural part of these molecules (relative to pantothenate) is expected to lie outside the active site of the three bioactivating enzymes,^{[16]-[18]} resistance development for these compounds is expected to emerge more slowly. This advantage of compounds **P-1a-e**, and the interesting activity of **P-1b-e** motivated us to pursue *in cellulo* and stability studies of **P-1a-e**.

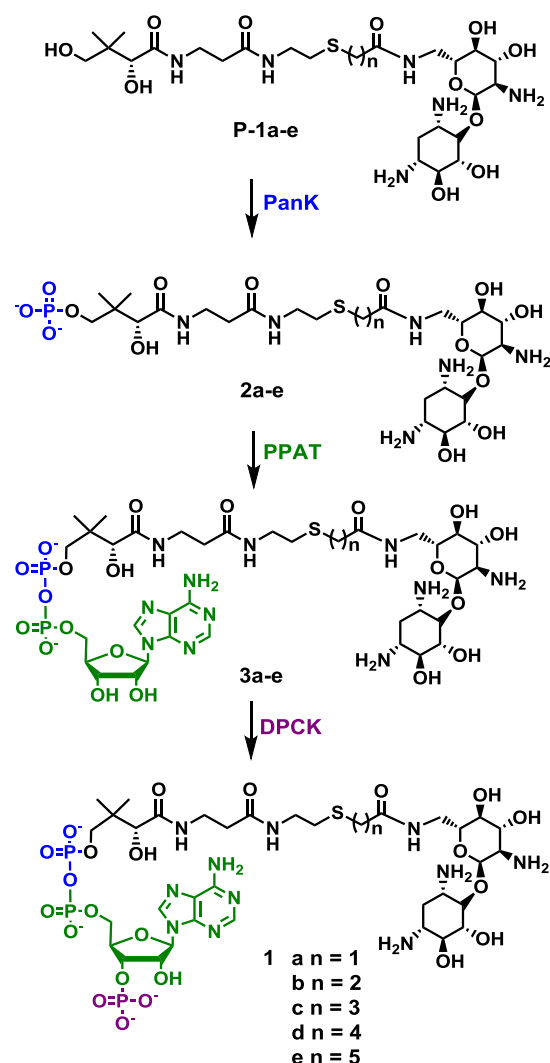


Figure 2. Proposed transformation of **P-1a-e** to the corresponding AAC(6')-II inhibitors **1a-e** by enzymes of the CoA biosynthetic pathway.

Results and Discussion

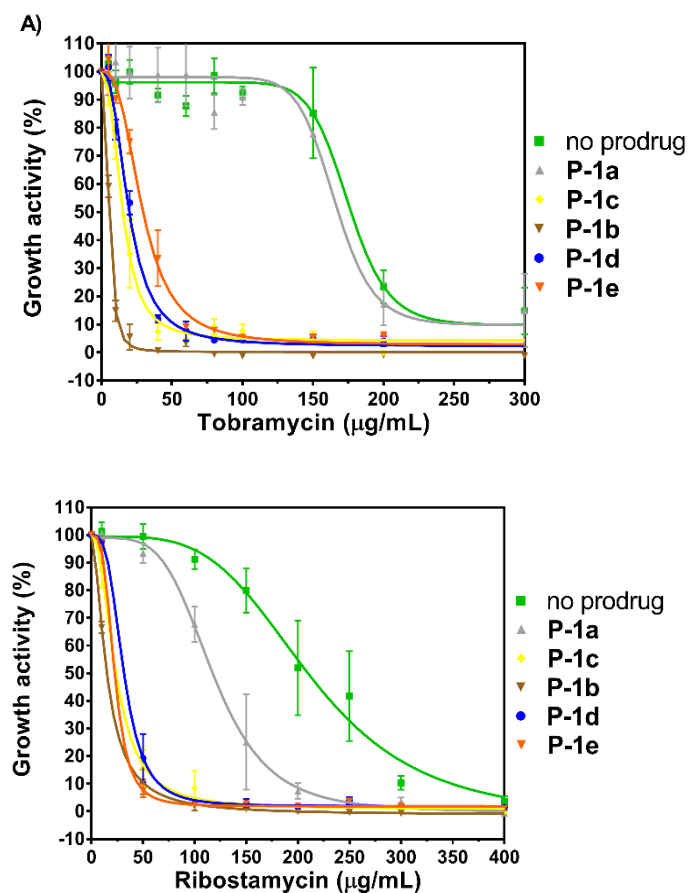
P-1a-e potentiate the antibacterial effect of different aminoglycosides

Compounds **P-1b-e** were reported to potentiate the antibacterial activity of kanamycin A, a 4,6-linked aminoglycoside, against the intrinsically resistant *E. faecium*.^[15] To investigate the scope of potentiation by **P-1a-e**, we tested them in combination with two additional aminoglycosides, tobramycin and ribostamycin, which were selected not only because they are 4,5-linked antibiotics but also because *E. faecium* ATCC 19434 is more resistant to these aminoglycosides (MIC values > 200 µg/mL, see Table S1) than to kanamycin A (128 µg/mL). While **P-1a** did not potentiate the activity of ribostamycin or tobramycin by much, **P-1b-e** greatly reduced the MIC of both aminoglycosides (Figure 3A). This trend is consistent with that reported for kanamycin A with **P-1a-e**,^[15] and demonstrates that **P-1b-e** can potentiate the activity of various aminoglycosides. Since compounds **P-1b-e** show a similar potentiating effect, the tobramycin-**P-1b** combination shows the best antibacterial activity, and the inhibitors **1b-c** are reported to be more potent than **1d-e**,^{[7],[15]} all subsequent experiments were performed with **P-1b** and/or **P-1c**.

Dose-response relationships were next examined. As shown in Figure 3B–C, the higher the concentration of **P-1b** or **P-1c** used, the less aminoglycoside is required to inhibit the growth of *E. faecium* ATCC 19434; and the higher the concentration of aminoglycoside, the less **P-1b** or **P-1c** is required to achieve the same growth inhibitory effect. This phenotype is within expectation since more **P-1b** or **P-1c** should lead to more AAC(6')-II inhibition in *E. faecium*, leaving the bacteria more sensitive to the aminoglycoside and consequently requiring less aminoglycoside. Furthermore, Figure 3B–C reveal that at

concentrations of tobramycin $\leq 15 \mu\text{g/mL}$, which is a pharmaceutically-relevant concentration,^{[19]-[21]} the minimal concentration of **P-1b** required to significantly inhibit the growth of *E. faecium* is 192 $\mu\text{g/mL}$.

It is worth noting that while **P-1a** did not potentiate the activity of kanamycin A or tobramycin, it does lower the MIC of ribostamycin towards *E. faecium* by ca. 100 $\mu\text{g/mL}$ (Figure 3A, right panel). This cannot be explained if compound **1a** is the active AAC(6')-II inhibitor since a previous *in vitro* study has shown that **P-1a** is only transformed into **2a** (5% yield) and **3a** (25% yield) by the CoA biosynthetic enzymes (Figure 2), with 70% of unmodified **P-1a**, and no **1a** observed.^[15] One possible explanation for this unexpected result may be a difference in enzyme activity between *in vitro* and *in cellulo* conditions, alternatively a difference in activity between the *E. coli* CoA biosynthetic enzymes (used for the *in vitro* studies)^[15] and the *E. faecium* enzymes (used for the *in cellulo* studies). The sequence identity of PanK, PPAT and DPCK between these two bacteria is 44%, 46% and 36%, respectively. To investigate this, we next looked at the metabolism of **P-1b** in *E. faecium* cells.



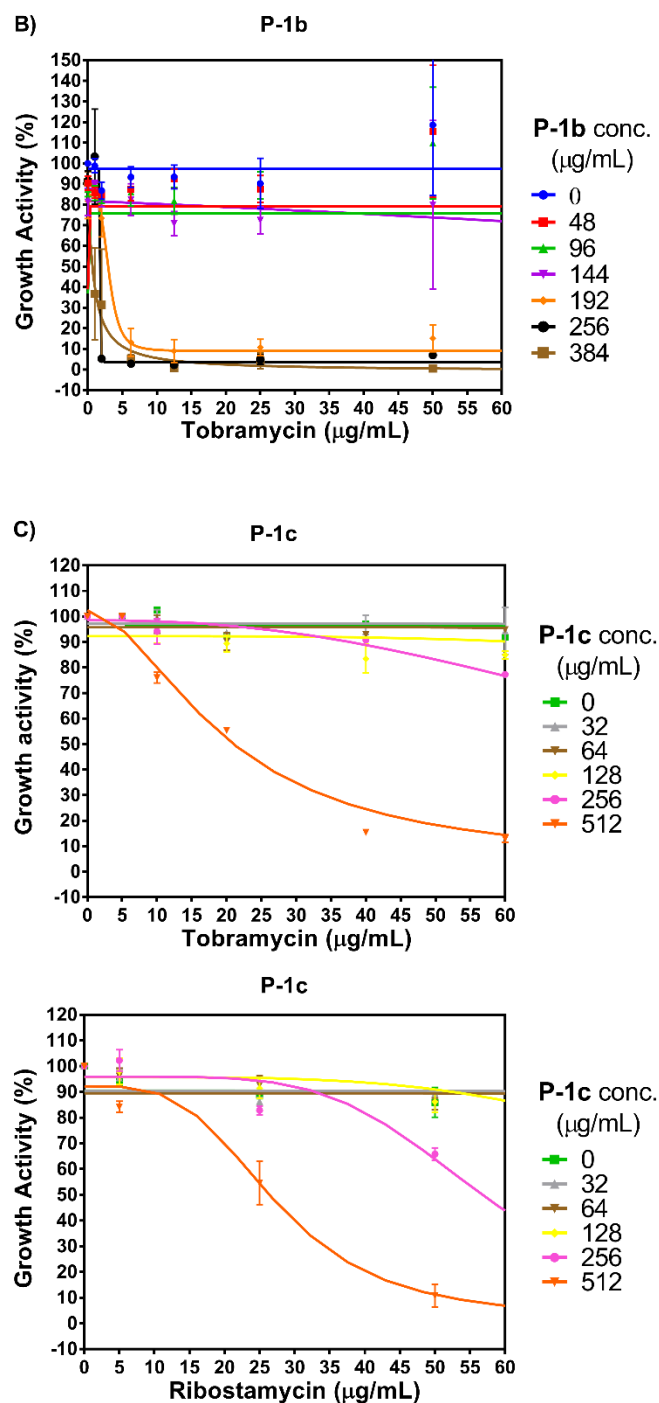


Figure 3. MIC plots reporting the antibacterial activity of different aminoglycosides (x-axis label) towards *E. faecium* ATCC 19434, in the presence or absence of potentiator, A) at 512 μg/mL of compounds **P-1a-e**; B) at different concentrations of **P-1b**, or C) at different concentrations of **P-1c**.

Time-kill studies with **P-1b**

To choose the time window for *in cellulo* investigations, time-kill studies were performed with an initial *E. faecium* culture of 10^5 CFU/mL. As shown in Figure 4, when no tobramycin nor **P-1b** are added to the growth medium, or when only **P-1b** is

added, *E. faecium* replicates exponentially and reaches 10^6 CFU/mL within ~2 h. In the presence of 100 µg/mL tobramycin, *E. faecium* grows slower, taking 4–6 h to reach the same cell density. In contrast, when both 384 µg/mL of **P-1b** and 25 µg/mL of tobramycin (a combination known to lead to 100% growth inhibition) are added to the growth medium, *E. faecium* remains in static growth for ~8 h. Finally, when the concentration of tobramycin in the medium is raised to 50 µg/mL or more (and **P-1b** is kept at 384 µg/mL), the number of *E. faecium* CFU/mL decreases slowly over the first 8 h, and takes more than 16 h to recover (based on the MIC studies). Because the drop in bacterial concentration is only ca. 20-fold, this combination of tobramycin and **P-1b** is considered bacteriostatic (drop less than 1000-fold), not bactericidal (drop more than 1000-fold).^[22] Interestingly, under these conditions, the cell concentration of *E. faecium* decreases continuously over 8 h, with a decrease rate proportional to the tobramycin concentration, suggesting that the **P-1b**-tobramycin combination is concentration-dependent, as reported for aminoglycosides when used alone.^[23] More importantly, when comparing the time-kill curves for tobramycin with or without **P-1b**, it is interesting to note that at time 4 h, the CFU/mL differ by almost 2 orders of magnitude, indicating that **P-1b** takes effect well before four hours. Therefore, the 0–4 h is a critical time window to study the *in cellulo* transformation of **P-1b**.

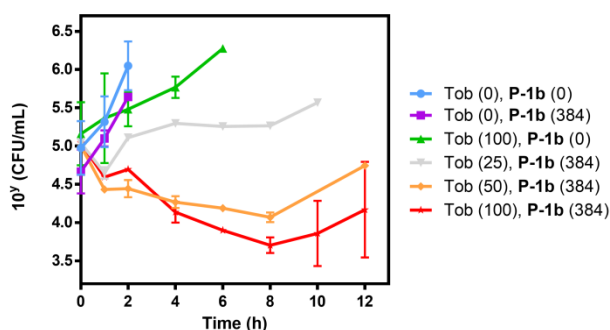


Figure 4. Time-kill curves of *E. faecium* ATCC 19434 in the presence of different concentrations of **P-1b** and tobramycin. Tob: tobramycin. The values in brackets in the legend are concentrations, in µg/mL.

Bioactivation of **P-1b** in *E. faecium* ATCC 19434

To investigate the bioactivation of **P-1b** in *E. faecium*, 17×10^9 colonies of *E. faecium* were collected every hour after the addition of **P-1b** (384 µg/mL) into the growth medium. The cells were washed, lysed and the metabolites were extracted at 0°C, before LCMS analysis to quantify **P-1b**, **2b**, **3b** and **1b**. Control experiments without **P-1b** were also analyzed, and no **P-1b**, **2b**, **3b** or **1b** was detected in these samples (data not shown). As shown in Figure 5, compounds **2b** and **3b** are produced in *E. faecium* within the first hour. The intracellular concentrations of **P-1b** and of **3b** are higher than that of **2b** for the entire experiment. This phenomenon is consistent with PanK being the rate-limiting enzyme in the bioactivation of **P-1b** to **3b**, as suggested by others for some microorganisms.^{[24],[25]} It is worth noting that even after 6 h, no inhibitor **1b** was detected (L.O.D = 0.05 pmol). Even though these studies were performed with *E. faecium* cells, the absence of detectable amounts of **1b** is consistent with the results obtained with purified *E. coli* PanK, PPAT and DPCK, where **P-1b** was transformed into 10% **1b** + 20% **2b** + 70% **3b**.^[15] Nevertheless, because little to no **1b** is detected, neither of these data sets can explain the time-kill results which suggest that **P-1b** has taken effect within the first hour, nor the potentiating effect of **P-1a** (which is not transformed to **1a** either) on the antibacterial activity of ribostamycin (Figure 3A, right panel).

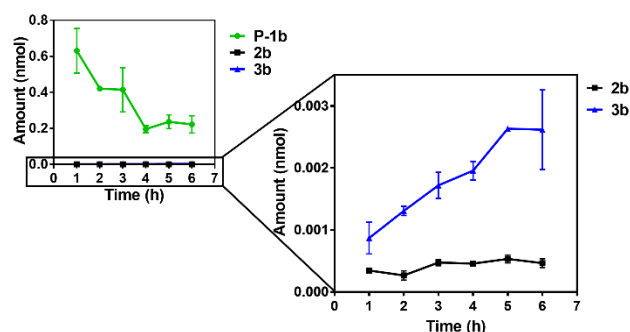
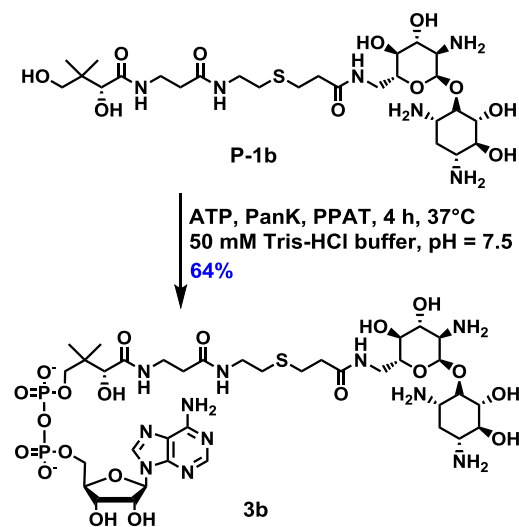


Figure 5. Transformation of **P-1b** in *E. faecium* measured by LCMS, with detection of **P-1b**, **2b** and **3b** in cells. No **1b** was detected (L.O.D = 0.05 pmol).

Since **3b** was found to rapidly accumulate during the first five hours, it was envisaged that the potentiating effect observed during the time-kill studies might be attributed in large part to **3b**. No K_i values are reported for the inhibition of AAC(6')-li by **2b** or **3b**, however, K_i values are known for **P-1a** ($\geq 500 \mu\text{M}$), the phosphorylated **2a** ($12 \mu\text{M}$), and the CoA derivatives **1a** ($0.076 \mu\text{M}$) and **1b** ($0.043 \mu\text{M}$).^{[7-8],[15]} These numbers suggest that the AAC(6')-li inhibitory activity of **3a** (which lacks only one phosphate group compared to **1a**) might be somewhere in between those of **1a** and **2a**, and considering that the AAC(6')-inhibitory activities of **1b** and **1a** are very similar, we expect compound **3b** to be a potent inhibitor of *E. faecium* AAC(6')-li.

Inhibition of AAC(6')-li by **3b**

Pure **3b** was prepared enzymatically from **P-1b** and ATP, using purified *E. coli* PanK and *E. coli* PPAT as the catalysts (Scheme 1). The resulting compound **3b** was purified by HPLC before enzyme inhibition studies. The K_i of **3b** for AAC(6')-li was measured using the previously reported method.^[7-8] As expected, compound **3b** is a competitive (vs AcCoA), tight-binding inhibitor of AAC(6')-li, with a K_i of $0.12 \pm 0.04 \mu\text{M}$. This is only ~3-fold higher than the K_i of **1b** ($0.043 \pm 0.023 \mu\text{M}$)^[7-8] and provides an explanation for the observed potentiating effect of **P-1b** in the absence of a significant amount of **1b** being produced. This may also explain the potentiating effect of **P-1a** on the antibacterial activity of ribostamycin. Since **1a** and **1b** show similar inhibitory activity towards AAC(6')-li, like **3b**, **3a** is likely a potent AAC(6')-li inhibitor. We therefore propose that the *in cellulo* bioactivation of **P-1b** leads to the formation of at least two AAC(6')-li inhibitors (**2b** and **3b**), and maybe a very small amount of **1b** (< 10% based on *in vitro* studies), with **3b** contributing the most to the potentiation effect.



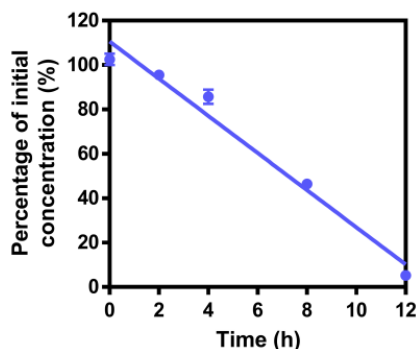
Scheme 1. Synthesis of compound **3b**.

Stability studies of P-1b

The stability of **P-1b** was next investigated. The half-life of **P-1b** in human serum is 7.2 ± 0.6 h (Figure 6A), which is significantly longer than its onset of action. Indeed our results show that **P-1b** takes effect within the first hour, its effect lasts at least 16 h based on MIC determinations, and the *in cellulo* bioactivation studies reveal the presence of AAC(6')-II inhibitor **3b** in cells within the first hour.

The stability of **P-1b** towards the action of P450 enzymes was also evaluated. We found that the metabolism of **P-1b** by rat liver microsomes is much slower than that of testosterone (Figure 6B). The relatively long half-life of **P-1b** in blood and its slow metabolism by P450 enzymes are both desirable traits for drug candidates.

A. Stability study of P-1b in human serum



B. Stability study towards rat liver microsomes

Time (min)	Turnover (pmol/min/mg protein)	
	P-1b	Testosterone
30	5.4 ± 0.2	128.7 ± 0.7

Figure 6. Stability studies of **P-1b**, A) in human serum, and B) towards P450 enzymes (rat liver microsomes).

The aminoglycoside potentiating effect of P-1b against other bacterial strains

Compound **P-1b** effectively potentiates the antibacterial activity of aminoglycosides towards the intrinsically resistant Gram-positive bacterium *E. faecium*. Its activity in Gram-negative bacteria was investigated next. An aminoglycoside-resistant *E. coli* strain was generated by transformation of competent *E. coli* BL21 cells with the plasmid pET22b(+)/AAC(6')-II. As expected, the resulting strain (abbreviated BL21-AAC(6')-II) is more resistant towards ribostamycin than the original strain (abbreviated BL21; Figure 7A). Interestingly, in the presence of **P-1b**, the MIC of ribostamycin towards BL21-AAC(6')-II decreased considerably. This result demonstrates that **P-1b** can enter Gram-negative bacterial cells and potentiate the activity of aminoglycosides.

Since **P-1b** itself does not inhibit AAC(6')-II, it can only take effect after being transformed by the CoA biosynthetic enzymes in bacteria. To confirm that the CoA biosynthetic enzymes of *E. coli* are involved in the bioactivation of **P-1b**, as is the case *in vitro* and in *E. faecium*, the aminoglycoside-potentiating activity of **P-1b** was studied towards BL21-AAC(6')-II in the presence of excess pantothenate. During bioactivation, **P-1b** must compete with pantothenate for the CoA biosynthetic enzymes. It was therefore hypothesized that excess pantothenate in the growth medium of BL21-AAC(6')-II may outcompete **P-1b**, thus decreasing its bioactivation by the CoA biosynthetic enzymes and reducing the aminoglycoside potentiation observed. As shown in Figure 7B, the MIC of the ribostamycin-**P-1b** combination for BL21-AAC(6')-II increased when the minimal growth medium (no pantothenate) was supplemented with 0.1 mM pantothenate. This supports our hypothesis that, as in *E. faecium*, the activity of **P-1b** in *E. coli* requires an input from the CoA biosynthetic enzymes.

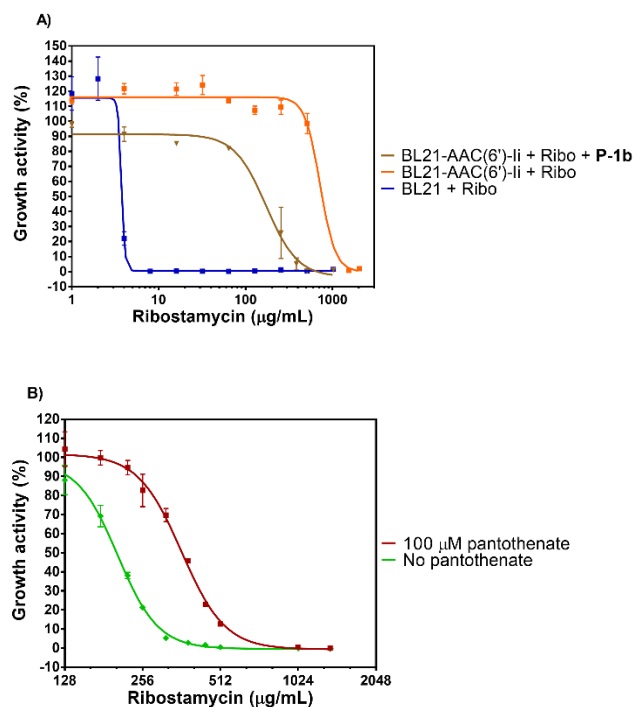


Figure 7. Potentiating effect of **P-1b** (512 μg/mL) on the antibacterial activity of ribostamycin towards different *E. coli* strains, A) in minimal medium (M9 + 0.2% glycerol) and B) in minimal medium (M9 + 0.2% glycerol) with or without 100 μM pantothenate added.

We next turned to vancomycin-resistant *Enterococci* (VRE) because of its rapidly increasing prevalence (as high as 44% in some countries) and the difficulty to treat VRE infections. Since the mechanisms evolved by bacteria to resist aminoglycosides are very different from those evolved to resist vancomycin (drug modification versus alteration to the structure of peptidoglycans), we expected **P-1b** to also resensitize VRE to aminoglycosides. Our results show that compound **P-1b** (16 μg/mL) can greatly decrease the MIC of different aminoglycosides towards VRE 2010A and VRE BAA-2316, two clinical strains (Table 1).

Table 1. Potentiating effect of compound **P-1b** (16 μg/mL) on the antibacterial activity of different aminoglycosides against two clinical VRE strains.

VRE strain	Compound	MIC (μg/mL)		
		Tobramycin	Kanamycin	Ribostamycin
2010A	No P-1b	128		
2010A	P-1b (16 μg/mL)	32		
BAA2316	No P-1b	128	256	512
BAA2316	P-1b (16 μg/mL)	32	64	64

Cell cytotoxicity studies

The cytotoxicity of **P-1b** was investigated using HeLa cells. As shown in Figure 8, even at a concentration of 1024 μg/mL, **P-1b** had little effect, if any, on the growth of HeLa cells.

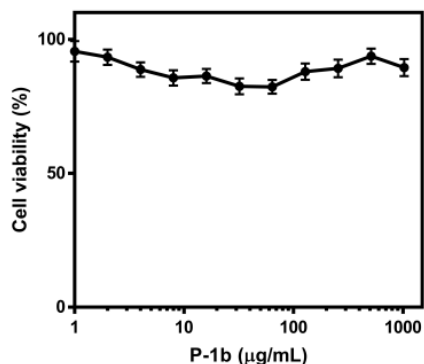


Figure 8. Effect of **P-1b** on the growth of HeLa cells.

Conclusions

Our group has demonstrated the use of CoA biosynthetic enzymes in prodrug activation.^{[15],[26]} Moreover, many pantothenate derivatives are known to act as antimetabolites and be transformed by the CoA biosynthetic enzymes in bacteria.^{[24],[25]} While it has been suggested in these studies that the active metabolite is the corresponding CoA derivative, the results presented here show that, at least for **P-1b**, an earlier intermediate (**3b** here) is the most important active molecule. Moreover, the cellular studies reported herein with **P-1b** reveal that bioactivation and AAC(6')-II inhibition occur within one hour.

Based on the absence of cell cytotoxicity observed at high concentrations of **P-1b**, its reasonable blood stability, slow metabolism, and ability to penetrate both Gram-positive and Gram-negative bacteria, we believe that this compound is a good starting point for the development of drugs to inhibit aminoglycoside resistance. Interestingly, because the unnatural part of our molecules (relative to the corresponding natural substrates) is expected to lie outside of PanK, PPAT and DPCK,^{[16]-[18]} we believe that the emergence of resistance may be slow.

Antibacterial resistance is growing to be a severe health issue around the world.^[27] Inhibiting resistance-causing enzymes is a validated approach for the design of new combination drugs to treat resistant infections.^[28] While several inhibitors of β -lactam resistance are used in the clinic, this is not the case for aminoglycoside resistance inhibitors. Our results suggest that compounds **P-1b-d** are promising hits for the design of such drugs. In particular, **P-1b-d** significantly reduce the MIC of different aminoglycosides towards *E. faecium*, an important nosocomial pathogen. Because enterococcal infections are intrinsically resistant to aminoglycosides, β -lactams, macrolides and clindamycin, their treatment typically requires drug combinations. The treatment of enterococcal infections is further complicated by the rapid spread of plasmids encoding additional resistance-causing genes.^[29] As many as 1/3 of all enterococcal infections in intensive care units are also vancomycin-resistant. Our results suggest that **P-1b** has potential for the treatment of VRE infections.

Experimental Section

Experimental details were described in the supporting information.

Acknowledgements

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Keywords: aminoglycoside • resistance inhibitor • coenzyme A • *Enterococcus faecium* • vancomycin-resistant *Enterococci*

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