The aminoglycoside-modifying enzyme Eis2 represents a new potential in vivo target for reducing antimicrobial drug resistance in Mycobacterium abscessus complex

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DOI: https://doi.org/10.1183/13993003.01541-2022

ERJ Letter

Article type	Word limit	Figures and tables	References	Online supplement	Abstract
Research letters	1200	1	15	Not accepted	No

TITLE: The aminoglycoside modifying enzyme Eis2 represents a new potential *in vivo* target for reducing antimicrobial drug resistance in *Mycobacterium abscessus* complex

Take-Home Message (256 char.)

Here we determined innovative therapeutic targets to reinforce existing current antibiotic treatments against *Mycobacterium abscessus* complex, exploiting murine preclinical model of respiratory infections and 727 public genomes from clinical isolates.

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Mycobacterium abscessus is an emerging opportunistic pathogen responsible for lung infections after lung colonisation in people with pulmonary disorders, like bronchiectasis or cystic fibrosis. It is becoming one of the most clinically relevant nontuberculous mycobacteria (REF) for severity of infections and poor response to antibiotic treatment. The *M. abscessus* complex (*MABSC*) is composed of three subspecies *abscessus*, *bolletii* and *massiliense*. MABSC pulmonary disease is characterized by the presence of specific microbiological, clinical and radiological features described in the ATS/ESCMID/ERS/IDSA consensus statement (REF). Infections are difficult to treat due to the high-level of antibiotic resistance conferred by an almost impermeable cell wall, drug efflux pumps, or drug-modifying enzymes (REF). Antibiotic regimens for treatment of MABSC pulmonary infections generally combine 3 to 4 antibiotics including Clarithromycin, Amikacin, Cefoxitin, Imipenem or Tigecycline for 12-24 months. Despite toxicity, the aminoglycoside Amikacin remains a key component in the regimen. Resistance to aminoglycosides is conferred mainly by modifying enzymes, such as *aac(2')*, *aph(3'')* and *eis2.* In a recent work the deletion of *eis2* from the genom of *M. abscessus*, encoding a promiscuous N-acetyltransferase, increased *in-vitro* susceptibility to Capreomycin, Kanamycin and Amikacin. The findings highlight that drug-modifying enzymes may provide new complementary therapeutic targets to fight antimicrobial resistance, through increasing the bacterial susceptibility to specific, licensed antibiotics thereby potentially improving treatment outcomes.

We aim to determine whether Eis2 is a relevant drug-modifying enzyme for bacterial resistance in preclinical model of *MABSC* and if it could be considered a clinically relevant target to reinforce existing antibiotic treatments in *MABSC* clinical isolates.

To understand the Eis2 relevance as MABSC drug resistance determinant *in-vivo* we tested *M. abscessus (Mabs)* Δ*eis2* mutant (REF) and its parental wt strain (ATCC 19977) in a preclinical model of lung infection (REF). As mentioned before, *in-vitro* ΔEis2 showed a 4-fold increase in the susceptibility (MIC) to Amikacin (REF) in comparison to the isogenic wt strain. We used a recently refined mouse model of *Mabs* respiratory infection (REF). Mice were infected with an intratracheal injection of 10^5 CFU embedded in agar beads, allowing persistence in the lung of C57BL/6N immunocompetent mice with a sustained bacterial load. Mice were treated daily by intraperitoneal injection of Amikacin (200 mg/kg) starting from 2 hours before bacterial challenge for the next six consecutive days (Figure 1A). Seven days post challenge, mice were sacrificed and their general health status, the local (lung homogenates) and systemic (spleen homogenates) bacterial burdens were evaluated. We observed that mice infected with Δ*eis2* mutant strain displayed a similar bacterial load as those challenged with wt strain ATCC19977, suggesting a similar bacterial capacity to multiply and persist in our model of infection and negating Eis2 as a virulence factor (Figure 1B). We confirmed that Amikacin treatment displays antibacterial activity (~1-Log CFU reduction) against the wt strain (ATCC19977) in our model of respiratory infection, as previous described (REF). Here, we determined that the bacterial burdens in the lung of mice infected with Δ*eis2* mutant strain and treated with Amikacin were significantly reduced in comparison to i) lungs of mice infected by Δ*eis2* mutant strain and treated with vehicle (~3-4 Log CFU reduction), and ii) lungs of mice infected by wt strain and treated with Amikacin (~2 Log CFU reduction) (Figure 1B). The same trend was also observed at the systemic level. In the spleen, mice infected with Δ*eis2* mutant strain and treated with amikacin displayed a significantly reduced bacterial burden in comparison to mice infected by wt strain and treated with the antibiotic (Figure 1C). Overall, these data proof that genetic inactivation of *eis2* increases Amikacin susceptibility at local and systemic compartments during lung *Mabs* respiratory infection in an *in vivo* preclinical model. We analysed Haematoxylin and Eosin (H&E) stained lung tissue slides at day 7 post challenge to evaluate the inflammatory foci in mice infected with Δ*eis2* strain with or without Amikacin treatment. As shown in Figure 1D, the lung infected with Δ*eis2* strain and treated with vehicle displayed more extended inflammatory foci (indicated by arrows), mainly composed by monocytes, than the lung infected with Δ*eis2* strain and treated with amikacin. This analysis was performed in three different mice/group (data not shown), confirming beneficial treatment of amikacin in limiting inflammatory foci in the lungs and potentially limiting the risk of the bacterial spreading at the systemic level. Overall *in vivo* data suggest that the deletion of *eis2* strongly enhances Amikacin antibacterial activity in the treatment of MABSC infections.

Previous studies focused their efforts in demonstrating *eis2* relevance, as MABSC drug resistance gene *in vitro*, through elegant mechanistic approaches (REF). To date, it is still unclear whether Eis2 target is conserved in *MABSC* clinical isolates. We determined the genomic conservation of *eis2* in *MABSC* publicly available genomes. Our analysis included clinical isolates from MABSC subsp. *abscessus*, *massiliense* or *bolletii*, and from different geographic regions (Europe and US). Non-synonymous mutations were found in 25 out of 535 MABSC subsp. *abscessus* strains (0.5 %), in 112 out of 144 strains of *Mabs* subsp. *massiliense* (77 %) and 48 out of 48 strains Mabs subsp. *bolletii* (100%) (FIGURE 1E). These data highlight that *eis2* genomic sequence is well conserved in MABSC subsp. *abscessus* strains, while the

biological relevance of nonsynonymous genomic variation observed in MABSC subsp. *massiliense* or *bolletii* remains unclear. We took advantage of the Eis2 crystal structure (REF) to determine whether the genomic variation observed among the MABSC public genomes could be associated with functional alteration of the catalytic site of Eis2 (PDB ID 6R FT, Figure 1F). We highlighted the mutated residues in the different subspecies, indicating that the mutations occur in distinct protein domains. In particular, most of the mutations mapped in MABSC subsp. *bolletii* are located close to the acetyl-CoA binding site, differently from MABSC subsp. *massiliense* and *abscessus*. While MABSC subsp. *abscessus* Eis2 structure is highly conserved, the knowledge of the structural consequences of mutations in *Mabs* subsp. *bolletii and massiliense* may pave the way to the design of Eis2-subspecies-specific inhibitors with the potential to counteract the intrinsic AMK resistance of *MABSC*.

Our work provides evidence that Eis2 represents a relevant drug-modifying enzyme for bacterial resistance *in vivo*. Our data proof that deletion of *eis2* strongly improves Amikacin antibacterial activity in our preclinical model of MABSC lung infections. Moreover, genomic analysis of *eis2* among clinical isolates suggest that sequencing approaches may promote future personalized medicine strategies based on the stratification of conserved drug-modifying enzymes of *MABSC.*

While the Eis2 crystal structure was already published, the relevance of non-synonymous mutations found in our analysis remains to be elucidated. Indeed, this highlights again the clinical relevance of genomic bacterial data for the design of future tailored personalized antimicrobial therapies. It's worth noting that potent inhibitors with a strong translational relevance have been identified against Eisof *Mycobacterium tuberculosis*. (REF) However, Amikacin is more important in the treatment of MABSC infection than in tuberculosis. Therefore, future identification of an Eis2 inhibitor could strongly enhance Amikacin antibacterial activity at least in the treatment of infections by MABSC subsp*. abscessus.*

In conclusion, our data, obtained from a preclinical model of respiratory infection and from the genome of clinical bacterial isolates, demonstrated that the aminoglycoside modifying enzyme Eis2 represents a new potential antimicrobial target for fighting multidrug resistance in *Mycobacterium abscessus* infections.

Figure Legend

Figure 1. (A) Expermental Design. Mice were infected with an intratracheal injection of 1×10^5 CFU of *Mabs* wt and Δ*eis2* embedded in agar beads at Day 0. AMK was daily administered through intraperitoneal injection until day 6. At day 7 mice were sacrificed and lungs and spleens have been collected. (*B*) *Mabs* burden in the lungs of mice treated with vehicle or AMK and infected with *M.abscessus* wt or Δ*eis2*. (**C**) *Mabs* burden in the spleens of mice treated with vehicle or AMK and infected with *M.abscessus* wt or Δ*eis2* (**D**) H&E stained lung tissue slides at day 7 post challenge. This panel compares lungs of mice infected with *Mabs* Δ*eis2* and treated either with AMK or with vehicle. Arrows indicate extended inflammatory foci mainly composed by monocytes. (**E**) Table showing the n of overall MABSC genome considered to determine the number and percentage of MABSC subspecies strains carrying *eis2* non-synonymous mutation as compared to *Mabs* ATCC 19977, among clinical MABSC *abscessus*, *bolletii* and *massiliense* strains. (**F**) The Eis2 protein (PDB ID 6RFT) is shown in cartoon/surface representation. The MABSC *abscessus*, *bolletii* and *massiliense* subspecies

mutations are shown in green, red and blue spheres, respectively. The cofactor acetyl-CoA is shown in yellow/blue spheres.

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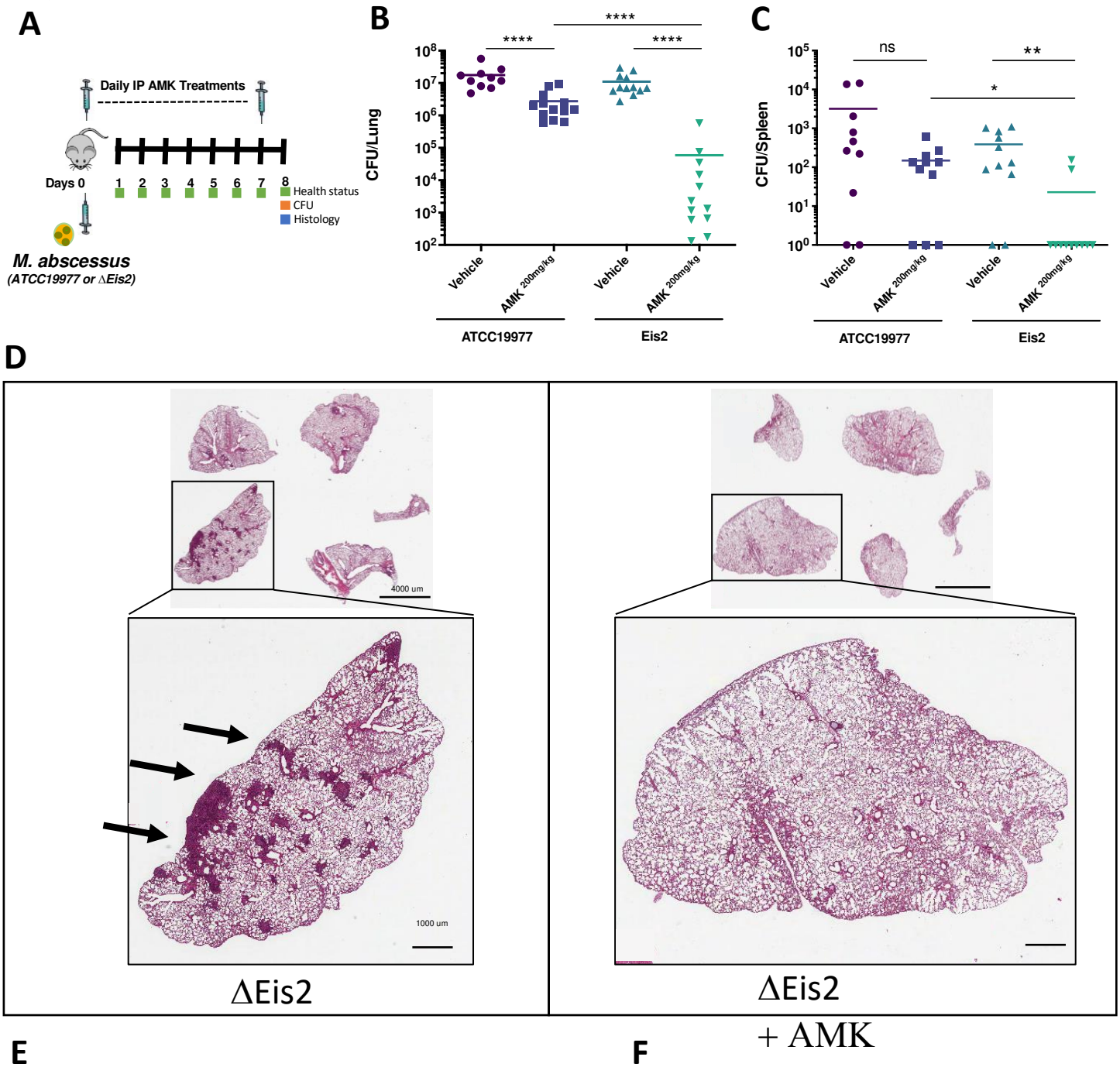
Conflict of interest: P. Sander reports grants from the Swiss National Science Foundation, Cystic Fibrosis Switzerland and Federal Office of Public Health during the conduct of the study. D. Schäfle declares no potential conflicht of interest.

Figure 1



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MABSC sup. species	n of analyzed genomes	n of genomes with non-synonomus mutations	% of genomes with non-synonomus mutations
abscessus	535	25	0,5 %
massiliense	144	112	77
bolletii	48	48	100

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