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# Evaluation of nitroxyl donors' effect on mycobacteria

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ABSTRACT

Nitroxyl (HNO) is a highly elusive and reactive molecule. Nitroxyl biological effects and pharmacological potential are becoming increasingly relevant. *Mycobacterium tuberculosis* infection needs new and more efficient drugs. Reactive Nitrogen and Oxygen Species (RNOS) are key compounds used by the immune system to fight intracellular infections, particularly *Mycobacterium tuberculosis*. In this context, we analyzed HNO potential to kill mycobacteria.

We evaluated the viability and biological response of mycobacteria towards HNO releasing compounds. Our results show that HNO donors can affect mycobacterial growth, for both *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. The effect can be observed using a single dose or with successive additions of lower concentrations of the donor, mimicking continuous HNO exposure. When analyzing the effect of the simultaneous addition of sub-inhibitory concentrations of HNO with antibiotics commonly used for *Mycobacterium tuberculosis* infection treatment we observed: a positive effect on Rifampicin, Kanamycin and Delamanid activity; and a negative effect on Isoniazid and Ethambutol activity. Regarding a possible mechanism of action, based on the recently developed fluoromycobacteriophage assay, we propose that HNO acts by interfering with general mycobacterial physiological state. The results of this study positions HNO donors as potential candidates as new drugs for a new tuberculosis treatment.

## 1. Introduction

Azanone (also called nitroxyl) or simply HNO, the one electron reduced species of Nitric Oxide (NO), is a highly elusive and reactive species, whose biological effects are becoming more and more relevant in the last two decades. HNO research main focus has been related to its protective cardiovascular effects [1], but a possible role in cancer therapy has also been explored due to its ability to inhibit glyceraldehyde-3-phosphate dehydrogenase, one of the main glycolytic enzymes [2]. HNO is a very unstable molecule: it reacts very fast in a dimerization reaction yielding innocuous $N_2O$. Previous work from our group [3] has shown that the relation between donor concentration and HNO concentration is not linear, since when the HNO production is increased (due to an increase in donor concentration) the dimerization rate increases with the square of [HNO], as described by the equation that follows (calculated at room temperature and pH 7):

$$[HNO] = \sqrt{\frac{k_{cat}\,[Donor]}{k_{dim}}}$$

This limits HNO concentration in solution to the micromolar level for only a few minutes, unless it is produced continuously. Because of that, nitroxyl study rely on the use of donors, compounds that spontaneously react releasing HNO, such as Angeli's salt (AS), hydroxamic acids [4] or NONOates [5]. Azanone main biochemical targets are oxygen, NO, and metal, particularly iron or thiol containing proteins, and many of these reactions result in the presence of several downstream Reactive Nitrogen and Oxygen Species (RNOS) [6]. Moreover, recent findings in our group showed that NO to HNO conversion, due to chemical reduction by thiols and/or aromatic alcohols (e.g. vitamin C, quinone and even tyrosine) is far more likely than previously suggested [7], and occurs *in-vivo* in neurons, endothelial cells and, most important, in lipopolysaccharide (LPS) activated macrophages [8]. Thus, azanone presence in the immune system could be far more important than commonly thought.

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**Abbreviations**

| | |
|---|---|
| MIC | Minimum inhibitory concentration |
| TB | Tuberculosis |
| AS | Angeli's salt, 3-amino-1,2,4-triazole |
| MSHA | Methanesulfohydroxamic acid |
| SNAP | S-nitroso-N-acetylpenicillamine |
| RIF | Rifampicin |
| KAN | Kanamycin |
| INH | Isoniazid |
| EMB | Ethambutol |
| DMN | Delamanid, OPC-68673 |
| ADC | Albumine dextrose complex |
| OADC | Oleic acid albumin dextrose complex |
| CFU | Colony forming unit |
| RFU | Relative fluorescence unit |

Infection with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), is one the world largest health problems [9]. TB treatment involves a long regimen of several drugs, which is increasingly hampered by the emergence of multi and extensive drug resistant strains, and negative drug-drug interactions (particularly in patients co-infected with HIV), revealing an urgent need for new (or complementary) therapeutic approaches [10]. RNOS are well documented bacterial killers, and the role of these species in prokaryotic biology is an area of intense research [11], especially in relation to the development of new therapeutic approaches [12]. In mycobacteria, for example, recent findings showed that bicyclic nitroimidazoles (PA-824, which is currently in clinical trials, and OPC-67683 which was approved in 2014 as a new antitubercular drug) exert their effect by means of intracellular NO release [13]; and vitamin C has been also shown to kill them by intracellular ROS generation [14].

In this context, we decided to investigate the HNO effect on mycobacteria. Our results show that HNO is able to impair mycobacterial growth (either with a single high concentration dose or with successive additions of lower concentrations), showing a complex interaction with known antibacterial drugs.

## 2. Results

### 2.1. HNO effect on M. smegmatis

To begin our analysis of HNO effect on mycobacteria, we performed a classical MIC (minimal inhibitory concentration) determination of HNO using AS and MSHA as donors. In a classical turbidimetric assay, the MIC for AS and MSHA was 60 mM and 2,4 mM, respectively (data not shown). These values were in the range of those obtained for SNAP (4 mM), a NO donor commonly used for comparative purposes. It should be noted, as previously reported [10], when working with R(N)OS releasing compounds, although we attempted to apply the same amount of RNOS generated stress by a rigorous control of the experimental conditions, we still obtained significant variation in bacterial growth among similar experiments.

To establish the mechanism of action for these compounds (bacteriostatic/bactericidal) on *M. smegmatis*, we calculated the number of colony forming units per ml (CFU/ml), after incubation for 24 and 48 h.

Fig. 1 represents the number of CFU/ml, in the presence of HNO as well as a NO donor. The results clearly show that HNO donors impact negatively on mycobacterial growth. When added at the MIC, a 2 log reduction in CFU/ml was observed in the presence of AS and a bacteriostatic effect was observed for the MSHA. For SNAP (a NO donor), at the MIC, a 2 log reduction in CFU/ml was obtained. Even though for the HNO donors a detrimental effect is observed after 24 h for concentrations lower than the MIC, the cells partially recover their growth capacity at 48 h reaching CFU/ml values similar to the control. When the cells where challenged with 3 times the MIC, a complete bactericidal effect was observed for AS and SNAP [15]. For MSHA, a 2 log reduction in CFU/ml was observed.

It is important to mention that since AS decomposition results in HNO and $NO_2^-$ we repeated the assay treating the cells with $NaNO_2$ as a control. Growth was similar to that observed in control experiments (data not shown), leading us to conclude that $NO_2^-$ doesn't exert an inhibitory effect on *M. smegmatis* growth and the observed AS effect is possibly due to HNO.

It is interesting to note that, due to its high dimerization rate, HNO displays a limiting concentration in the order of μM, and is rapidly consumed unless continuously produced by the donor. Both compounds (AS and MSHA) display a half-life of ca. 15 min, and therefore in the described experiments azanone must have reacted with its targets to exert the observed effect during the first 1 or 2 h after addition of the donor to the cells. But strikingly, as shown in Fig. 1A and B, the detrimental effect remains for at least 24–48 h.

### 2.2. Cysteine partially blocks HNO inhibitory effect

To provide further evidence that the observed effect is HNO specific, we performed azanone blocking experiments. Thiols are preferred HNO targets and cysteine is commonly used as an HNO scavenger [16]. Thus, we analyzed the effect of the addition of cysteine excess in combination with HNO donors on bacterial growth. While, as expected, the addition of cysteine didn't show any inhibitory effect on cell growth *per se*, the detrimental effect caused by AS was partially reduced by cysteine and bacterial cells completely recovered after 48 h (Fig. 2), supporting our interpretation that the observed inhibitory effect on cell growth was mediated by HNO.

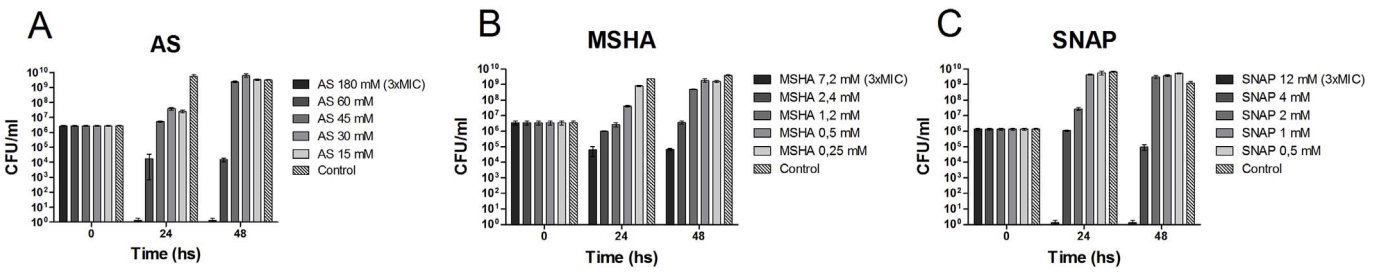


**Fig. 1. Effect of HNO and NO on survival of *M. smegmatis*.** *M. smegmatis* $mc^2155$ was inoculated in 7H9 medium supplemented with ADC and Tween 80 to an initial $OD_{600nm} \approx 0{,}02$ in the presence of increasing concentrations of AS **(A)**, MSHA **(B)** and SNAP **(C)**. Samples were collected at t = 0 h, 24 h and 48 h and the number of live cells was estimated by colony formation in 7H10 plates. Results are presented as CFU/ml. Experiments were done in triplicate. Error bars indicate standard deviation.

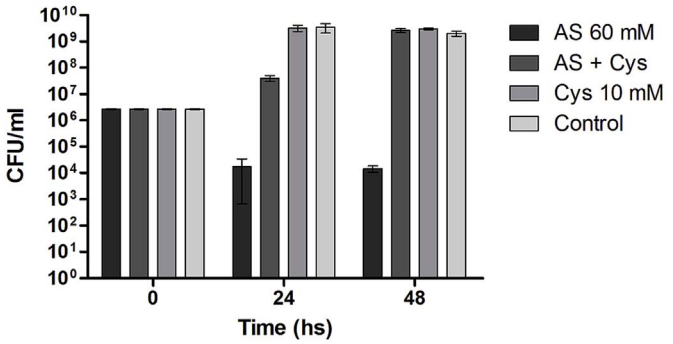


**Fig. 2. Cysteine partially blocks HNO inhibitory effect.** *M. smegmatis* mc$^2$155 was inoculated in 7H9 medium supplemented with ADC and Tween 80 to an initial $OD_{600nm} \approx 0{,}02$ and treated with AS alone at the MIC (60 mM) or in combination with cysteine (10 mM) as a blocking agent. One culture was treated only with cysteine (10 mM) and kept as control. Samples were collected at t = 0 h, 24 h and 48 h and surviving cells were estimated by colony formation in 7H10 plates. Results are presented as CFU/ml. Experiments were done in triplicate. Error bars indicate standard deviation.

### 2.3. Successive sub-inhibitory HNO additions lead to inhibition of mycobacterial growth

As previously mentioned, due to donor half-life in current conditions (15 min) and HNO reactivity, the time window for HNO reactivity is short. Therefore, in an alternative experimental design that mimics continuous exposure to lower HNO levels, we analyzed its effect on mycobacterial growth by adding 8 successive pulses of AS, at one-hour intervals, at 1/8th of the MIC to an exponentially growing culture of *M. smegmatis* ($OD_{600nm} = 0{,}3$). As shown in Fig. 3, continuous exposure to HNO in low doses was also able to arrest mycobacterial growth and the effect was similar to that observed for a single high concentration dose. Moreover, when 8 pulses of an even lower donor concentration (corresponding to 1/15th of the determined MIC) were applied, the fraction of surviving cells at 24 h was similar to that obtained with one dose of AS 60 mM (data not shown). These results strongly suggest that the effective concentration of AS can be lowered when the cells are continuously exposed to the nitroxyl.

### 2.4. HNO effect in combination with antimycobacterial agents

Having shown that HNO exerts an inhibitory effect on mycobacterial growth, we evaluated its effect when applied in combination with commonly frontline drugs used in tuberculosis treatment. We analyzed bacterial growth in the presence of HNO (at a concentration

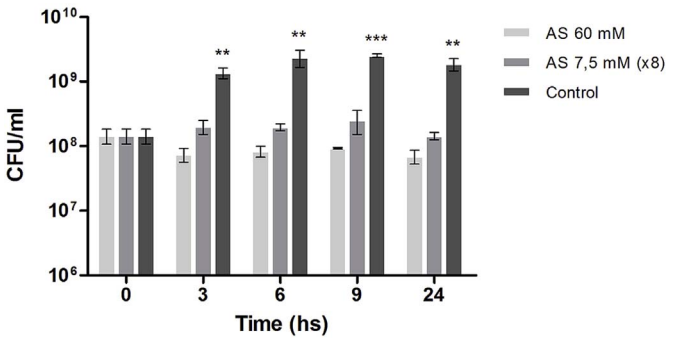


**Fig. 3. Successive HNO additions also lead to an inhibition on mycobacterial growth.** *M. smegmatis* mc$^2$155 was inoculated in 7H9 medium supplemented with ADC and Tween 80 to an initial $OD_{600nm} \approx 0{,}3$ and treated with 8 pulses of AS 7,5 mM (1/8th of the MIC), at one hour intervals. One culture was treated with one dose of AS 60 mM (MIC) and one kept as control. Samples were collected at t = 0 h, 3 h, 6 h, 9 h and 24 h and surviving cells were estimated by colony formation in 7H10 plates. Results are presented as CFU/ml. Experiments were done in triplicate. Error bars indicate standard deviation. (**) indicate P value ≤ 0,01; (***) indicate P value ≤ 0,001.

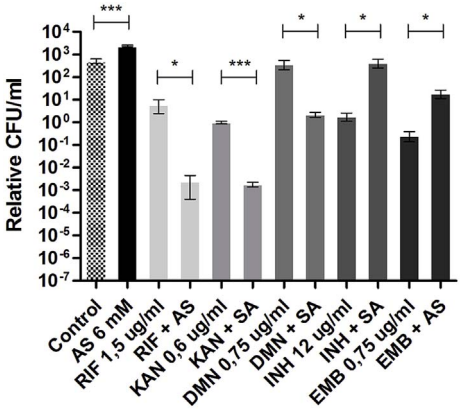


**Fig. 4. Effect of joint addition of HNO and anti-mycobacterial agents.** *M. smegmatis* mc$^2$155 was inoculated in 7H9 medium supplemented with ADC and Tween 80 to an initial $OD_{600nm} \approx 0{,}02$ and after 48 h surviving cells were estimated by colony formation in 7H10 plates. One culture was kept as control; one was treated with AS 6 mM and the others with the following antibiotics alone or in combination with AS 6 mM: Rifampicin (RIF), Kanamycin (KAN), Isoniazid (INH), Ethambutol (EMB) and Delamanid (OPC-68673, DMN). The relative CFU/ml were normalized to the control at the initial time point (before the addition of the donor and the antibiotics). Experiments were done in triplicate. Error bars indicate standard deviation. (*) indicate P value ≤ 0,05; (***) indicate P value ≤ 0,001.

10 times below its MIC) and the following antibiotics (at a concentration half of the calculated MIC in *M. smegmatis* mc$^2$155): Rifampicin, Isoniazid, Kanamycin, Ethambutol and Delamanid. In each experiment, as control, we also tested the effect of HNO and the antibiotics individually, at the concentrations used in the combined assay (Fig. 4). We observed two types of effects: a positive effect was evidenced for Rifampicin, Kanamycin and Delamanid. For these antibiotics, in the presence of low concentrations of AS a significant reduction in cell viability (CFU/ml) was observed in comparison to the antibiotic alone. Quite the contrary, as shown in Fig. 4, when Isoniazid and Ethambutol were applied in combination with AS, a reversion of the effect of the antibiotic alone was observed, reflected in an increase of the CFU number.

### 2.5. HNO effect on M. tuberculosis

Since HNO was able to inhibit *M. smegmatis* growth, we decided to evaluate its effect on *M. tuberculosis* using our recently developed fluoromycobacteriophage based WCS (whole cell screening) assay [17]. Fluoromycobacteriophages, reporter mycobacteriophages that carry fluorescent genes, can rapidly and easily reveal the metabolic state of *M. tuberculosis* and in consequence, determine its response to different compounds [18]. Briefly, we used a $mCherry_{bomb}\phi$ to infect *M. tuberculosis* mc$^2$6230 [19] treated with increasing concentrations of AS, $NaNO_2$, MSHA and SNAP. Then, we followed the expression kinetics of $mCherry_{bomb}$ using an automated multiwell plate reader fluorimeter. Only viable cells can be infected and express the reporter gene. As previously reported [18], drugs and fluoromycobacteriophages can be added simultaneously if the drug of interest has an immediate effect on bacterial metabolism (e.g. transcription or translation). On the other hand, when bacterial duplication is needed for the drug to exert its effect (e.g. cell wall synthesis), cells have to be preincubated for 24 h with the tested compound prior to the addition of fluoromycobacteriophages. At present, the physiological mechanism of HNO on bacterial viability is still unknown. However, previous studies in *M. tuberculosis* showed how the microorganism's proteome is affected after treating the cells with NO [20]. The authors observed that 29 proteins were S-nitrosilated as a result of the treatment with NO and they were involved both in intermediary metabolism and in macromolecular biosynthesis. Based on this, we decided to evaluate both protocols.

For each experiment, we first obtained RFU (relative fluorescence units) vs time plots for all the tested concentrations of HNO, NO donors and $NaNO_2$. As shown in Fig. 5A a time and concentration dependent effect on cell viability (as evidenced by RFU values) can be clearly observed, in this case for AS. To summarize all the results (shown in Supplementary material, Fig. S1) we plotted RFU values vs concentration of donors at the end point (18 h) using both experimental designs (preincubation and simultaneous addition of the compound). As shown in Fig. 5B–D, we observed an inhibitory effect in *M. tuberculosis* metabolic state for all the tested compounds (evidenced as a decrease in the fluorescence signal with increasing concentrations of the tested donors) with MIC-like values of 30–60 mM for the AS, 5 mM for the MSHA and 4 mM for the SNAP. However, $NO_2^-$ seems to have an inhibitory effect in *M. tuberculosis* as well. Only when AS and $NO_2^-$ were added simultaneously with the reporter fluoromycobacteriophage (without preincubation of the compound) a slight difference between their effect could be distinguished (Fig. 5B). Anyway, the observed effect of nitrite was not unexpected since its antibacterial effect on *M. tuberculosis* has been previously reported [21].

Concerning the possible mechanism of action, for all tested compounds, we saw that preincubation of the cells with the donors prior to exposure to fluoromycobacteriophages results in growth inhibition at 2–5 times lower donor concentrations. This observation suggests that even though HNO, and also NO, could have an effect on proteins involved on gene expression, most probably has a general effect on proteins related to mycobacterial metabolism.

## 3. Discussion

HNO is the most recent addition to the RNOS family and its biological -and notorious pharmacological-properties, particularly in humans, are under intensive study. HNO has been shown to be well tolerated by mammalian cells, is already in clinical trials for heart failure treatment and has also been proposed for cancer therapy [1,2]. Regarding microbiological studies, while NO effects have been vastly studied using *E. coli* and *B. subtilis* [11] and also *M. tuberculosis* [22,23], those of HNO on bacterial cells, on the contrary, are scarce.

In this work, we have explored the role of HNO on mycobacterial growth and we have shown that two commonly used azanone donors, AS and MSHA can have a detrimental effect on *M. smegmatis* and strongly affect *M. tuberculosis* metabolism at donor concentrations that are similar to those for NO donors, which are in the mM range. The effect can be observed either with a single dose or with successive additions of lower concentrations, which mimic continuous HNO production. It is important to note that due to its high dimerization rate, HNO peak concentrations are limited and even at mM donor concentrations real HNO concentration is below μM and is completely depleted in 1–2 h, unless continuously produced. On the other hand, for NO, higher concentrations can be achieved [3]. Thus the fact that the inhibitory effect is observed at similar donor concentrations, could suggest that HNO is a more potent antimycobacterial agent, in agreement with its higher reactivity. The differences observed between AS and MSHA (their MIC and bactericidal/bacteriostatic effect), could be explained due to their different physicochemical properties (charge, lipophilicity) which in turn would determine their permeability towards the mycobacterial cell.

To get further insights into HNO possible mechanism of action, first, we analyzed the effect on bacterial growth when adding simultaneously sub-inhibitory concentrations of AS with antibiotics commonly used for tuberculosis treatment. We observed a positive effect on the activity of Rifampicin, Kanamycin and Delamanid. Contrarily, when AS was used in combination with Isoniazid and Ethambutol, a reversion of the effect of the antibiotic was observed. The additive effect observed for Rifampicin and Kanamycin, two antibiotics that target gene expression and protein synthesis, can be rationalized assuming that HNO has not a specific protein target, but instead affects (or stresses) general

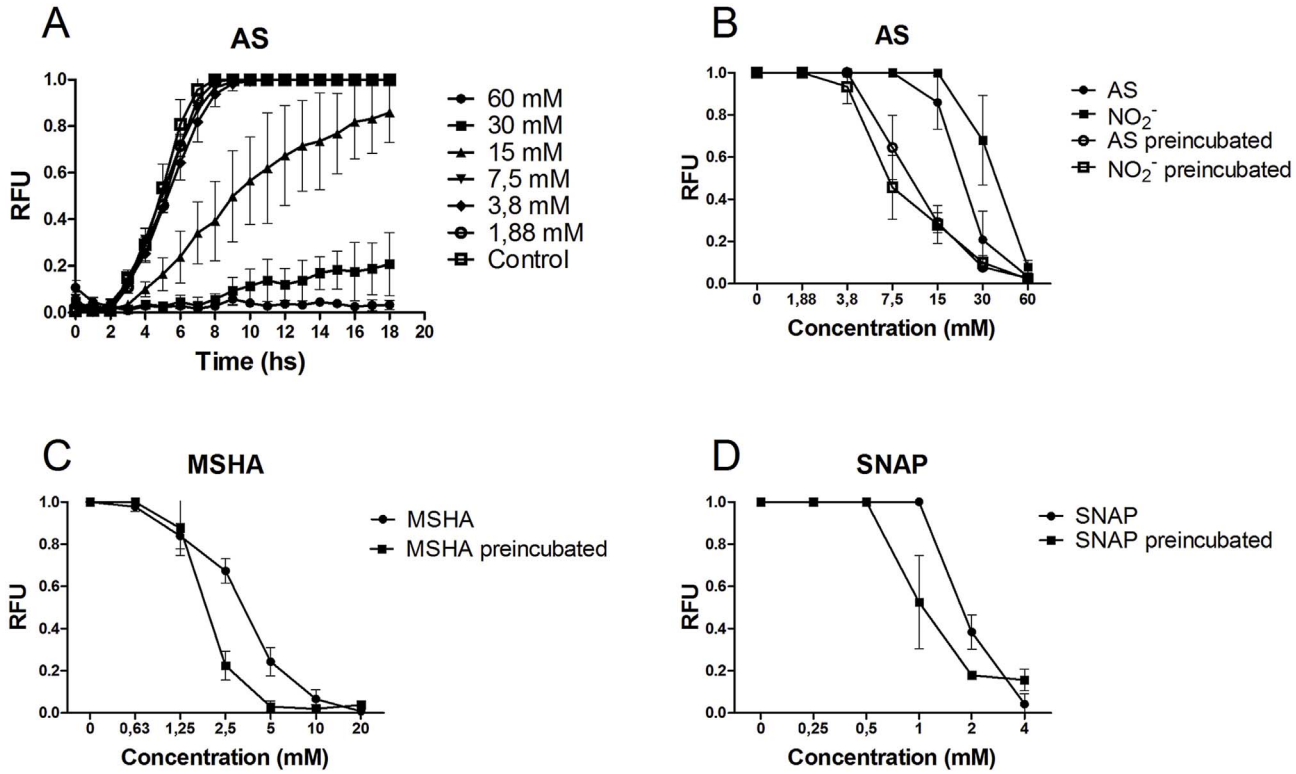


**Fig. 5. Effect of HNO and NO on *M. tuberculosis*. (A)** Monitoring of fluorescence after infection of *M. tuberculosis* (treated with AS) with fluoromycobacteriophages. *M. tuberculosis* $mc^26230$ cells were infected with $mCherry_{bomb}\phi$ in the presence of increasing concentrations of AS or $NaNO_2$ **(B)**, MSHA **(C)** and SNAP **(D)**. Donors were added simultaneously with $mCherry_{bomb}\phi$ or preincubated with cells 24 h before infection. The expression kinetics of $mCherry_{bomb}$ was followed and, for the time point 18 h, we plotted RFU (relative fluorescence units) vs donor concentration. Experiments were done in triplicate. Error bars indicate standard deviation.

mycobacterial physiological state. A point of view that is consistent with the results showing that growth inhibition can be achieved with small but continuous exposure to azanone (pulse experiment) and the observed effect of HNO donors without preincubation in the fluoromycobacteriophage assay.

For the negative effects, in the case of Isoniazid this can be explained by the known inhibitory deleterious effect of HNO on heme proteins like catalase (the product of *katG*), necessary for activation of the pro-drug to its active form [24]. On the other hand, for Ethambutol it is still unclear the reason of the antagonism observed in this work and further studies are needed.

The positive effect observed on Delamanid activity is most interesting since its proposed mechanism of action involves intracellular NO release. Given the overlapping reactivity of NO and HNO it is not unexpected that they show additive effects on mycobacterial growth. Also interesting, is the possibility of intracellular NO to HNO conversion (or reduction). Recent work from our group [8] showed that common aromatic alcohols, such as ascorbic acid as well as thiols, especially $SH_2$, are able to reduce NO to HNO inside cells. Mycobacteria are known to produce mycothiol, a low weight thiol whose function is analogous to that of glutathione in Gram-negative bacteria and eukaryotes, and whose absence renders the bacteria highly sensitivity to the immune system's nitrosative attack [25]. The mentioned thiol, found at relatively high concentrations, is a good candidate for converting NO to HNO inside mycobacterial cells. This internal conversion can explain the additive effect observed on Delamanid activity and the similar pattern of results obtained for AS, MSHA, SNAP and $NO_2^-$ in *M. tuberculosis* with the fluoromycobacteriophage based assay.

A final interesting point which could hint to azanone mechanism of action is related to the observation, that sub-inhibitory concentrations of AS result in less flocculated, smoother cultures which is also evidenced in a slightly increased of the $OD_{600nm}$ and the CFU count in comparison to the control. This observation, may suggest that some of the HNO targets could be related to cell wall synthesis, what could also explain the observed negative effect observed on Ethambutol and Isoniazid antimycobacterial activity.

Overall, the presented results open several lines of future azanone research related to mycobacteria. There are currently many known HNO donors that vary not only in their chemical properties (solubility, lipophilicity, charge, size, etc) but also in both their release kinetics and pH activity range, allowing optimization for specific therapeutic purposes [4]. Other, more basic, goals concern the use of HNO to unravel mycobacterial biological response to RNOS and azanone potential role, as an active player in the immune response in a reductive environment.

## 4. Conclusions

In summary, our results show that HNO kills mycobacteria at micromolar concentrations, possibly by interfering with general bacterial metabolism.

## 5. Materials and methods

### 5.1. Strains and reagents

The strains used in this study were *Mycobacterium smegmatis* $mc^2155$ and *Mycobacterium tuberculosis* $mc^26230$ (Δ*RD1* Δ*panCD*) [19]. Both strains were grown in Middlebrook 7H9 medium, supplemented with 0,05% Tween 80 and 10% ADC or OADC at 37 °C. *M. tuberculosis* $mc^26230$ was grown in the presence of Pantothenic Acid (50 μg/ml) due to its Δ*panCD* genotype. Rifampicin (3 μg/ml and 1,5 μg/ml), Kanamycin (1,25 μg/ml and 0,6 μg/ml), Isoniazid (24 μg/ml and 12 μg/ml), Ethambutol (1,5 μg/ml and 0,75 μg/ml) and Delamanid (1,5 μg/ml and 0,75 μg/ml) were used for the interaction assay. Rifampicin, Kanamycin, Isoniazid and Ethambutol were from Sigma-Aldrich. Delamanid was from MedChem Express. AS, $NaNO_2$, MSHA and SNAP were synthesized in our laboratory or purchased from Cayman Chemicals.

### 5.2. Determination of mycobacterial growth and MIC values

*M. smegmatis* was grown in 7H9 supplemented with ADC and Tween 80 to an $OD_{600nm}$ of ≈1. The bacterial suspension was diluted to an $OD_{600nm}$ of 0,02 and then treated with the indicated concentrations of AS, MSHA and SNAP.

Donors were dissolved right before their addition to the cultures. AS was dissolved in phosphate buffer pH 10 to a stock concentration of 400 mM. MSHA was dissolved in phosphate buffer pH 6 to a stock concentration of 80 mM. SNAP was dissolved in DMSO to a stock concentration of 100 mM. Cultures were incubated at 37 °C. Samples were collected at t = 0 h (before the addition of the drugs), t = 24 h and t = 48 h and plated on drug-free 7H10 plates for CFU calculation (CFU/ml). Experiments were performed in triplicate and average values were used for calculations.

### 5.3. HNO pulse assay

*M. smegmatis* was grown in 7H9 supplemented with ADC and Tween 80 until reaching mid-exponential growth phase ($OD_{600nm}$ ≈ 0,3). Then, 8 successive AS pulses were added spaced at one-hour intervals, at the indicated concentrations. Samples were collected at t = 0 h (before the addition of AS) and at the indicated time points and plated on drug-free 7H10 plates for CFU calculation (CFU/ml). Experiments were performed in triplicate and average values were used for calculations.

### 5.4. Fluoromycobacteriophage based MIC assay

For WCS assay with fluoromycobacteriophages we followed the protocol previously described [17]. Briefly, *M. tuberculosis* $mc^26230$ was grown in 7H9 supplemented with OADC and Pantothenic acid (50 μg/ml) to an $OD_{600nm}$ ≈ 1.100 μl of cells were added in triplicate to a black, flat, clear bottom 96-well microplates (Greiner BioOne) together with 100 μl of serial half dilutions of the tested donors (AS, $NaNO_2$, MSHA and SNAP) at the indicated concentrations. When a preincubation step was performed, the indicated dilutions of the donors were incubated at 37 °C with the cells for 24 h before infection with fluoromycobacteriophages. $mCherry_{bomb}\phi$ was used to infect *M. tuberculosis* cells at a MOI (multiplicity of infection) of 100 and infection was evaluated by monitoring the expression kinetics of $mCherry_{bomb}$ by excitation at 584 nm and emission at 620 nm with an OPTIMA Fluostar multiwell plate reader at 37 °C. During reading of fluorescence, microplates were covered using a black light-absorbing sealing film (AbsorbMax, Excel Scientific Inc.).

Results were represented as relative fluorescence units (RFU) versus time. RFUs were calculated as follows:

$$\text{RFU} = \frac{\text{mean fluorescence units} - \text{background}}{\text{maximal fluorescence reading units} - \text{background}}$$

When RFU values were plotted against donors' concentration, the end point time corresponding to 18 h was used.

### 5.5. Statistical analysis and replicas

Statistical analysis were performed with the GraphPad Prism program. In all cases, values from at least three independent experiments were analyzed. Differences were tagged as statistical significant using *t*-test with a $p < 0.05$.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2018.01.006.

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