Redox homeostasis as a target for new antimycobacterial agents

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Abbreviations:
GSH: reduced glutathione
NAC: N-acetyl-cysteine
M. avium: *Mycobacterium avium*
ERG: ergothioneine
MSH: mycothiol
Mtb: *Mycobacterium tuberculosis*
IL: interleukin
IFN: interferon
ROI: reactive oxygen intermediates
RNI: reactive nitrogen intermediates
PBMC: peripheral blood mononuclear cells
g.u.: genomic units
MOI: multiplicity of infection

Abstract

Despite early treatment with anti-mycobacteria combination therapy, drug resistance continues to emerge. Maintenance of redox homeostasis is essential for *Mycobacterium Avium* (*M. avium*) survival and growth. The aim of the present study was to investigate the antimycobacterial activity of two pro-glutathione (GSH) drugs, which are able to induce redox stress in *M. avium* and to modulate cytokine production from macrophages. Hence, we investigated two molecules shown to possess antiviral and immunomodulatory properties: C4-GSH, an N-butanoyl GSH derivative, and I-152, a pro-drug of N-acetyl-cysteine (NAC) and beta-mercaptoethylamine (MEA). Both molecules showed activity against replicating *M. avium*, both in the cell-free model and inside macrophages. Moreover, they were even more effective in reducing the viability of the bacteria that had been kept in water for seven days, proving to be active against both replicating and non-replicating bacteria. By regulating the macrophage redox state, I-152 modulated cytokine production. In particular, higher levels of IFN-γ, IL-1b, IL-18, and IL-12, which are known to be crucial for the control of intracellular pathogens, were found after I-152 treatment. Our results show that C4-GSH and I-152, by inducing perturbation of redox equilibrium, exert bacteriostatic and bactericidal activity against *M. avium*. Moreover, I-152 can boost the host reponse by inducing the production of cytokines which serve as key regulators of the Th1 response.

Keywords: *M. avium*, redox homeostasis, glutathione, pro-glutathione molecules, anti-mycobacterial compounds

1. Introduction

Mycobacterial infections are major causes of worldwide morbidity and mortality. *Mycobacterium avium* (*M. avium*), a member of the Mycobacterium avium complex (MAC), is an opportunistic pathogen, normally present in the environment, that has now emerged the main bacterial infection in patients with AIDS [1], [2]. A common characteristic shared by many mycobacterial species is that upon host invasion, these bacteria arrange an army of factors, which circumvent macrophage defences to elude macrophage killing and to replicate within these phagocytes [3]. Moreover, *M. avium* can manipulate its host's complex immune signalling pathways by influencing the cytokine environment and inhibiting essential immune functions [4], [5]. Treatment with conventional antimycobacterial drugs does not ensure the elimination of intracellular bacteria [6]. Most current bactericidal antimicrobials inhibit DNA synthesis, RNA synthesis, cell wall synthesis, or protein synthesis [7]. The identification of new antibacterial compounds with novel mechanisms of action has become an urgent need in light of the growing threat of drug-resistant infections [8]. The bactericidal potential of a drug can be influenced significantly by its mode of action within cellular metabolic and signalling networks. One susceptible pathway in the bacterial cell is the thiol-based redox metabolism, which plays a key role in many cellular processes, including protection of the bacterial cell against endogenous and exogenous reactive oxygen species, proper protein folding, and DNA synthesis [9]. While glutathione (GSH) is rarely detected in pathogenic prokaryotes, ergothioneine (ERG) and mycothiol (MSH) are the most important low molecular weight thiols in mycobacteria [9], [10]. They preserve cellular homeostasis by maintaining a reducing environment, and function as detoxification agents against antibiotics, alkylating agents, electrophiles, and other reactive intermediates [10]. Several studies have shown that MSH is also important to the virulence and survival of Mycobacterium tuberculosis (Mtb), as the loss of MSH biosynthesis and MSH-dependent detoxification inhibit the growth of mycobacteria [11]. Moreover, MSH is an important component that combats acidic stress during Mycobacterium infection [11], [12]. Due to their beneficial role, ERG and MSH can be considered valid targets for the development of antibacterial drugs. Indeed, several studies have demonstrated that affecting the redox balance in Gram-positive bacteria results in bacterial cell death, and depletion of thiol pools is a promising target to promote Mtb killing and potentiation of antimicrobials [13].

On the other hand, it is known that redox modulation of macrophage pro-inflammatory innate immune signalling pathways may play a critical role in the immune response [14]. Previously, we have shown that it is possible to shift the intra-macrophage environment towards a more reduced state by using pro-glutathione (GSH) molecules, namely the N-butanoyl derivative of GSH (C4-GSH) or I-152, a precursor of N-acetyl-cysteine (NAC) and cysteamine (MEA). We established that these molecules increase the intracellular GSH content [15], while other studies have shown that Mycobacterium growth is sensitive to GSH and NAC [16], [17]. Hence, we hypothesized that C4-GSH and I-152 could unbalance oxidation-reduction reactions orchestrated by *M. avium* to maintain metabolic homeostasis. Moreover, they could modulate the production of anti/pro-inflammatory cytokines in the macrophage host to make the immune response more effective. In fact, C4-GSH and I-152 have been shown to influence macrophage cytokine production in both *in vitro* and *in vivo* models, favouring Th1 responses, although by different mechanisms and by influencing the signalling pathways involved in cytokine production in different ways [15], [18], [19], [20]. In this paper, we report preliminary results regarding the bacteriostatic and bactericidal activity of C4-GSH and I-152 against *M. avium.* Moreover, we show that these molecules can regulate the production of macrophage cytokines involved in the immune and inflammatory processes.

2. Materials and methods

2.1. The preparation of pro-GSH molecules

I-152 was synthesized as previously described [21] and C4-GSH was kindly provided by GLUOS S.r.l. Both molecules were dissolved in culture medium at the highest concentration and then serially diluted.

2.2. Bacterial strain, media and culture preparation

M. *avium* strain 662*,* obtained from the blood of AIDS patients (L. Sacco Hospital, Milan, Italy) who had never been treated for mycobacterial infection, was used. The strain was identified by polymerase chain reaction (PCR) as described by Schiavano et al [22].

The bacteria were grown in Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI, USA) with oleic acid albumin dextrose catalase (OADC) and in Middlebrook 7H9 broth with ADC enrichment at 35°C for eight to ten days. Before the experiments, all the bacterial suspensions were sonicated for 6 s, washed and quantified by spectrophotometry to an optical density of 0.12 at 650 nm. Ten µl of each dilution suspension was plated in triplicate on Middlebrook 7H10 agar and incubated at 35°C for eight to ten days for the enumeration of colony-forming units (CFUs)/ml.

2.3. Evaluation of I-152 and C4-GSH efficacy on *M. avium* growth and viability

The effect of I-152 or C4-GSH on *M. avium* growth and viability was determined in mycobacterial suspensions in supplemented Middlebrook 7H9 broth or in sterile distilled water respectively. Following a seven-day incubation at 35°C, each sample was two-fold serially diluted and 10 µl of the diluted sample was plated in triplicate on Middlebrook 7H10 agar for the enumeration of CFUs/ml. A control without compounds was included in each series.

2.4. Separation of monocytes from human blood and infection with *M. avium*

Monocyte-derived macrophages were prepared from leukocyte buffy coats obtained from healthy volunteers and purified as previously described [6], [22].

The macrophage cultures were exposed for four hours to bacteria at a multiplicity of infection (MOI) of 60:1 (bacilli:macrophage). After removal of extracellular bacilli, one sample of infected macrophages was lysed to assess the intracellular CFUs (infection at time 0). The final macrophage lysate suspension was serially diluted and plated onto 7H10 agar for the enumeration of CFUs. For the assay of antimycobacterial activity, the compounds were added at the concentration of 10 mM to the macrophage culture in RPMI supplemented with 10% FCS without penicillin/streptomycin. After four days, the supernatants were removed, replaced with fresh medium containing the molecules and processed for the CFU counts. On the seventh day of the culture, supernatants and adherent macrophages were processed to evaluate the viable extracellular and intracellular mycobacteria. Total viable counts were expressed by adding the supernatant and intracellular CFU counts on day seven to the supernatant CFU counts on day four. Control samples were run in parallel and treated in the same manner as the experimental macrophage cultures; no detachment of cells from the wells of the culture plate was observed. Total macrophage protein content was quantified on the supernatant and adherent cells.

2.5. Determination of GSH and cysteine in *M. avium*-infected macrophages

Macrophages, exposed to *M. avium* as described in the previous section (2.4), were washed and processed for thiol determination as previously described [15]. GSH and cysteine levels were determined by high performance liquid chromatography (HPLC) [15]. The procedure was performed at different times from *M. avium* exposure and then repeated seven days after *M. avium* removal.

2.6. *M. avium* quantification by real-time PCR

M. avium-infected macrophages, treated with 10 mM of I-152 and lysed as described for bacterial plate counts, were used for total DNA extraction as described by Pathak et al. [23]. Five µl were amplified by qPCR with the *M. avium* trasposon element (IS1245) Advanced kit (genesig, Primerdesign, Camberley, UK) according to the manufacturer's instruction. The amplification reaction included an *M. avium* specific primer/probe mix targeting the insertion sequence IS1245 and an endogenous control primer/probe mix (human β-actin) used to normalize the number of *M. avium* genomic units (g.u.) against the macrophage number. The declared detection sensitivity was less than 100 copies of target template.

2.7. Magnetic multiplex immunoassay of cytokines

Cytokine concentrations in supernatants from macrophages (collected after four and seven days of infection) were determined through a multiplex suspension immunomagnetic assay, as previously detailed [19]. Levels of analytes were determined using a Bio-Plex 200 array reader (Bio-Rad Labs, Hercules, CA, USA). Data were collected and analyzed using a Bio-Plex 200 instrument equipped with BioManager analysis software (Bio-Plex Manager Software v. 6.1). Levels of cytokines (IFN-γ, IL-1b, IL-8, IL-18, IL-12p40, IL-10, IL-6 and TNF-α) were determined in the supernatants of three separate experiments plated in duplicate. Final values represent the sum of the fourth and seventh day values. Comparable levels were obtained in two experiments, while in the third experiment, although the trend was similar to the other two, the values were lower, and IL-10 and IL-12 were not detectable. Hence, the results obtained from this experiment were not considered in the final results.

2.8. Statistical analysis

Statistical analysis was performed with GraphPad Instat. Data were analyzed with the two-tail unpaired Student's t test. A P value <0.05 was considered significant.

3. Results

3.1. Pro-GSH molecules possess bacteriostatic and bactericidal activity vs *M. avium*

3.1.1 Effect of I-152 and C4-GSH on *M. avium* growth

N-acetyl-cysteine is the molecule of choice to increase intracellular GSH levels. It has shown potent anti-mycobacterial activity and significant improvement in mycobactericidal responses when combined with antibiotic treatment [17], [24]. Moreover, several pro-GSH molecules with improved bioavailability and targeting capacity have been synthesized [25].

The antimycobacterial activity of I-152 and C4-GSH was evaluated by incubating *M. avium* in Middlebrook 7H9 broth in the presence of different concentrations of I-152 (5, 10 and 20 mM) or C4-GSH (5 and 10 mM). The 20 mM concentration of C4-GSH was not tested because C4-GSH concentrations higher than 10 mM did not dissolve in the medium. Mycobacterium replication was compared to that of mycobacteria grown in the absence of the molecules. The results showed that both I-152 (Figure 1a) and C4-GSH (Figure 1b) significantly inhibited the growth of the bacterium at all the tested doses. I-152 yielded similar results when used in 10 and 20 mM concentrations; therefore, subsequent experiments were performed with 10 mM or 5 mM I-152. On the other hand, C4-GSH interfered with *M. avium* replication in a concentration dependent manner. It was found that 10 mM of GSH exerted a significantly lower activity than C4-GSH administered at the same concentration (Figure 1b, white column). These experiments showed that I-152 and C4-GSH inhibited *M. avium* proliferation, indicating bacteriostatic activity.

3.1.2 Effect of I-152 and C4-GSH on *M. avium* viability

Most antibiotics that are used against Mycobacterium target biosynthetic processes essential for cell growth. Inhibition of nucleic acid, protein, or cell wall synthesis leads to cessation of growth [6], [7]. Indeed, we proposed a model in which an imbalance in the redox homeostasis may be at least partially responsible for cell death. Hence, we investigated whether I-152 and C4-GSH have bactericidal activity. To this end, bacterial suspensions were maintained in sterile distilled water at 35°C for seven days in the presence or absence of I-152 or C4-GSH, as described in section 2.3. Dose-dependent effects were observed. Indeed, the highest concentration of I-152 (10 mM) reduced the number of viable bacteria by about 4.5 logs, while a 5 mM concentration of I-152 resulted in a 2 log reduction (Figure 2a). Similar results were obtained with C4-GSH treatment (Figure 2b). Thus, exposure to I-152 or C4-GSH markedly reduced the number of bacterial cells, indicating bactericidal activity.

3.2. Pro-GSH molecules inhibit intra-macrophage replication of *M. avium* and modulate cytokine production

3.2.1. The effect of I-152 and C4-GSH on *M. avium* growth in human macrophages

To assess the ability of I-152 and C4-GSH to affect the intracellular growth of *M. avium* and the macrophage cytokine response, macrophages were exposed to *M. avium* for four hours and then treated with I-152 or C4-GSH, as described in section 2.4. GSH has been shown to have direct antimycobacterial effects [16], [17]. Moreover, the addition of NAC to antibiotics improves their mycobactericidal responses against Mtb infection [24]. Upon infection, macrophages undergo morphological and functional changes, including variations in redox equilibrium [26], [27], [28]. Thus, we first measured GSH levels after infection with *M. avium*. We found that, although differences were observed in basal GSH levels in macrophages exposed to *M. avium*, the infection caused an intracellular GSH increase that was significant compared to the uninfected cells after four hours of infection, and it was still appreciable after seven days (Figure 3a). On the contrary, we found no differences in the GSH content at early times of infection (within 1 hour) or in cysteine levels at

all the tested times (not shown). To evaluate the antimycobacterial activity of the pro-GSH molecules, I-152 and C4-GSH were added to *M. avium*-infected macrophages at a concentration of 10 mM. In this case, we observed a marked reduction in bacterial loads, demonstrating the ability of the drugs to limit mycobacterial growth within macrophages (Figure 3b).

To quantify *M. avium* inside macrophages real-time PCR was performed following the procedure described in section 2.6. In this preliminary study, we chose to investigate this parameter only in the cells having received the most effective treatment in terms of growth inhibition, i.e. 10 mM of I-152. The $\Delta\Delta C_T$ method was used to estimate fold differences in intracellular *M. avium* g.u. between treated and untreated macrophages by relative quantification. The results, reported in Table 1, show that *M. avium* g.u. were more than three times higher in I-152-treated macrophages than in untreated cells.

3.2.2. Modulation of cytokine production in human macrophages by I-152 and C4-GSH

Macrophage cytokines play a major role in determining the outcome of mycobacterial infections [4], [5], [24], [28], [29], [30]. In the present study, we assessed a panel of eight cytokines secreted from macrophages upon *M. avium* infection examining how they were modulated by treatment with pro-GSH molecules. Infection of macrophage cells with *M. avium* strain 662 resulted in a high production of IL-6, IL-8 and TNF-α, a medium level production of IL-12 and IL-10 and a low production of IFN-γ, IL-1b and IL-18 (Figure 4). When the macrophages were treated with I-152, although the bacterial growth rate was significantly inhibited, IFN-γ, IL-1b, IL-18, IL-8, IL-10 and IL-12 levels showed a three- to six-fold increase compared to infected/untreated cells (I). By contrast, IL-6 concentration was not affected by the treatment and TNF-α concentration was decreased (Figure 4, light grey columns). Cytokine production was less influenced by C4-GSH, as reported in Figure 4 (dark grey columns).

4. Discussion

C4-GSH and I-152 have been used as cellular redox modifiers for controlling *M. avium* infection on the one hand and to modulate macrophage cytokine production on the other hand.

It was not our aim to compare the antibacterial activity of the two molecules, but it is interesting to note that, although both C4-GSH and I-152 are GSH-boosting drugs, their activity against *M. avium* was found to be different. Indeed, I-152 was shown to be more effective than C4-GSH in all the experimental conditions under study. We think that, as has already been reported for other pro-GSH molecules [16], [17], [31], the antimycobacterial activity of I-152 and C4-GSH can be ascribed to GSH. Extracellular GSH is converted to dipeptide (Cys-Gly) due to the action of transpeptidase, and the dipeptide is then transported into the bacterial cells [32]. However, while this mechanism can explain the inhibitory effects of the molecules within macrophages, it does not explain the antimycobacterial activity found in cell-free systems. In fact, it is known that mycobacteria do not synthesize GSH. Hence, we can hypothesize that the accumulation of reducing equivalents is sensed through mycobacteria redox sensors causing DNA and protein synthesis inhibition as well as modifications in metabolic pathways [33], [34]. The proposed mechanism also suggests that eukaryotic thiol pools may be targets for C4-GSH and I-152. Indeed, this assumption has been demonstrated in macrophage cells treated with high I-152 concentrations, in which temporary GSH depletion was shown, but not in cells treated with C4-GSH [15], [20]. I-152 and C4-GSH are pro-GSH molecules that act through different mechanisms. C4-GSH, as it is, can enter the cell or act through a mechanism similar to the one described above for GSH, i.e., it can be a substrate for the enzyme gamma-glutamyl transpeptidase. The cysteine derived from C4-GSH metabolism can be used to synthesize GSH in a eukaryotic cell or may interfere with MSH within the Mycobacterium. On the other hand, I-152 induces a transient GSH depletion since it may be considered xenobiotic and conjugated with GSH. However, no significant macrophage cytotoxicity has been observed for I-152, probably because the thiol species provided by I-152, in the form of NAC and MEA, are promptly used to restore the GSH pool [19], [20]. We can hypothesize that I-152 may have a similar effect inside the mycobacterium, where the MSH concentration and/or activity may be affected by I-152 itself and by the aberrant increase in reducing equivalents provided by the molecule. On the other hand, the initial GSH depletion within the macrophage could contribute to creating an adverse environment for mycobacterium replication, while subsequently, high levels of GSH could modulate the cytokine production. It is known that reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) are generated by phagocytic cells to inhibit growth of intracellular pathogens [28]. When ROI and RNI are generated, there is a simultaneous synthesis of GSH, which protects against the toxic effects of the reactive species. In fact, we found a significant GSH increase upon *M. avium* infection, regardless of different basal macrophage GSH levels, which depended on the blood sample from which macrophages derived. Despite these differences in basal GSH levels, we preferred to use primary cells since they provide a more representative result than immortalized macrophage cell-lines. Monocyte-derived macrophages matured in absence of any stimuli give rise to a mixed culture of both the M1 and M2 phenotype, and such cultures have been reported to be suitable for infection studies with *M. avium.* In fact, it is not known whether *M. avium* infects a particular phenotype [26], [35].

In this study, we demonstrate that the antimicrobial efficacy of C4-GSH and I-152 is not limited to growing bacteria, but extends to quiescent bacterial cells, suggesting a functional linkage between redox changes and survival. High concentrations of C4-GSH and I-152 may create a reductive stress which the bacterium cannot control, leading to cell death [32], [33]. In short, our experiments show that pro-GSH molecules could exert bactericidal activity by weakening redox homeostasis in *M. avium*. Of course, further studies are necessary to delineate the precise mechanisms of action and in particular, to investigate the significance of the preliminary result obtained by the molecular analysis of infected macrophages treated with I-152. In fact,

this assay revealed higher amounts of intra-macrophage *M. avium* DNA, possibly deriving from both live and dead cells. On the other hand, the amounts of total viable *M. avium* cells were found to be significantly lower. We can thus hypothesize that most intracellular bacteria were dead. Indeed, as previously reported [23], while the plating method is a measure of live bacteria, the qPCR measures the total number of bacterial genomes, regardless of whether they derive from dead or live cells. Future studies will aim to establish the subcellular localization of mycobacteria in infected macrophages treated with I-152 and the fate of those cells.

By altering the glutathione-redox balance in macrophages, it is possible to modulate cytokine production [31], [34], [35], [36]. Hence, we investigated inflammatory and anti-inflammatory cytokine production. Both GSH and NAC have been shown to diminish tuberculosis pathology and inflammation by immune-modulation [24], [34]. In our experimental conditions, infected macrophages produced high concentrations of IL-6, IL-8 and TNF-α, medium levels of IL-12 and IL-10 and low levels of IFN-γ, IL-1b and IL-18. It is known that high levels of intra-macrophage GSH favor the Th1 response, mainly by modulating IL-12 production [36]. All the cytokines that were examined were found to be present at higher levels in I-152-treated macrophages, with the exception of IL-6 and TNF-α, a finding which is partially in agreement with the reported data on NAC therapy for tuberculosis infection [30]. Increased levels of IL-12, whose biological functions are pivotal in both innate and adaptive immunity [37], confirm previous findings regarding the capacity of I-152 to induce the production of macrophage cytokines able to support the Th1 response [18], [19]. Regarding the IL-18 effect, although there is conflicting evidence in the literature, most studies support its role in synergizing with IL-12 to induce IFN-γ production in dendritic cells, macrophages, and B cells [38]. It is generally accepted that IL-1 has a role in increasing survival and decreasing pulmonary bacterial loads in Mtb infection, probably through different mechanisms described for IL-12 or IFN-γ [36]. Hence, we can generally conclude that I-152, by increasing the levels of the cited cytokines, could be useful in controlling mycobacteria infection. It is more difficult to interpret the effect exerted on TNF-α, IL-10 and IL-6 because these cytokines are induced by mycobacteria but have different effects according to the nature of the challenge and the experimental conditions [38]. It is therefore difficult to foresee the final effect of I-152 because it will likely differ according to the experimental conditions and should be analyzed in concert with the other cytokines. In the present study, C4-GSH treatment did not markedly influence cytokine production although we have previously found that production of all the main inflammatory cytokines is blocked by C4-GSH through inhibition of NFkB mediated signaling [39]. We hypothesize that these differences may be due to the different experimental models, in particular, in relation to the intracellular redox state and the stimulus triggering the cytokine response. Moreover, we have previously demonstrated that I-152 and C4-GSH can regulate cytokine production by modulating different signaling pathways. Hence, there is no doubt that the tested pro-GSH molecules can modulate cytokine production, but the exact effect on the single cytokine cannot be foreseen.

In conclusion, C4-GSH and I-152 limit *M. avium* infection through direct antimicrobial activity and regulation of the cytokine response. Our most interesting finding was the inhibitory activity of the molecules on the survival of *M. avium*. In fact, most bacteria can survive inside macrophages by eluding the killing mechanisms of the host, and conventional antimicrobial agents are effective against actively growing bacteria.

Conclusions: these data suggest that the redox state of mycobacteria could be a novel target for killing dormant bacteria and pro-GSH drugs may target the bacteria in a persistent/latent infection. Moreover, thanks to its immune modulation, the addition of I-152 to the existing anti-Mycobacterium drugs could be a valid strategy for clearance of *M. avium*.

DECLARATIONS

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References

[1] Nightingale SD, Byrd LT, Southern PM, Jockusch JD, Cal SX, Wynne BA. Incidence of *Mycobacterium avium-intracellulare* complex bacteremia in human immunodeficiency virus-positive patients. J Infect Dis 1992;165:1082-85. https://doi.org/10.1093/infdis/165.6.1082.

[2] Karakousis PC, Moore RD, Chaisson RE. Mycobacterium avium complex in patients with HIV infection in the era of highly active antiretroviral therapy. Lancet Infect Dis 2004;4:557-65. https://doi.org/10.1016/S1473-3099(04)01130-2.

[3] Queval CJ, Brosch R, Simeone R. The Macrophage: A Disputed Fortress in the Battle against Mycobacterium tuberculosis. Front Microbiol 2017;8:2284. https://doi.org/10.3389/fmicb.2017.02284.

[4] Vankayalapati R, Wizel B, Samten B, Griffith DE, Shams H, Galland MR, et al. Cytokine Profiles in Immunocompetent Persons Infected with Mycobacterium avium Complex. J Infect Dis 2001;183:478-84. https://doi.org/10.1086/318087.

[5] Wagner D, Sangari FJ, Kim S, Petrofsky M, Bermudez LE. Mycobacterium avium infection of macrophages results in progressive suppression of interleukin-12 production in vitro and in vivo. J Leukoc Biol 2002;71:80-8. https://doi.org/10.1189/jlb.71.1.80.

[6] Schiavano GF, Celeste AG, Salvaggio L, Sisti M, Brandi G. Efficacy of macrolides used in combination with ethambutol, with or without other drugs, against Mycobacterium avium within human macrophages. Int J Antimicrob Agents. 2001;18:525-30. https://doi.org/10.1016/S0924-8579(01)00461-7.

[7] Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol 2010;8:423-35. https://doi.org/10.1038/nrmicro2333.

[8] Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 2009;48:1-12. https://doi.org/10.1086/595011.

[9] Ritz D, Beckwith J. Roles of thiol-redox pathways in bacteria. Annu Rev Microbiol 2001;55:21-48. https://doi.org/10.1146/annurev.micro.55.1.21.

[10] Sao Emani C, Gallant JL, Wiid IJ, Baker B. The role of low molecular weight thiols in Mycobacterium tuberculosis. Tuberculosis (Edinb) 2019;116:44-55. https://doi.org/10.1186/s12929-018-0458-9.

[11] Pacl HT, Reddy VP, Saini V, Chinta KC, Steyn AJC. Host-pathogen redox dynamics modulate Mycobacterium tuberculosis pathogenesis. Pathog Dis 2018;76:fty036. https://doi.org/10.1093/femspd/fty036.

[12] Harbut MB, Vilchèze C, Luo X, Hensler ME, Guo H, Yang B, et al. Auranofin exerts broad-spectrum bactericidal activities by targeting thiol-redox homeostasis. Proc Natl Acad Sci USA 2015;112:4453-8. https://doi.org/10.1073/pnas.1504022112.

[13] Coulson GB, Johnson BK, Zheng H, Colvin CJ, Fillinger RJ, Haiderer ER, et al. Targeting Mycobacterium tuberculosis sensitivity to thiol stress at acidic pH kills the bacterium and potentiates antibiotics. Cell Chem Biol 2017;24:993-1004.e4. https://doi.org/10.1016/j.chembiol.2017.06.018.

[14] Gostner JM, Becker K, Fuchs D, Sucher R. Redox regulation of the immune response. Redox Rep 2013;18:88-94. https://doi.org/10.1179/1351000213Y.0000000044.

[15] Fraternale A, Crinelli R, Casabianca A, Paoletti MF, Orlandi C, Carloni E, et al. Molecules altering the intracellular thiol content modulate NF-kB and STAT-1/IRF-1 signalling pathways and IL-12 p40 and IL-27 p28 production in murine macrophages. PLoS One 2013;8:e57866. https://doi.org/10.1371/journal.pone.0057866.

[16] Venketaraman V, Rodgers T, Linares R, Reilly N, Swaminathan S, Hom D, et al. Glutathione and growth inhibition of Mycobacterium tuberculosis in healthy and HIV infected subjects. AIDS Res Ther 2006;3:5. https://doi.org/10.1186/1742-6405-3-5.

[17] Amaral EP, Conceição EL, Costa DL, Rocha MS, Marinho JM, Cordeiro-Santos M, et al. N-acetyl-cysteine exhibits potent anti-mycobacterial activity in addition to its known anti-oxidative functions. BMC Microbiol 2016;16:251. https://doi.org/10.1186/s12866-016-0872-7.

[18] Fraternale A, Paoletti MF, Dominici S, Caputo A, Castaldello A, Millo E, et al. The increase in intra-macrophage thiols induced by new pro-GSH molecules directs the Th1 skewing in ovalbumin immunized mice. Vaccine 2010;28:7676-82. https://doi.org/10.1016/j.vaccine.2010.09.033.

[19] Brundu S, Palma L, Picceri GG, Ligi D, Orlandi C, Galluzzi L, et al. Glutathione Depletion Is Linked with Th2 Polarization in Mice with a Retrovirus-Induced Immunodeficiency Syndrome, Murine AIDS: Role of Proglutathione Molecules as Immunotherapeutics. J Virol 2016;90:7118-30. https://doi.org/10.1128/JVI.00603-16.

[20] Crinelli R, Zara C, Smietana M, Retini M, Magnani M, Fraternale A. Boosting GSH Using the Co-Drug Approach: I-152, a Conjugate of N-acetyl-cysteine and β-mercaptoethylamine. Nutrients 2019;11.pii:E1291. https://doi.org/10.3390/nu11061291.

[21] Oiry J, Mialocq P, Puy JY, Fretier P, Dereuddre-Bosquet N, Dormont D, et al. Synthesis and biological evaluation in human monocyte-derived macrophages of N-(N-acetyl-L-cysteinyl)-S-acetylcysteamine analogues with potent antioxidant and anti-HIV activities. J Med Chem 2004;47:1789-95. https://doi.org/10.1021/jm030374d.

[22] Schiavano GF, De Santi M, Sisti M, Amagliani G, Brandi G. Disinfection of *Mycobacterium avium* in drinking tap water using ultraviolet germicidal irradiation. Environ Technol 2018;39:3221-7. https://doi.org/10.1080/09593330.2017.1375028.

[23] Pathak S, Awuh JA, Anders Leversen N, Flo TH, Åsjø B. Counting Mycobacteria in Infected Human Cells and Mouse Tissue: A Comparison between qPCR and CFU. PLoS One 2012;7:e34931. https://doi.org/10.1371/journal.pone.0034931.

[24] Teskey G, Cao R, Islamoglu H, Medina A, Prasad C, Prasad R, et al. The Synergistic Effects of the Glutathione Precursor, NAC and First-Line Antibiotics in the Granulomatous Response Against Mycobacterium tuberculosis. Front Immunol 2018;9:2069. https://doi.org/10.3389/fimmu.2018.02069.

[25] Cacciatore I, Cornacchia C, Pinnen F, Mollica A, Di Stefano A. Prodrug approach for increasing cellular glutathione levels. Molecules 2010;15:1242-64. https://doi.org/10.3390/molecules15031242.

[26] Eligini S, Crisci M, Bono E, Songia P, Tremoli E, Colombo GI, et al. Human monocyte-derived macrophages spontaneously differentiated in vitro show distinct phenotypes. J Cell Physiol 2013;228:1464-72. https://doi.org/10.1002/jcp.24301.

[27] Greenwell-Wild T, Vázquez N, Sim D, Schito M, Chatterjee D, Orenstein JM, et al. Mycobacterium avium infection and modulation of human macrophage gene expression. J Immunol 2002;169:6286-97. https://doi.org/10.4049/jimmunol.169.11.6286.

[28] Venketaraman V, Dayaram YK, Talaue MT, Connell ND. Glutathione and nitrosoglutathione in macrophage defense against Mycobacterium tuberculosis. Infect Immun 2005;73:1886-9. https://doi.org/10.1128/II.73.3.1886-1889.2005.

[29] Shi L, Jiang Q, Bushkin Y, Subbian S, Tyagi S. Biphasic dynamics of macrophage immunometabolism during Mycobacterium tuberculosis infection. mBio 2019;10:e02550-18. https://doi.org/10.1128/mBio.02550-18.

[30] Cooper AM, Mayer-Barber KD, Sher A. Role of innate cytokines in mycobacterial infection. Mucosal Immunol 2011;4:252–60. https://doi.org/10.1038/mi.2011.13.
[31] Guerra C, Morris D, Sipin A, Kung S, Franklin M, Gray D, et al. Glutathione and adaptive immune responses against Mycobacterium tuberculosis infection in healthy and HIV infected individuals. PLoS One 2011;6:e28378. https://doi.org/10.1371/journal.pone.0028378.
[32] Dayaram YK, Talaue MT, Connell ND, Venketaraman V. Characterization of a glutathione metabolic mutant of Mycobacterium tuberculosis and its resistance to glutathione and nitrosoglutathione. J Bacteriol 2006;188:1364-72. https://doi.org/10.1128/JB.188.4.1364-1372.2006.
[33] Mavi PS, Singh S, Kumar A. Reductive Stress: New Insights in Physiology and Drug Tolerance of Mycobacterium. Antioxid Redox Signal 2019; https://doi.org/10.1089/ars.2019.7867.
[34] Young C, Walzl G, Du Plessis N. Therapeutic host-directed strategies to improve outcome in tuberculosis. Mucosal Immunol 2020;13:190-204. doi: 10.1038/s41385-019-0226-5.
[35] Rocco JM, Irani VR. Mycobacterium avium and modulation of the host macrophage immune mechanisms. Int J Tuberc Lung Dis 2011;15:447-52. https://doi.org/10.5588/ijtld.09.0695.
[36] Domingo-Gonzalez R, Prince O, Cooper A, Khader S. Cytokines and Chemokines in Mycobacterium tuberculosis infection. Microbiol Spectr 2016;4. https://doi.org/10.1128/microbiolspec.TBTB2-0018-2016.
[37] Alam K, Ghousunnissa S, Nair S, Valluri VL, Mukhopadhyay S. Glutathione-redox balance regulates c-rel-driven IL-12 production in macrophages: possible implications in antituberculosis immunotherapy. J Immunol 2010;184:2918-29. https://doi.org/10.4049/jimmunol.0900439.
[38] Méndez-Samperio P. Role of interleukin-12 family cytokines in the cellular response to mycobacterial disease. Int J Infect Dis 2010;14:e366-71. https://doi.org/10.1016/j.ijid.2009.06.022.
[39] Limongi D, Baldelli S, Checconi P, Marcocci ME, De Chiara G, Fraternale A, et al. GSH-C4 Acts as Anti-inflammatory Drug in Different Models of Canonical and Cell Autonomous Inflammation Through NFκB Inhibition. Front Immunol 2019;10:155. https://doi.org/10.3389/fimmu.2019.00155.

Legends to the figures

Figure 1. Inhibition of *M. avium* growth by I-152 (a) or C4-GSH (b).

Bacteria suspensions, grown in Middlebrook 7H9 broth, were exposed to different concentrations of the molecules for seven days at 35°C. Inhibition growth was evaluated by plating, at the beginning and at the end of the experiment, 10 µl of suspensions on Middlbrook 7H10 agar for the enumeration of CFUs/ml. Mean values ± standard deviations for three experiments are shown. Significant *M. avium* growth inhibition vs not treated is indicated as follows: *p<0.05; **p<0.01; ***p<0.001.

Figure 2. Inhibition of *M. avium* viability by I-152 (a) or C4-GSH (b).

Viability was determined in mycobacterial suspensions in sterile distilled water containing or not containing the molecules (5 and 10 mM) at 35° C. Cell viability was evaluated by plating in triplicate 10 µl of each suspension on Middlebrook 7H10 agar for the enumeration of CFUs/ml. The results are the mean of two independent experiments.

Figure 3. Glutathione (GSH) levels in uninfected and *M. avium*-infected macrophages (a). Intracellular growth of *M. avium* in human macrophages in the presence of 10 mM of I-152 or 10 mM of C4-GSH (b).

The macrophage cultures were exposed for four hours to bacteria at a MOI of 60:1 (bacilli:macrophage). At this time and seven days after bacterium suspension removal, GSH was determined in the macrophage cells by HPLC. For the assay of antimycobacterial activity, I-152 and C4-GSH were dissolved in the culture medium at a concentration of 10 mM and added to the macrophages. After four days, the medium was replaced with fresh medium containing the pro-GSH molecules. CFUs, determined as described in section 2.4 , were evaluated per total macrophage proteins. The results of antimycobacterial activity are given as percentage of growth inhibition vs infected and untreated macrophages. Mean values ± standard deviations for three experiments are shown. Significant *M. avium* growth inhibition is indicated as follows: *p<0.05; **p<0.01.

Figure 4. Modulation of cytokine production in *M. avium*-infected macrophages untreated or treated with I-152 or C4-GSH.

The cytokines were determined in the supernatants collected after four and seven days of infection by a multiplex biometric ELISA-based immunoassay. The interleukin concentrations were calculated using a standard curve and software provided by the manufacturer (Bio-Plex manager software, v.6.1). Final values represent the sum of the fourth day values and the seventh day values. The results represent the mean of two separate experiments and are given as cytokine pg/CFU.

Figure 1

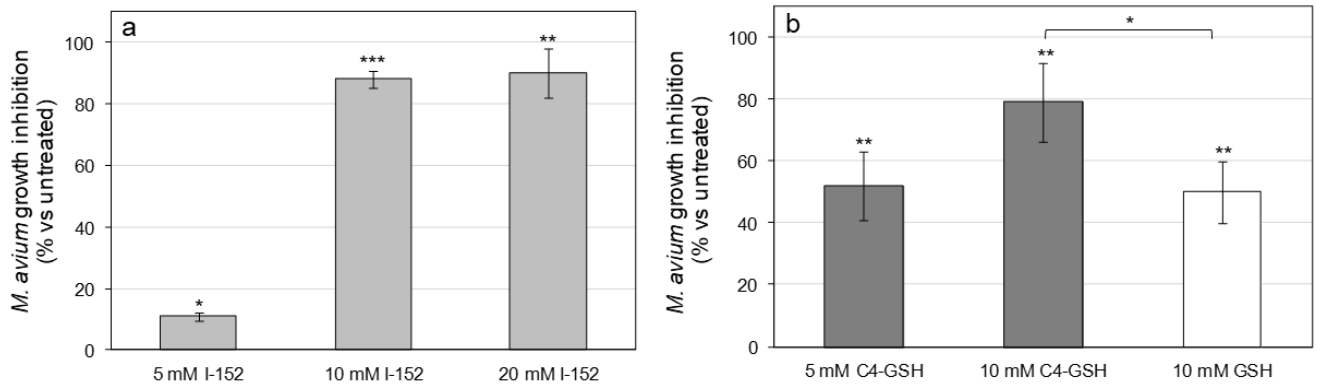

a
M. avium growth inhibition (% vs untreated)
100
80
60
40
20
0
*
***
**
5 mM I-152
10 mM I-152
20 mM I-152
b
M. avium growth inhibition (% vs untreated)
100
80
60
40
20
0
*
**
**
**
5 mM C4-GSH
10 mM C4-GSH
10 mM GSH

Figure 2

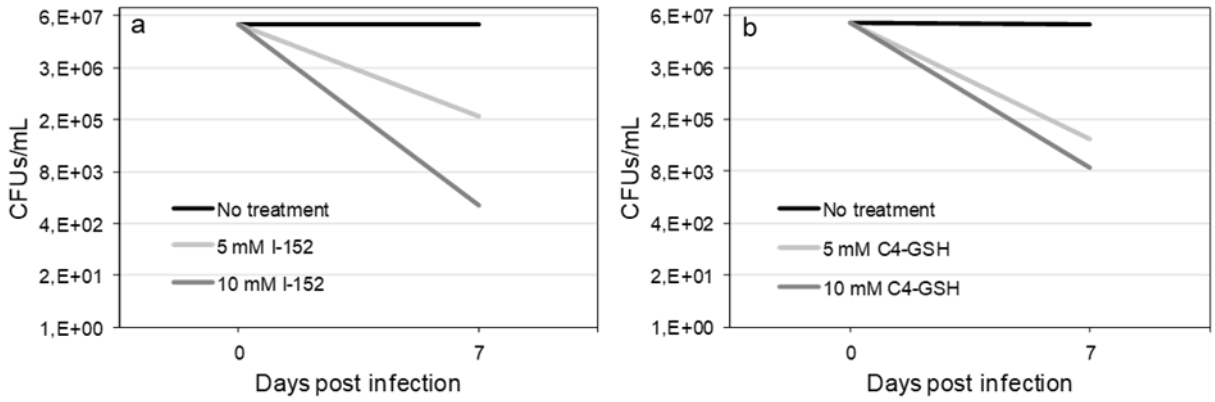

a
6,E+07
3,E+06
2,E+05
8,E+03
4,E+02
2,E+01
1,E+00
CFUs/mL
No treatment
5 mM I-152
10 mM I-152
0
7
Days post infection
b
6,E+07
3,E+06
2,E+05
8,E+03
4,E+02
2,E+01
1,E+00
CFUs/mL
No treatment
5 mM C4-GSH
10 mM C4-GSH
0
7
Days post infection

Figure 3

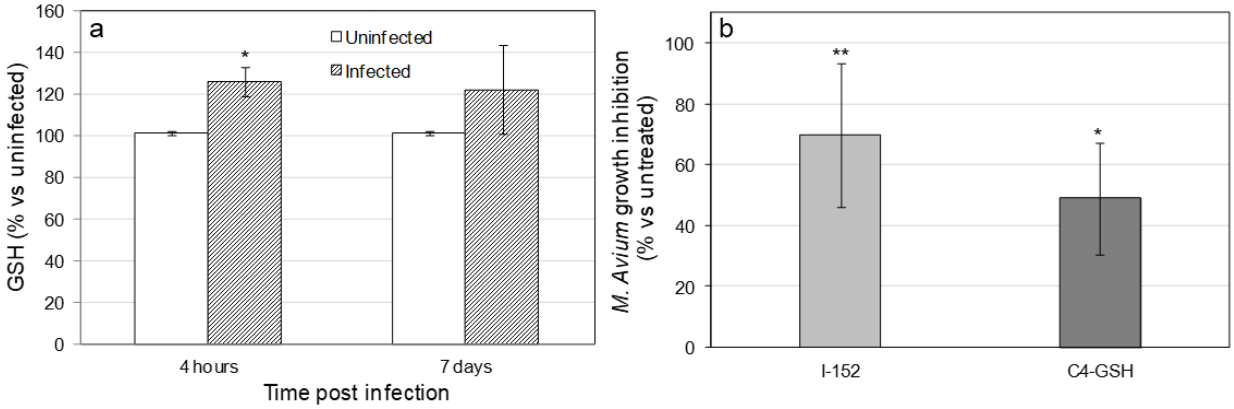
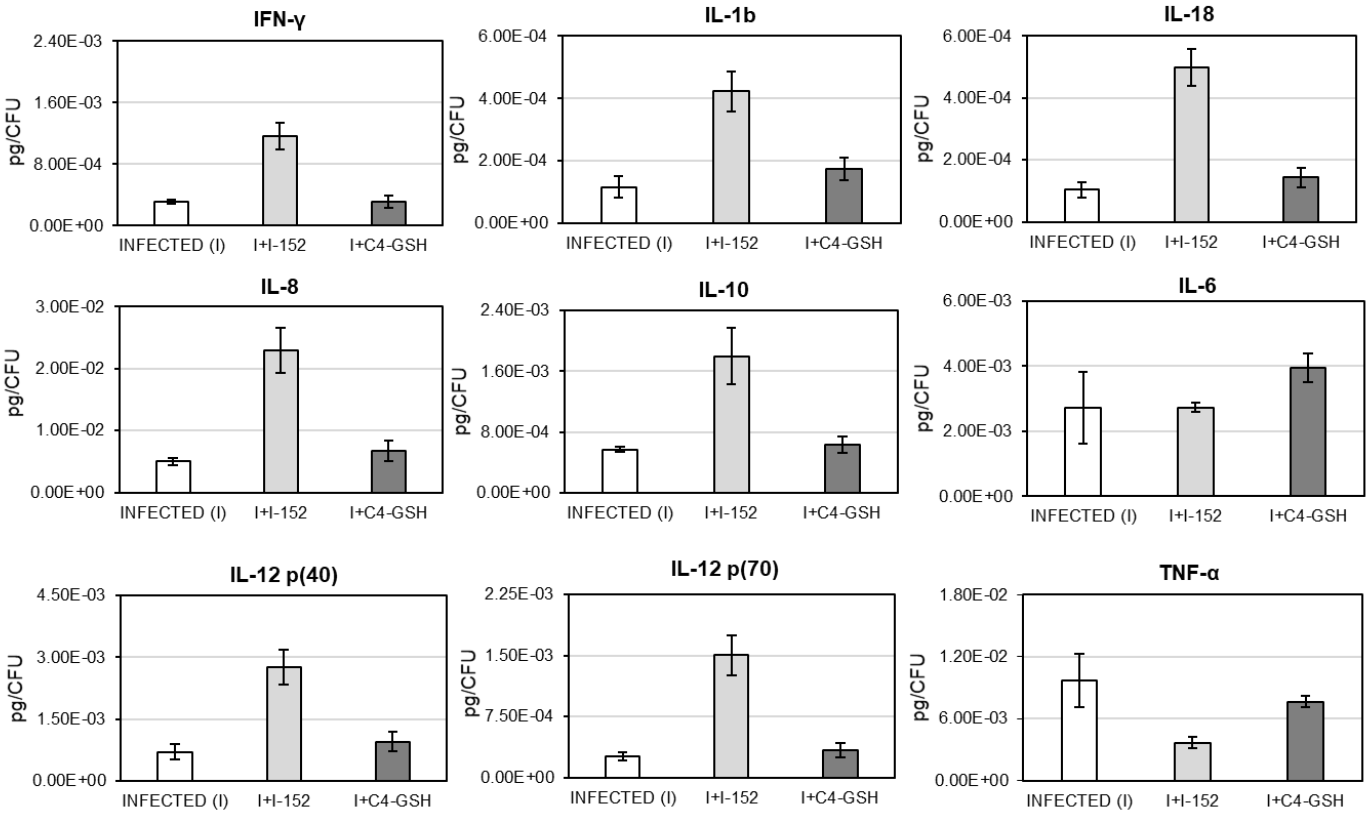

a
Uninfected
Infected
GSH (% vs uninfected)
160
140
120
100
80
60
40
20
0
*
4 hours
7 days
Time post infection
b
M. Avium growth inhibition (% vs untreated)
100
80
60
40
20
0
**
*
I-152
C4-GSH

Figure 4


IFN-γ
2.40E-03
1.60E-03
8.00E-04
0.00E+00
pg/CFU
INFECTED (I)
I+I-152
I+C4-GSH
IL-1b
6.00E-04
4.00E-04
2.00E-04
0.00E+00
IL-18
IL-8
3.00E-02
2.00E-02
1.00E-02
IL-10
IL-6
6.00E-03
4.00E-03
2.00E-03
IL-12 p(40)
4.50E-03
3.00E-03
1.50E-03
IL-12 p(70)
2.25E-03
7.50E-04
TNF-α
1.80E-02
1.20E-02