International Journal of Antimicrobial Agents 40 (2012) 182–185

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Short communication

Activity of IQG-607, a new orally active compound, in a murine model of *Mycobacterium tuberculosis* infection

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ARTICLE INFO

Article history:
Received 16 March 2012
Accepted 29 April 2012

Keywords:
Tuberculosis
Drug development
Pre-clinical test
InhA inhibitor
IQG-607
Mycobacterium tuberculosis

ABSTRACT

We have previously demonstrated a potent in vitro inhibitory activity for two pentacyano(isoniazid)ferrate(II) compounds, namely IQG-607 and IQG-639, against the *Mycobacterium tuberculosis* enoyl-acyl carrier protein reductase enzyme. In this study, the activity of these compounds was evaluated using an in vivo murine model of tuberculosis. Swiss mice were infected with *M. tuberculosis* H37Rv strain and then IQG-607 or IQG-639 (250 mg/kg) was administered for 28 days or 56 days. In addition, a dose–response study was performed with IQG-607 at 5, 10, 25, 50, 100, 200 and 250 mg/kg. The activity of test compounds was compared with that of the positive control drug isoniazid (INH) (25 mg/kg). After 28 days or 56 days of treatment, both IQG-607 and INH significantly reduced *M. tuberculosis*-induced splenomegaly as well as significantly diminishing the colony-forming units in the spleen and lungs. IQG-607 and INH ameliorated the lung macroscopic aspect, reducing lung lesions to a similar extent. However, IQG-639 did not significantly modify any evaluated parameter. Experiments using early and late controls of infection revealed a bactericidal activity for IQG-607. IQG-607 might well represent a good candidate for clinical development as a new antimycobacterial agent.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a major global health concern. According to the World Health Organization (WHO), TB was responsible for 1.3 million deaths in 2009 and currently represents the main cause of human death due to a single pathogen [1,2]. Increasing HIV–TB co-infections [2] as well as the emergence of multidrug-resistant, extensively drug-resistant [1] and totally drug-resistant [3] strains have increased the need for the development of new anti-TB drugs. Novel anti-TB drugs should be effective against resistant strains, decreasing the length of treatment, with a lower dose frequency, minimal drug–drug interactions and reduced toxicity [1]. Isoniazid (INH) is the most prescribed drug for active TB and prophylaxis. Its primary target is the *M. tuberculosis* 2-*trans*-enoyl-acyl carrier protein (CoA) reductase enzyme (InhA) [4]. INH is a pro-drug activated by the mycobacterial *katG*-encoded catalase–peroxidase enzyme in the presence of manganese ions, NAD(H) and oxygen [5].

Our group has described a new approach to the rational design of an INH analogue based on an inorganic group (a pentacyanoferrate III/II) attached to the nitrogen atom of the heterocyclic ring of INH, which inhibits a validated target (InhA) [6]. The metal centre can promote an electron transfer reaction that mimics the in vitro activation of INH by the KatG enzyme [6]. Our group has also proved that this new compound, pentacyano(isoniazid)ferrate(II), named IQG-607 (Supplementary Fig. S1), does not require

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http://dx.doi.org/10.1016/j.ijantimicag.2012.04.019

activation by KatG or any other enzyme to bind to its probable molecular target, the *M. tuberculosis* InhA [7]; this might help to overcome an important mechanism of INH resistance, i.e. mutations in the *katG* gene. Moreover, we demonstrated that IQG-607 is able to inhibit the in vitro activity of wild-type and INH-resistant (I21V, I47T and S94A) *M. tuberculosis* InhA enzymes [8]. Another new compound containing a pentacyanoferrate and an oxadiazole moiety, pentacyano[2-metil-5-(piridin-4-il)-1,3,4-oxadiazole]ferrate(II), denoted IQG-639 (Supplementary Fig. S1), was also found to inhibit the in vitro activity of wild-type and INH-resistant (S94A) *M. tuberculosis* InhA enzymes (data not shown).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2012.04.019.

IQG-607 and IQG-639 were active against cultures of *M. tuberculosis* H37Rv as well as two INH-resistant clinical isolates. For IQG-607, the minimum inhibitory concentration (MIC) was 0.25 μg/mL for the H37Rv strain, whereas for the strains containing mutations in the structural (S94A) or promoter region [C(−15)T] of the *inhA* gene, MIC values were 1.0 μg/mL and 4.0 μg/mL, respectively [9]. For the same strains, the MIC values obtained for IQG-639 were 0.5, 2.0 and 4.0 μg/mL, respectively. Noteworthy, for these resistant strains, the MICs for INH were reported to be ≥16 μg/mL [10]. Importantly, preliminary toxicological studies in rodents revealed a very favourable outcome for both compounds, thus it appears warranted to examine their potential in vivo anti-TB activity. Accordingly, the present work aimed at describing the pre-clinical evaluation of IQG-607 and IQG-639 in a murine model of TB.

2. Materials and methods

2.1. Drugs and reagents

INH was purchased from Acros Organics (Geel, Belgium). IQG-607 was synthesised according to Oliveira et al. [6]. IQG-639 had its 2-metil-5-(piridin-4-il)-1,3,4-oxadiazole complex synthesised as previously described [11], and the product was further coordinated to the pentacyanoferrate(II) moiety according to Oliveira et al. [6]. IQG-607 and IQG-639 had their chemical structures characterised as reported previously [6]. All drugs were dissolved in saline solution and were prepared freshly, being protected from light and heat.

2.2. Bacterial strain

Virulent *M. tuberculosis* H37Rv reference strain (ATCC 27294) was cultured in Ogawa medium at 37 °C in a 5% CO_2 environment for 5 weeks. *Mycobacterium tuberculosis* colonies were carefully scraped and suspended in sterile 0.9% saline solution containing 0.05% Tween 80 (Sigma-Aldrich, St Louis, MO). This cell suspension was vortexed with sterile glass beads (4 mm) for 5 min to disrupt clamps and was allowed to settle for 30 min. The supernatant was measured spectrophotometrically at an absorbance of 600 nm. Subsequently, the suspension was appropriately diluted to achieve an optical density at 600 nm of 0.8, which corresponds to 2×10^8 cells/mL. The *M. tuberculosis* suspension was aliquoted and stored at −20 °C.

2.3. Infection procedures

Male Swiss mice (25–30 g) obtained from the Central Biotery of Universidade Federal de Pelotas (Brazil) were infected intravenously through the retro-orbital venous plexus according to a previously described technique [12]. Groups of six mice were anaesthetised by intraperitoneal injection of a mixture containing ketamine (100 mg/kg) (Cristália, Itapira, SP, Brazil) and xylazine (10 mg/kg) (Vetbrands, Jacareí, SP, Brazil) and were subsequently infected with 10^6–10^7 viable *M. tuberculosis* H37Rv cells suspended in 200 μL of saline solution. For each experiment, a non-infected control group of six animals received saline solution by the intravenous route. Efficiency of the infection protocol was confirmed by Ziehl–Neelsen staining of lung sections.

2.4. Treatment schemes

Following infection, mice were randomly divided into treatment groups with six animals each. Treatment was started 7 days post infection and drugs were administered daily by oral gavage. The first chemotherapeutic experiment consisted of a 28-day schedule of treatment. To assess the impact of increasing the period of drug administration, a second experiment was designed that consisted of 56 days of treatment. For both the first and second regimens, treatment groups received 250 mg/kg IQG-607 (corresponding to 560 μmol/kg) or 250 mg/kg IQG-639 (532 μmol/kg).

Experiment 3 evaluated the dose–response pattern of compound IQG-607. Different groups of *M. tuberculosis*-infected mice received different daily doses of IQG-607 at 5, 10, 25, 50, 100, 200 and 250 mg/kg (corresponding to 11, 22, 56, 112, 224, 448 and 560 μmol/kg, respectively) for 28 days. INH (25 mg/kg, or 182 μmol/kg) [12,13] was given as a positive treatment control. Non-infected mice and the disease-positive group (infected and not treated) received saline solution during the treatment period.

2.5. Assessment of treatment efficacy

To assess lung and spleen CFU counts and splenomegaly, mice were euthanised by isoflurane (Cristália) inhalation 5 days after the last dose of drugs. The spleen and left lung were removed under aseptic conditions. The weight of the spleen was measured aseptically. The lung and spleen were placed in 3 mL of saline solution and were disrupted in a glass tissue homogeniser (Góes Vidros Especiais, Porto Alegre, RS, Brazil). The number of viable organisms was determined by plating serial dilutions of homogenates on Middlebrook 7H10 agar (Difco, Sparks, MD) plates containing 10% Middlebrook OADC (oleic acid–albumin–dextrose–catalase) enrichment (Becton Dickinson, Franklin Lakes, NJ). Plates were incubated at 37 °C for 28 days in a 5% CO_2 environment prior to counting of viable *M. tuberculosis* cells.

2.6. Evidence of bactericidal effects

To investigate the bactericidal activity of IQG-607, a fourth experimental set was performed. Seven days after infection, infected mice received INH (25 mg/kg) or IQG-607 (150 mg/kg, or 336 μmol/kg) for 28 days. A group of six infected mice was sacrificed at the start of treatment as pre-treatment controls (early control group) [12] and the numbers of viable organisms in the spleen and lungs were determined.

2.7. Statistical analysis

For *M. tuberculosis* cell counts, the numbers were first converted into logarithms of CFU ($\log_{10}$ CFU). Spleen weight was previously corrected with the body weight from each animal. Data were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Differences were considered significant at the 95% level of confidence.

Table 1
Colony-forming unit (CFU) counts in organ homogenates from mice infected with *Mycobacterium tuberculosis* H37Rv and treated for 28 days or 56 days with isoniazid (INH), IQG-607 or IQG-639.

Treatment	Log_{10} CFU/organ (mean ± SEM)[a]			
	28 days		56 days	
	Lungs	Spleen	Lungs	Spleen
Untreated	5.73 ± 0.23	5.84 ± 0.07	6.37 ± 0.05	5.34 ± 0.21
INH (25 mg/kg)	3.36 ± 0.08*	4.07 ± 0.20*	3.37 ± 0.36*	3.13 ± 0.23*
IQG-607 (250 mg/kg)	3.36 ± 0.27*	4.44 ± 0.17*	3.17 ± 0.20*	3.18 ± 0.12*
IQG-639 (250 mg/kg)	5.72 ± 0.25	5.65 ± 0.08	6.35 ± 0.09	5.38 ± 0.24

SEM, standard error of the mean.
[a] Results represent the mean ± SEM of four to five mice per group.
* $P < 0.001$ compared with the untreated group.

3. Results

3.1. Evaluation of the activity of IQG-607 and IQG-639 after 28 days of treatment

A marked splenomegaly was observed in the infected group, as indicated by a 261% increase in spleen weight ($P < 0.001$) compared with the non-infected group. Furthermore, it was possible to macroscopically visualise the lung tissue damage, with many tubercles compared with the non-infected group (Supplementary Fig. S2). Ziehl–Neelsen staining of histological lung sections was also performed, which confirmed the typical granuloma structure formation with acid-fast bacilli within macrophages surrounded by lymphocytes (Supplementary Fig. S3).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2012.04.019.

Spleen weights were 58% ($P < 0.001$) and 50% ($P < 0.001$) lower in INH- and IQG-607-treated animals, respectively, compared with untreated controls. Both INH and IQG-607 treatments ameliorated the lung macroscopic aspect by reducing the lung lesions in relation to the untreated group (Supplementary Fig. S2). Furthermore, CFU counting results from INH-treated mice were 2.37 $\log_{10}$ ($P < 0.001$) and 1.77 $\log_{10}$ ($P < 0.001$) lower than those from the untreated group in the lungs and spleen, respectively (Table 1). Importantly, significant differences in bacterial loads were observed between IQG-607-treated mice and untreated controls: 2.37 $\log_{10}$ ($P < 0.001$) in the lungs and 1.4 $\log_{10}$ ($P < 0.001$) in the spleen (Table 1). No significant difference in CFU loads was observed following IQG-639 treatment (Table 1).

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3.2. Evaluation of the activity of IQG-607 and IQG-639 after 56 days of treatment

In this experiment, spleen weights were 66% ($P < 0.001$) and 67% ($P < 0.001$) lower in INH- and IQG-607-treated animals, respectively, compared with untreated controls. On the other hand, 56 days of treatment with IQG-639 was not sufficient to obtain a significant difference of this parameter (data not shown). Treatment with INH resulted in 3 $\log_{10}$ ($P < 0.001$) and 2.21 $\log_{10}$ ($P < 0.001$) CFU lower than those from the untreated group in the lungs and spleen, respectively (Table 1). The bacterial loads in the lungs and spleen from IQG-607-treated mice were 3.2 $\log_{10}$ ($P < 0.001$) and 2.16 $\log_{10}$ ($P < 0.001$) lower than those of untreated controls (Table 1). Treatment with IQG-639 for 56 days did not affect the CFU counts in either the lungs or spleens (Table 1).

Table 2
Colony-forming unit (CFU) counts in organ homogenates from mice infected with *Mycobacterium tuberculosis* H37Rv and treated with isoniazid (INH) or different doses of IQG-607 for 28 days.

Treatment	Log_{10} CFU/organ (mean ± SEM)[a]	
	Lungs	Spleen
Untreated	7.03 ± 0.11	6.10 ± 0.15
INH (25 mg/kg)	3.42 ± 0.15*	5.03 ± 0.07*
IQG-607		
5 mg/kg	6.46 ± 0.12	5.98 ± 0.06
10 mg/kg	4.44 ± 0.08*	5.31 ± 0.08*
25 mg/kg	4.25 ± 0.08*	5.34 ± 0.11*
50 mg/kg	4.11 ± 0.32*	5.07 ± 0.14*
100 mg/kg	3.74 ± 0.23*	5.15 ± 0.09*
200 mg/kg	3.67 ± 0.27*	5.16 ± 0.07*
250 mg/kg	3.76 ± 0.18*	5.26 ± 0.14*

SEM, standard error of the mean.
[a] Results represent the mean ± SEM of five to six mice per group.
* $P < 0.001$ compared with the untreated group.

3.3. Evaluation of the activity of different doses of IQG-607

Spleen weights were significantly lower in IQG-607-treated animals at doses of 10, 25, 50, 100, 200 and 250 mg/kg as well as INH-treated animals compared with the untreated controls ($P < 0.001$ for all treatments). In INH-treated mice, splenomegaly was 47% lower than controls, whereas at doses of 10, 25, 50, 100, 200 and 250 mg/kg IQG-607 spleen weights were 38%, 43%, 40%, 45%, 52% and 51% lower, respectively. Treatment with 5 mg/kg IQG-607 did not significantly affect spleen weights ($P > 0.05$).

The INH-treated group showed a decrease of 3.61 $\log_{10}$ ($P < 0.001$) in CFU counts in the lungs and 1.07 $\log_{10}$ ($P < 0.001$) in the spleen compared with untreated group (Table 2). Treatment with doses of 10, 25, 50, 100, 200 and 250 mg/kg of IQG-607 resulted in a range of 2.59 to 3.36 $\log_{10}$ ($P < 0.001$ for all doses) CFU units lower than the untreated group in the lungs (Table 2). In the spleen, these doses reduced the bacterial counts in a range of 0.76 to 1.03 $\log_{10}$ ($P < 0.001$ for all treatments) compared with untreated mice (Table 2). No significant reduction in spleen and lung CFU loads was observed after 28 days of treatment with 5 mg/kg IQG-607 (Table 2).

3.4. Assessment of bactericidal activity

In this experiment, 25 mg/kg INH reduced splenomegaly by 55% ($P < 0.001$) and 51% ($P < 0.01$) compared with early and late untreated controls, respectively. Treatment with 150 mg/kg IQG-607 decreased the spleen weights by 46% ($P < 0.01$) and 41% ($P < 0.05$) compared with early and late controls, respectively. Treatment with INH or IQG-607 significantly reduced the bacterial load from the lungs and spleen compared with the early and late control groups ($P < 0.001$) (Table 3).

Table 3
Colony-forming unit (CFU) counts in organ homogenates from mice infected with *Mycobacterium tuberculosis* H37Rv and treated with isoniazid (INH) or IQG-607 for 28 days, showing data on early and late controls of infection.

Treatment	Log_{10} CFU/organ (mean ± SEM)[a]	
	Lungs	Spleen
Early control[b]	5.32 ± 0.13	7.34 ± 0.12
Late control[c]	6.88 ± 0.16	5.88 ± 0.09
INH (25 mg/kg)	3.11 ± 0.12[*,†]	5.03 ± 0.13[*,†]
IQG-607 (150 mg/kg)	3.28 ± 0.35[*,†]	4.86 ± 0.14[*,†]

SEM, standard error of the mean.
[*] $P < 0.001$ compared with the early control group.
[†] $P < 0.001$ compared with the late control group.
[a] Results represent the mean ± SEM of four to six mice per group.
[b] Early control represents mice sacrificed at the start of treatment.
[c] Late control represents mice sacrificed at the end of the treatment.

4. Discussion

Both compounds IQG-607 and IQG-639 have shown marked inhibitory activities on the *M. tuberculosis* InhA enzyme in vitro [6,8]. These two new compounds also displayed in vitro antimicrobial activities against cultures of *M. tuberculosis* H37Rv strain and INH-resistant clinical isolates [9]. This study evaluated the in vivo efficacy of both compounds in a murine model of TB.

Infection of mice with *M. tuberculosis* induced severe splenomegaly, as also observed in previous mycobacterial infection studies [13,14]. In addition, macroscopic visualisation of the lung aspects suggested a marked inflammatory response and the consistent development of lesions. Moreover, the morphological features of granuloma formation [15] were observed in lungs from infected mice. Importantly, this work shows that treatment with either the reference drug INH or the test compound IQG-607 effectively resolved visible lung lesions according to macroscopic evaluation.

Regarding CFU counts, the bacterial loads both in the lungs and the spleen from IQG-607-treated mice were markedly lower than those from untreated controls after 28 days and 56 days of treatment. The efficacy of IQG-607 at 250 mg/kg was virtually similar to that observed for INH (25 mg/kg) when considering the CFU counts of lungs and spleen after 28 days or 56 days of treatment. We believe that IQG-607 is absorbed and reaches the lungs following oral administration, being able to reach the bacilli and killing them within the phagosome of the macrophages. However, 250 mg/kg IQG-639 was not capable of significantly modifying any evaluated parameters of infection even after 56 days of treatment. It could be speculated that IQG-639 does not reach the intracellular compartments of the macrophages or that it is not absorbed following oral administration. Furthermore, IQG-639 might also be inactivated by some additional unknown mechanism, which remains to be evaluated. Next we evaluated whether IQG-607 activity follows a dose–response pattern. In this protocol, 10 mg/kg IQG-607 was the lowest dose able to show significant activity. The dose of IQG-607 used for treating infected mice for 28 days and 56 days (250 mg/kg) has been chosen according to the acute oral toxicity assays, as both 250 mg/kg and 500 mg/kg did not cause any toxic clinical sign or mortality in male and female mice, showing favourable toxicological features. INH, in turn, caused 80% deaths in mice following single oral administration of 250 mg/kg [9]. Of note, IQG-607 was also found to display bactericidal activity. Based on the efficacy data presented in this work, we have solid evidence that IQG-607 is absorbed following oral administration without causing any apparent toxicity in mice. Altogether, these results allow us to suggest that IQG-607 might represent a good candidate for clinical development as a new antimycobacterial agent.

Funding: This work was supported by Banco Nacional de Desenvolvimento Econômico e Social (BNDES/FUNTEC) and the National Institute of Science and Technology on Tuberculosis (Decit/SCTIE/MS-MCT-CNPq-FNDTC-CAPES), Brazil. DSS and LAB also acknowledge grants awarded by FINEP (PUCRSINFRA-01.11.0014-00) and FAPERGS-CNPq-PRONEX-2009. DSS, JBC, LAB, LGFL, AAS and MMC are research career awardees of the National Council for Scientific and Technological Development of Brazil (CNPq).

Competing interests: None declared.

Ethical approval: All animal procedures were reviewed and approved by the Ethics Committee for Animal Use of Pontifícia Universidade Católica do Rio Grande do Sul (Porto Alegre, Brazil) (permit no. 09/94).

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