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In vitro and *in vivo* antimycobacterial activities of ketone and amide derivatives of quinoxaline 1,4-di-*N*-oxide

Raquel Villar[1], Esther Vicente[1], Beatriz Solano[1], Silvia Pérez-Silanes[1], Ignacio Aldana[1], Joseph A. Maddry[2], Anne J. Lenaerts[3], Scott G. Franzblau[4], Sang-Hyun Cho[4], Antonio Monge[1] and Robert C. Goldman[5]*

[1]Unidad en Investigación y Desarrollo de Medicamentos, Centro de Investigación en Farmacobiología Aplicada (CIFA), Universidad de Navarra, C/Irunlarrea s/n, 31080 Pamplona, Spain; [2]Southern Research Institute, Birmingham, AL 35225-53053, USA; [3]Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523, USA; [4]Institute for Tuberculosis Research, University of Illinois at Chicago, IL 60612, USA; [5]Division of AIDS, National Institute of Allergy and Infectious Diseases, Therapeutics Research Program, Bethesda, MD 20892, USA

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***Objectives*: To evaluate a novel series of quinoxaline 1,4-di-*N*-oxides for *in vitro* activity against *Mycobacterium tuberculosis* and for efficacy in a mouse model of tuberculosis (TB).**

***Methods*: Ketone and amide derivatives of quinoxaline 1,4-di-*N*-oxide were evaluated in *in vitro* and *in vivo* tests including: (i) activity against *M. tuberculosis* resistant to currently used antitubercular drugs including multidrug-resistant strains (MDR-TB resistant to isoniazid and rifampicin); (ii) activity against non-replicating persistent (NRP) bacteria; (iii) MBC; (iv) maximum tolerated dose, oral bioavailability and *in vivo* efficacy in mice; and (v) potential for cross-resistance with another bioreduced drug, PA-824.**

***Results*: Ten compounds were tested on single drug-resistant *M. tuberculosis*. In general, all compounds were active with ratios of MICs against resistant and non-resistant strains of $\leq$4.00. One compound, 5, was orally active in a murine model of TB, bactericidal, active against NRP bacteria and active on MDR-TB and poly drug-resistant clinical isolates (resistant to 3–5 antitubercular drugs).**

***Conclusions*: Quinoxaline 1,4-di-*N*-oxides represent a new class of orally active antitubercular drugs. They are likely bioreduced to an active metabolite, but the pathway of bacterial activation was different from PA-824, a bioreducible nitroimidazole in clinical trials. Compound 5 was bactericidal and active on NRP organisms indicating that activation occurred in both growing and non-replicating bacteria leading to cell death. The presence of NRP bacteria is believed to be a major factor responsible for the prolonged nature of antitubercular therapy. If the bactericidal activity and activity on non-replicating bacteria *in vitro* translate to *in vivo* conditions, quinoxaline 1,4-di-*N*-oxides may offer a path to shortened therapy.**

Keywords: antitubercular drugs, resistance, *in vivo* efficacy

Introduction

Tuberculosis (TB) is a contagious disease with high mortality worldwide. It is estimated that 1.7 million deaths resulted from TB in 2006, and there are an estimated 8 million new cases each year. Moreover, up to 50 million people are infected with drug-resistant forms of TB.[1] Although drug-resistant TB has existed since the introduction of the anti-TB chemotherapy, the global magnitude of drug-resistant TB was not adequately studied until recently.[2] The magnitude and extent of drug-resistant strains have increased concern that TB may once again become an incurable disease and emphasized the need for new drugs to treat this infection. The recent appreciation of the widespread existence of extensively drug-resistant tuberculosis (XDR-TB) has further

*Corresponding author. Tel: +1-301-496-8424; Fax: +1-301-451-5481; E-mail: rgoldman@niaid.nih.gov

heightened the awareness of the need for new anti-tubercular agents with novel modes of action and full activity on poly drug-resistant strains (resistant to 3–5 antitubercular drugs), including multidrug resistance (MDR; resistant to isoniazid and rifampicin) and XDR-TB.[3–9]

Our group has previously reported on the synthesis and biological evaluation of a large number of quinoxaline and quinoxaline 1,4-di-*N*-oxide derivatives.[10–15] Various 2-acetyl, 2-benzoyl and 2-carboxamide quinoxaline derivatives have been evaluated as anti-*Mycobacterium tuberculosis* agents.[16,17] Specific analogues showed good *in vitro* parameters in cytotoxicity assays and in a TB-infected macrophage model. We later confirmed the antimicrobial activity of this class of compounds, and report here on the *in vitro* activity of ketone and amide derivatives of quinoxaline 1,4-di-*N*-oxide against different strains of drug-resistant *M. tuberculosis* and in the rapid *in vivo* mouse efficacy model conducted as part of the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF).

Materials and methods

Synthesis of compounds

The methods for the synthesis of quinoxaline-2-acetyl, 2-benzoyl and 2-carboxamide 1,4-di-*N*-oxide derivatives were reported elsewhere.[16,17] A list of compounds, their structures and significant values from the earlier assays are presented in Table 1 and Figure 1. The criteria used by the TAACF for moving a compound on to *in vivo* testing includes a selectivity index, defined as the ratio of the measured IC_{50} in VERO cells to the MIC, of >10. In the same way, the concentrations which cause a 90% reduction in residual mycobacterial growth in the macrophage assay should be lower than $16\times$ the MIC.[16,17]

Determination of MICs against single drug-resistant M. tuberculosis and MBC

MICs were determined in the microplate Alamar Blue assay (MABA)[18,19] for strains of single drug-resistant (SDR) *M. tuberculosis* (each strain resistant to a single TB drug): isoniazid (ATCC 35822), rifampicin (ATCC 35838), thiacetazone (ATCC 35829), ethambutol (ATCC 35837), *p*-aminosalicylic acid (ATCC 35821), ethionamide (ATCC 35839) and ciprofloxacin (laboratory stock collection) as well as the drug-susceptible $H_{37}Rv$ strain (ATCC 35801). Generally, MICs for SDR strains should not be $>10\times$ the MIC for non-resistant strains to continue compound evaluation.

The MBC was then determined for *M. tuberculosis* $H_{37}Rv$ strain by subculturing onto drug-free solid medium and enumeration of Cfu after drug exposure in Middlebrook 7H9 medium supplemented with drug concentrations equivalent to and higher than the previously determined MICs against the respective strains. Samples were incubated for 7 days at 37°C and then plated for change in cfu. Cfu was read after 10 days of incubation and followed for any changes in cfu for a total of 21 days. The MBC was the lowest concentration of drug that killed $>99\%$ of the bacterial population present when the drugs were added.

Potential for cross-resistance with PA-824

PA-824 is a nitroimidazole agent in clinical trials for treating TB. *Mycobacterium bovis* strains resistant to PA-824 were obtained from Dr Lacy Daniels (Texas A&M College of Pharmacy, Kingsville, TX, USA). *M. bovis* strains were used because: (i) these represented the best genetically and biochemically characterized mycobacterial strains resistant to PA-824; and (ii) *M. bovis* is 99.9% identical to *M. tuberculosis* at the genetic level.[20] MICs were determined using microbroth dilution. PA-824 was obtained from the Global Alliance for Tuberculosis Drug Development (New York, NY, USA).

Determination of MICs against four poly drug-resistant and MDR-TB strains

MDR-TB strains obtained from Dr R. C. Chan (Department of Microbiology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, China)[21] were cultured in 7H9 broth supplemented with ADC (Middlebrook ADC Enrichment containing bovine albumin fraction V, glucose and catalase) until an OD_{600} of 0.6–0.8 was obtained, and then frozen in aliquots at −80°C until required. Bacterial suspensions were prepared to reach an inoculum of 10^5 cfu per well in a total volume of

Table 1. Structures and previous *in vitro* activity against *M. tuberculosis*

Compound	R6	R7	R2	MIC (mg/L)	SI[a]	IC_{90}/MIC[b]	TAACF #
1	H	H	CH_3	3.13	>20.0	0.87	149520
2	H	CH_3	CH_3	3.13	>20.0	0.80	151989
3	H	OCH_3	CH_3	1.56	37.82	4.29	118850
4	H	F	CH_3	3.13	>20.0	0.79	150568
5	H	Cl	CH_3	0.78	20.13	3.13	118845
6	CH_3	CH_3	CH_3	6.25	>10.0	0.44	148142
7	H	H	Ph	6.25	>10.0	ND	150355
8	H	H	NH-Ph	3.13	>20.0	0.89	150354
9	H	H	NH-Ph-(*o*)CH_3	6.25	>10.0	0.42	150356
10	H	Cl	NH-Ph-(*o*)CH_3	6.25	>10.0	0.14	151986

[a]SI is the selectivity index calculated as IC_{50} (concentration inhibiting growth of VERO cells in culture by 50% following 72 h of exposure and assessed using the CellTiter 96® Non-Radioactive Cell Proliferation Assay reagent from Promega) divided by the MIC.
[b]IC_{90}/MIC is a measure of the activity against intracellular *M. tuberculosis* taken up by mouse bone marrow macrophages (concentration required to inhibit growth of intracellular *M. tuberculosis* by 90%) divided by the MIC.

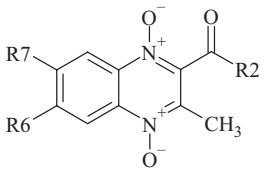


Figure 1. Quinoxaline 1,4-di-*N*-oxide core structure.

100 μL 7H9 medium. Drugs were dissolved in 100% DMSO to a 50× stock concentration. A 1:2 dilution series of both compounds were made in a separate 96-well plate using DMSO as the diluent. Two microlitres of the drugs were transferred to the appropriate wells of the microtitre plate. Only the inner 60 wells of the 96-well plate were used in the assay; the surrounding wells were filled with water to maintain humidity. Microtitre plates were incubated at 37°C for at least 14 days and observed every 3–4 days to determine changes in growth. Growth of the bacteria was scored at different days by a visual read and by a terminal spectrophotometer reading at OD_{600}.

Maximum tolerated dose (MTD) and oral bioavailability

All experiments using mice were approved by the Animal Care and Use Committee at Colorado State University (approval 06-221A-02, expiration date 17 October 2008) and under the Animal Welfare Assurance number A3572-01. C57BL/6 female mice were orally administered (by gavage) a single dose of drug at 100, 300 and 500 mg/kg, using three mice per dose. Mice were observed post-administration at 4 and 6 h, and then twice daily for the duration of the study (1 week). Oral bioavailability was determined by bioassay as described.[22]

Rapid in vivo screen

Eight- to 10-week-old female specific pathogen-free C57BL/6-Ifngtm1ts mice [gamma interferon gene-disrupted (GKO) mice] were purchased from Jackson Laboratories, Bar Harbor, ME, USA.[23] The mice were infected via low-dose aerosol exposure to *M. tuberculosis* Erdman using a Middlebrook aerosol generation device (Glas-Col Inc., Terre Haute, IN, USA), and the short-course mouse model was performed as described previously.[24] Briefly this involves a delivery of ~50–100 cfu to each mouse and this is confirmed 1 day post-infection by sacrificing three mice to verify the uptake of 50–100 cfu of bacteria per mouse. Treatment is initiated 15–18 days post-infection for nine daily treatments of one single oral dose (at 300 mg/kg). Bacterial load is determined the day after the nineth daily dose of drug in the lungs and spleens of the mice by serial dilution of the tissue homogenates on nutrient Middlebrook 7H11 agar plates (GIBCO-BRL, Gaithersburg, MD, USA). The plates were incubated at 37°C in ambient air for 4 weeks prior to the counting of viable *M. tuberculosis* colonies (cfu). The viable bacterial numbers were converted to logarithms, which were then evaluated by multiple-comparison analysis of variance by a one-way Dunnett test (SigmaStat software program). Differences were considered significant at the 95% level of confidence. Negative control mice remained untreated. An isoniazid control group, administered via oral gavage at 25 mg/kg/day, was included in each study. Each treatment group consisted of five mice. Five infected mice were killed at the start of treatment as pre-treatment controls. Drugs were administered daily by oral gavage.

Dose–response in the rapid in vivo screen

The *in vivo* activity of compound **5** (TAACF 118845) was examined in the rapid screening model as a repeat of the efficacy testing and to determine the minimal effective dose. Doses of 25, 100 and 300 mg/kg were tested using the same methods as in the initial *in vivo* test.

Statistical analysis

The viable counts were converted to logarithms, which were then evaluated by a one-way ANOVA followed by a multiple comparison analysis of variance by a one-way Tukey test (SigmaStat software program). Differences were considered significant at the 95% level of confidence.

Activity against non-replicating persistent M. tuberculosis

The activity of compound **5** against non-replicating *M. tuberculosis* was determined by measuring inhibitory activity under anaerobic conditions against *M. tuberculosis* adapted to low oxygen.[25] This Low Oxygen Recovery Assay (LORA) quantifies antibacterial activity by measuring the subsequent ability of a recombinant, containing a plasmid with an acetamidase promoter driving a bacterial luciferase gene, to produce a luminescent signal when placed back into an environment with ambient oxygen. The activity against the same reporter strain is also tested under aerobic conditions as follows. The microplate cultures with added drug were placed in an incubator under ambient gaseous conditions (5% CO_2-enriched air) for 7 days and 100 μL culture was transferred to white 96-well microtitre plates to determine the luminescence.

Results and discussion

The most potent compounds from our previous studies were subjected to the following set of tests: determination of MIC against different SDR strains of *M. tuberculosis*, MBC, oral bioavailability, MTD and *in vivo* efficacy in mice.

Table 2 shows the MIC values obtained against SDR strains of *M. tuberculosis*, including those resistant to isoniazid, rifampicin, thiacetazone, ethambutol, ciprofloxacin, kanamycin, ethionamide and *p*-aminosalicylic acid. MIC was also retested against a susceptible strain. In general, all the compounds showed good MIC values against resistant strains. Results showed that the most moderate activity was observed against the ciprofloxacin-resistant strain, and MICs ranged between 6.25 and 12.5 mg/L, although compound **9** revealed the poorest activity against an isoniazid-resistant strain, with an MIC value of 100 mg/L. The susceptibilities of rifampicin, thiacetazone, ethambutol and *p*-aminosalicylic acid-resistant strains can be considered comparable to those of $H_{37}Rv$, as was indicated by the ratios of MICs against resistant and non-resistant strains (Table 3), which were generally ~1. This indicates that there is a little, if any, cross-resistance with the current anti-TB drugs thereby supporting a novel mechanism of action. These results are promising for the development of new effective compounds against the growing number of drug-resistant strains. Only compound **9** showed resistance with the isoniazid-resistant strain, with a ratio >31.9. The reason for this finding is unknown.

Compound **5** was also active against MDR-TB strains, including strains with resistance to additional TB drugs and

Table 2. Determination of MIC against strains of SDR *M. tuberculosis*

Compound	MIC (mg/L)[a]								
	$H_{37}Rv$	INH-R	RIF-R	TAC-R	EMB-R	CIP-R	KAN-R	ETA-R	PAS-R
1	3.13	6.25	3.13	3.13	3.13	6.25	3.13	6.25	ND
2	3.13	6.25	3.13	ND	3.13	12.5	6.25	ND	ND
3	≤1.56	6.25	3.13	ND	≤1.56	12.5	6.25	ND	ND
4	≤1.56	ND[b]	≤1.56	0.2	ND	3.13	ND	3.13	≤1.56
5	0.78	1.56	≤0.39	ND	≤0.39	1.56	0.78	ND	ND
6	≤3.13	6.25	≤3.13	6.25	≤3.13	12.5	6.25	6.25	ND
7	≤3.13	ND	≤3.13	0.2	0.39	6.25	ND	6.25	≤3.13
8	3.13	6.25	3.13	3.13	3.13	6.25	3.13	3.13	ND
9	3.13	100	≤3.13	≤3.13	≤3.13	6.25	6.25	6.25	ND
10	6.25	6.25	≤3.13	≤3.13	ND	6.25	ND	6.25	ND

[a]INH-R, isoniazid-resistant strain; RIF-R, rifampicin-resistant strain; TAC-R, thiacetazone-resistant strain; EMB-R, ethambutol-resistant strain; CIP-R, ciprofloxacin-resistant strain; KAN-R, kanamycin-resistant strain; ETA-R, ethionamide-resistant strain; PAS-R, *p*-aminosalicylic-acid-resistant strain.
[b]ND, no data available.

Table 3. Ratios of MICs against resistant and non-resistant strains

Compound	Resistant strain MIC: $H_{37}Rv$ MIC[a]							
	INH-R	RIF-R	TAC-R	EMB-R	CIP-R	KAN-R	ETA-R	PAS-R
1	2.00	1.00	1.00	1.00	2.00	1.00	2.00	ND
2	2.00	1.00	ND	1.00	4.00	2.00	ND	ND
3	≥4.00	≥2.00	ND	ND	≥8.00	≥4.00	ND	ND
4	ND[b]	ND	≥0.13	ND	≥2.00	ND	≥2.00	ND
5	2.00	≤0.50	ND	≤0.50	2.00	1.00	ND	ND
6	≥2.00	ND	≥2.00	ND	≥4.00	≥2.0	≥2.0	ND
7	ND	ND	≥0.06	≥0.13	≥2.00	ND	≥2.00	ND
8	2.00	1.00	1.00	1.00	2.00	1.00	1.00	ND
9	31.9	≤1.00	≤1.00	≤1.00	2.00	2.00	2.00	ND
10	1.00	≤0.5	≤0.5	ND	1.00	ND	1.00	ND

[a]INH-R, isoniazid-resistant strain; RIF-R, rifampicin-resistant strain; TAC-R, thiacetazone-resistant strain; EMB-R, ethambutol-resistant strain; CIP-R, ciprofloxacin-resistant strain; KAN-R, kanamycin-resistant strain; ETA-R, ethionamide-resistant strain; PAS-R, *p*-aminosalicylic-acid-resistant strain. Ratios of MICs against resistant and non-resistant strains with values of 1–4 indicate activity against the resistant strain within experimental error.
[b]ND, no data available, or endpoints not attained in Table 2 data.

quinolones (Table 4). The activity on the drug-susceptible strain and the four MDR-TB strains varied only 2-fold, which is within the variation of MIC determinations. One of the strains tested was resistant to isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide, indicating that quinoxaline 1,4-di-*N*-oxides will maintain activity on MDR and poly drug-resistant strains. Such activity is particularly important in light of the recent reports of XDR strains of TB.[3–5,7–9,26] Overall, compound **5** was tested and found to be active on *M. tuberculosis* $H_{37}Rv$, *M. tuberculosis* strain Erdman, *M. bovis* strain BCG Montreal (also a human pathogen), four independent clinical isolates of *M. tuberculosis* from China with multiple resistance phenotypes and eight SDR strains of *M. tuberculosis* (Table 2).

Compound **5** is likely activated via bioreduction in bacteria, similar to the reduction observed for other substituted *N*-oxides.[27] Since PA-824, a nitroimidazole in clinical trials for treating TB,[28] is bioreduced to an active intermediate,[29–31] we tested the activity of compound **5** against an isogenic set of *M. bovis* strains with defined resistance to PA-824 (Table 5). PA-824 is bioreduced to an active form by a pathway involving the deazaflavin F_{420} cofactor-dependent glucose dehydrogenase (Fdg1)[29] and other cellular factors including molybdopterin, a cofactor for many oxido-reductase enzymes. Thus, loss of function of Fdg1 or loss of the ability to synthesize the F_{420} cofactor leads to resistance to PA-824 (Table 5). Compound **5** was active on all PA-824-resistant *M. bovis* strains tested, thus showing the lack of cross-resistance and supporting a different pathway of drug activation.

The antitubercular activity of compound **5** was concurrently tested against *M. tuberculosis* strain $H_{37}Rv$ using MABA and strain Rv containing the luciferase reporter, both under aerobic conditions; activity was comparable in both cases (Table 5). Niclosamide was included as another control compound that is structurally different from PA-824 and activated by a different

Table 4. Activity of compound **5** and moxifloxacin control (mg/L) on drug-resistant clinical isolates of *M. tuberculosis*

	Strain[a]				
	M10	M13	M14	M70	$H_{37}Rv$
DNA gyrase A mutation	none	Asp-94→Gly	none	Asp-94→Gly	none
INH[b]	R	R	R	R	S
RIF	R	S	R	R	S
STR	R	R	R	S	S
ETH	R	S	S	R	S
PZA	R	S	R	R	S
Moxifloxacin MIC (mg/L)	0.5	2	0.25	2	0.2
Compd. **5** MIC (mg/L)	1.25	1.25	0.625	1.25	0.625

[a]Strains M10, M13, M14 and M70 were obtained from Dr A. Cheng, Department of Microbiology, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, China.[21]
[b]INH, isoniazid; RIF, rifampicin; STR, streptomycin; ETH, ethionamide; PZA, pyrazinamide.

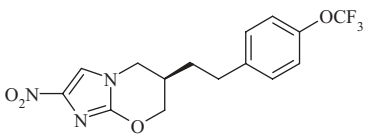
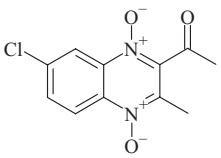
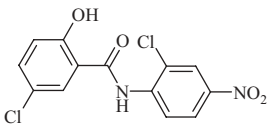
pathway. Table 5 also shows that compound **5** is equally active on growing bacteria and non-replicating persistent (NRP) bacteria adapted to low oxygen in the LORA test. In this test, activity was 1.65 and 1.21 μM (0.42 and 0.31 mg/L) against growing and NRP bacteria, respectively. This is another unique and important property of quinoxaline 1,4-di-*N*-oxides that may translate to a faster sterilization of infected tissues. The long continuation phase for the treatment of TB is believed to be in part due to the presence of non-replicating organisms that persist even in the presence of anti-tubercular drugs. PA-824 is an experimental nitroimidazole that is in Phase I clinical trials. PA-824 is bioreduced by *M. tuberculosis* to an active component but unlike compound **5**, PA-824 is only about 1/10 as active against NRP bacteria compared with aerobically growing cells.

The MBCs of several compounds against $H_{37}Rv$ were determined (Table 6). A compound is generally considered to be bactericidal if the ratio of MIC to MBC is ≤ 4;[32] so, compounds **1**, **5** and **8** could be considered to be bactericidal due to the low ratios obtained. On the other hand, compounds **3**, **6** and **9**, which showed higher MBC/MIC ratios for $H_{37}Rv$, may be less bactericidal.

Compound **5** was chosen for evaluation in *in vivo* assays. The MTD of compound **5** was determined by using an escalating dose of drug given to mice by oral gavage. No adverse effects or reactions were observed at a dose of 500 mg/kg in this test of acute, single dose toxicity. Compound **5** was orally bioavailable as assessed in the bioassay method[22] with an estimated blood level of ~5 mg/L at 30 min post-oral dosing of mice with 200 mg/kg.

Preliminary *in vivo* evaluation of compound **5** was made at a dose of 300 mg/kg in infected GKO C57BL/6 mice.[24] This compound afforded significant reductions of 2.7 and 2.82 $\log_{10}$ cfu in the lung and spleen tissues, respectively, versus the untreated controls. Compound **5** was bactericidal *in vivo* because the cfu present in the lung and spleen at the start of therapy (day 15) are

Table 5. Activity of compound **5** (μM) in the LORA and on *M. bovis* strains with defined resistance to PA-824, and *M. tuberculosis* strain $H_{37}Rv$ with and without a reporter gene

Compound	PA-824	Compd. **5**	Niclosamide
Structure			
LORA (RLU)[a]	1.40 (4.85)[b]	0.42 (1.65)	0.32 (0.99)
Aerobic (RLU)[c]	0.10 (0.35)	0.31 (1.21)	0.29 (0.87)
MTB $H_{37}Rv$[d]	0.032 (0.11)	0.44 (1.74)	0.64 (1.97)
BCG[e] WT	0.035 (0.12)	0.41 (1.63)	1.02 (3.13)
fgd (F_{420}-dependent)	>37.0 (>128)	0.81 (3.19)	0.53 (1.61)
moaA (molybdopterin synthesis)	>37.0 (>128)	0.50 (1.97)	0.61 (1.86)
fbi C (F_{420} synthesis)	>37.0 (>128)	0.47 (1.84)	0.51 (1.57)
moaD (molybdopterin synthesis)	>37.0 (>128)	0.98 (3.87)	0.62 (1.90)
pil 8[f] (Rv2627)	>37.0 (>128)	0.75 (2.96)	0.61 (1.87)
Comment	active *in vivo*; some activity in LORA	active *in vivo*; active in LORA	inactive *in vivo*; active in LORA

[a]LORA (RLU): low oxygen recovery assay using relative light units.
[b]Values are in mg/L with micromolar values given in parentheses.
[c]Aerobic (RLU): the same strain used in the LORA (MTB $H_{37}Rv$ containing a plasmid with an acetamidase promoter driving a bacterial luciferase gene) but incubated aerobically (5% CO_2 enriched air) for 7 days and 100 μL culture transferred to white 96-well microtitre plates for determination of luminescence.
[d]*M. tuberculosis* strain Rv measured in MABA.
[e]BCG: *M. bovis* strain BCG Montreal measured in MABA as were all other *M. bovis* strains.
[f]pil 8: Rv2627, function unknown.

Table 6. MBCs against $H_{37}Rv$ and SDR strains[a]

	MTB $H_{37}Rv$			INH-resistant			RIF-resistant		
Compound	MIC (mg/L)	MBC (mg/L)	MBC/MIC	MIC (mg/L)	MBC (mg/L)	MBC/MIC	MIC (mg/L)	MBC (mg/L)	MBC/MIC
1	3.13	6.25	2.00	3.13	3.13	1.0	3.13	3.13	1.0
3	≤1.56[b]	12.5	≥8.00	6.25	6.25	1.0	3.13	3.13	1.0
5	0.78	0.78	1.00	1.56	0.78	0.5	≤0.39	0.78	≥2.0
6	≤3.13	12.5	≥4.0	<3.13	12.5	≥4.0	6.25	6.25	1.0
8	3.13	6.25	2.0	6.25	6.25	1.0	3.13	3.13	1.0
9	≤3.13	12.5	≥4.0	100	50	0.5	≤3.13	6.25	≥2.0

[a]INH, isoniazid; RIF, rifampicin.
[b]On occasion, the MIC dilution series was not diluted down far enough to capture the MIC in a given test, thus giving limits of the differential.

lowered by greater than 2 logs (i.e. 99% killing) following 9 days of treatment. Visual inspection showed little lung pathology with a few small granulomas. Spleens appeared visibly normal and mice appeared normal and active. The efficacy of the compound was statistically better than controls ($P < 0.05$) and equivalent to the efficacy of isoniazid ($P < 0.05$). The protection shown by compound **5** is similar to clinically available compounds.[33] In this same *in vivo* run, isoniazid at 25 mg/kg/day gave a reduction in cfu of 3.1 and 4.37 $\log_{10}$ in the lungs and spleen, respectively, and was bactericidal.

In a second experiment, the dose–response of compound **5** *in vivo* was determined using the GKO mouse model at doses of 25, 100 and 300 mg/kg (Table 7). Compound **5** was active in the lung and spleen at 100 and 300 mg/kg ($P < 0.001$), respectively. At 25 mg/kg, it was active in the spleen ($P < 0.05$) but not statistically active in the lung. Activity at 300 mg/kg dosing was striking in that it lowered the cfu by 4.04 and 5.19 log cfu, respectively, in the lung and spleen. Bactericidal activity was detected at the higher doses of 100 and 300 mg/kg (Table 7). No clear toxicity was apparent in the first *in vivo* experiment, while

Table 7. Activity of compound **5** (TAACF 118845) in the mouse low-dose aerosol model

Sample (dose mg/kg)	Organ	Log cfu/organ (SD)	Log cfu decrease versus controls	Comments
Exp. 1				
untreated—d15	lung	6.81 (±0.09)		normal
	spleen	5.37 (±0.18)		normal
untreated—d24	lung	7.57 (±0.11)		normal
	spleen	6.57 (±0.17)		normal
Isoniazid (25)	lung	4.47 (±0.12)	3.10	normal
	spleen	2.20 (±0.45)	4.37	normal
118845 (300)	lung	4.88 (±0.21)	2.70	normal
	spleen	3.75 (±0.18)	2.82	normal
Exp. 2				
untreated—d15	lung	6.62 (±0.14)		normal
	spleen	4.42 (±0.52)		normal
untreated—d24	lung	7.76 (±0.10)		normal
	spleen	6.86 (±0.16)		normal
Isoniazid (25)	lung	4.43 (±0.06)	3.33	normal
	spleen	1.82 (±0.31)	5.04	1 mouse w/o cfu
118845 (25)	lung	6.97 (±0.17)	0.79	normal
	spleen	6.43 (±0.15)	0.43	normal
118845 (100)	lung	6.15 (±0.13)	1.61	normal
	spleen	5.62 (±0.17)	1.24	normal
118845 (300)	lung	1.82 (±0.95)	4.04	toxicity[a]
	spleen	1.67 (±0.55)	5.19	toxicity[b]

d15, day 15 post-infection; d24, day 24 post-infection.
[a]Data from only three mice. Two deaths due to apparent drug toxicity. Only one mouse with cfu, other two mice culture negative. Therapy stopped at 7 days. Slightly lethargic, hunched posture.
[b]Data from only three mice. Two deaths due to apparent drug toxicity. Two mice with cfu, other mouse culture negative. Therapy stopped at 7 days. Slightly lethargic, hunched posture.

in the dose–response experiment, some toxicity was observed at the highest dose; two mice in the 300 mg/kg group died during treatment, which was truncated to 7 days instead of the usual 9 days. No cfu was recovered from the lungs of two of the surviving mice or from the spleen of one of the surviving mice dosed at 300 mg/kg, indicating that the organs may have been sterilized. Preliminary studies indicate that both *in vitro* (cytotoxicity) and *in vivo* toxicity can be separated from the antitubercular activity.

In conclusion, an extended evaluation of the *in vitro* and *in vivo* antimycobacterial activities of quinoxaline 1,4-di-*N*-oxide derivatives was performed. All of them displayed good inhibitory activity against resistant strains and only compound **9** showed a significant resistance in an isoniazid-resistant strain. Compounds **1**, **5** and **8** can be considered to be bactericidal due to the low MBC/MIC ratios. Furthermore, compound **5** showed strong *in vivo* activity comparable to clinically used TB drugs, although a relatively high dose of compounds was required to obtain equivalent reductions in lung cfu. Overall, these data also suggest the importance of the chlorine group in position 7 of the benzene moiety. The activity of compound **5** is unique in that it is active on: (i) SDR strains; (ii) poly drug-resistant clinical isolates, including MDR-TB; and (iii) NRP mycobacteria. This latter activity may prove important for attaining cures in a shorter amount of time, since the presence of NRP bacteria is believed to be a major factor responsible for the prolonged nature of antitubercular therapy. Additional studies are planned to further assess the *in vivo* efficacy of compound **5** alone and in combination with other clinically used and antitubercular drugs in the standard mouse model of TB.

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