J Antimicrob Chemother 2010; **65**: 1424–1427
doi:10.1093/jac/dkq145 Advance Access publication 30 April 2010

Journal of Antimicrobial Chemotherapy

Killing of non-replicating *Mycobacterium tuberculosis* by 8-hydroxyquinoline

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Received 3 February 2010; returned 23 March 2010; revised 5 April 2010; accepted 6 April 2010

Objectives: To determine the effect of 8-hydroxyquinoline (8HQ) on non-replicating *Mycobacterium tuberculosis* (Mtb) in comparison with its reported effect on replicating Mtb.

Methods: The MIC of 8HQ for replicating H37Rv Mtb was determined by microdilution in 7H9 broth. Bactericidal activity was determined by exposing H37Rv Mtb to 8HQ for 4 days under conditions that otherwise allowed exponential replication (20% O_2, pH 6.6) and conditions under which replication was precluded: 1% O_2, pH 6.6; 20% O_2, pH 5.5; or 20% O_2, pH 5.5, 0.5 mM sodium nitrite. Serial dilutions were plated on 7H11 agar to quantify cfu. Frequency of resistance (FOR) was determined with $>10^9$ bacteria plated on 7H9 agar plates containing 2× MIC 8HQ.

Results: 8HQ was active against replicating Mtb (MIC 2.5 μM, 0.36 mg/L). Under both replicating and non-replicating conditions, cfu were reduced in 4 days by $\geq$5 $\log_{10}$ at the highest concentration tested (10 μM). Bactericidal activity was maximal at low pH, where 8HQ reduced cfu by 1–1.5 $\log_{10}$ at 1 μM. We were unable to recover any 8HQ-resistant colonies.

Conclusions: This study demonstrates that 8HQ has bactericidal activity of comparable potency against non-replicating and replicating Mtb, a property not observed for anti-infective agents currently approved for treatment of tuberculosis, and a very low FOR. Drugs with these properties are urgently needed to shorten the course of treatment for both active and latent tuberculosis.

Keywords: *M. tuberculosis*, TB, hydroxyquinolines

Introduction

Non-replicating populations of *Mycobacterium tuberculosis* (Mtb) are phenotypically more tolerant to most anti-infective agents than replicating Mtb. This is thought to be one reason why cure of tuberculosis requires prolonged treatment. Latent tuberculosis is usually treated with isoniazid, which is inactive against non-replicating Mtb *in vitro*, and treatment requires 6 months of daily administration. Treatment of clinically active tuberculosis also requires at least 6 months, even with multiple drugs to which the infecting strain is susceptible. Inability to complete such a prolonged, complex course of treatment allows the emergence and spread of drug-resistant tuberculosis, whose treatment may require 2 years. Treatment of drug-resistant tuberculosis is difficult in resource-poor settings and is becoming increasingly ineffective. Thus, there is intense interest in identifying compounds that can rapidly kill non-replicating Mtb.

8-Hydroxyquinolines (8HQs) are known for their antimicrobial activity. 8HQ itself (Figure 1) was inhibitory to replicating *Mycobacterium bovis* BCG (a non-pathogenic vaccine strain) (MIC 2 μM, 0.3 mg/L) and weakly bactericidal against BCG under non-replicating conditions (1 $\log_{10}$ reduction in cfu at 250 μM).[1] A mono-halogenated 8HQ, cloxyquin (5-chloroquin-8-ol), was active against drug-resistant Mtb, suggesting that it has a different mechanism of action from current anti-tuberculosis drugs.[2] A di-halogenated 8HQ, clioquinol (5-chloro-7-iodo-quinolin-8-ol), had a dramatic beneficial effect on tuberculosis in the guinea pig.[3] Clioquinol was widely used to treat diarrhoea from 1929 through to the 1970s and has been studied in Phase II trials for Alzheimer's disease.[4] Recently, >200 8HQ-like compounds were reported to inhibit replicating Mtb.[5] The most potent compound in the class was 8HQ itself. This led us to evaluate the activity of 8HQ against non-replicating Mtb. In addition, given that weak activity against BCG[1] usually

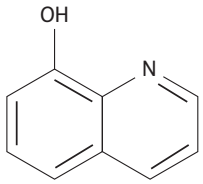

Figure 1. Structure of 8-hydroxyquinoline (8HQ).

foretells weak or even weaker activity against Mtb, it was important to seek independent confirmation of the report of potent activity against replicating Mtb.[5]

Materials and methods

Chemicals

Chemicals were from Sigma (St Louis, MO, USA). Stock solutions were prepared in DMSO.

Strains and media

Mtb H37Rv was cultivated in Middlebrook 7H9 at pH 6.6 with 0.2% glycerol, 0.5% BSA, 0.2% dextrose, 0.085% NaCl and 0.05% Tween 80 (7H9 medium) or on Middlebrook 7H11 plates containing 0.5% glycerol and 10% OADC (oleic acid/albumin/dextrose/catalase supplement) (Difco). Frequency of resistance (FOR) was assessed on 7H9 agar plates (7H9 medium, 1.5% Bacto agar and 10% OADC) containing 5 μM (0.72 mg/L) 8HQ. When indicated, 7H9 medium was adjusted to pH 5.5 with or without 0.5 mM sodium nitrite and an atmosphere of 1% O_2 was maintained in a BioSpherix box.

MIC determination

Mid-log phase cultures were washed with 7H9 and centrifuged at 120 **g** for 10 min. Supernatants were used as single cell suspensions and were adjusted to an optical density at 580 nm (OD_{580}) of 0.01 ($\sim 5\times10^6$ cfu/mL) in 7H9 and exposed to 2-fold dilutions of 8HQ in a final volume of 200 μL in 96-well microtitre plates. Each concentration was tested in triplicate in three independent experiments. Nickel chloride, magnesium sulphate, copper sulphate, zinc sulphate or ferric chloride (each 50 μM) were added where specified. After 2 weeks at 37°C, each well was resuspended and OD_{580} was measured using a microtitre plate reader. The MIC was defined as the lowest concentration at which there was no increase in OD beyond the starting value of 0.01.

Bactericidal assays

Single cell suspensions at an OD_{580} of 0.01 were dispensed into wells containing 0, 0.1, 1.0, 3.3 or 10 μM 8HQ at 37°C at: 20% O_2, pH 6.6; 1% O_2, pH 6.6; 20% O_2, pH 5.5; or 20% O_2, pH 5.5, 0.5 mM sodium nitrite. After 4 days, samples were serially diluted and plated on 7H11 agar. Colonies were counted after 2 weeks. DMSO (1% final) served as a vehicle control.

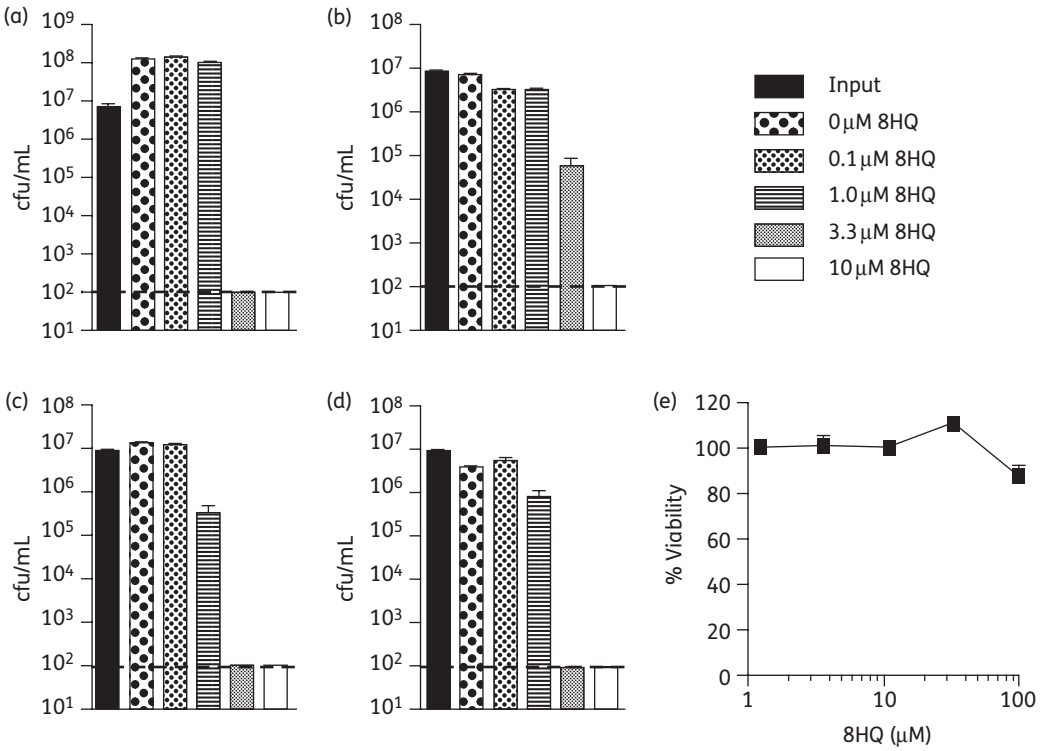

Figure 2. Effect of 8HQ on the viability of replicating and non-replicating Mtb and on Vero green monkey kidney cells. Mtb was exposed to indicated concentrations of 8HQ under normal growth conditions (a), 1% oxygen (b), pH 5.5 (c) and pH 5.5 plus 0.5 mM sodium nitrite (d) for 4 days at 37°C. Horizontal broken lines indicate the limit of detection. Results represent two independent experiments, each performed in triplicate. Vero green monkey kidney cells were exposed to varying concentrations of 8HQ for 2 days at 37°C and viability assessed microscopically and by an MTS reduction assay (e). Results represent three independent experiments, each performed in triplicate. Mean values and standard deviations are shown for all experiments.

FOR determination

Single cell suspensions containing 5.5×10^9 or 7.5×10^9 bacteria were plated onto 7H9 plates containing 2× MIC (5 μM) 8HQ, or 1×10^9 bacteria were plated onto 7H11 plates containing 1 mg/L rifampicin. Plates were incubated at 37°C for 5 weeks for determination of cfu.

Mammalian cell toxicity

Vero green monkey kidney cells (ATCC) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/L streptomycin, 0.5 mg/L gentamicin, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 mM HEPES. Confluent cells were trypsinized, counted and seeded in 200 μL in 96-well plates at 1×10^4 cells/well and incubated at 37°C for 2 days. The medium was removed, the cells washed once with PBS and medium containing 2% FBS and 8HQ was added (final DMSO, 0.25%). After 2 days at 37°C viability was assessed microscopically and by a tetrazolium (MTS) reduction assay (Promega).

Results and discussion

The MIC of 8HQ for H37Rv Mtb was 2.5 μM (0.36 mg/L), comparable to the reported MIC of 8HQ for BCG (0.3 mg/L) and the MIC of rifampicin (0.5 mg/L), the most potent of the approved anti-tuberculosis agents.[6] 8HQ was bactericidal to Mtb under replicating conditions, reducing cfu by $\geq$5 $\log_{10}$ (below the limit of detection) after 4 days of exposure to a concentration of 3.3 μM (Figure 2a).

To determine whether the activity of 8HQ is limited by the replicative state of Mtb, we tested 8HQ against Mtb under three conditions that markedly restrict its replication; low oxygen, mild acid and the generation of small fluxes of NO from mildly acidified nitrite. These conditions are physiologically relevant: Mtb-containing granulomas are hypoxic;[7,8] the Mtb-containing phagosome is acidic;[9] and pulmonary alveolar and lesional macrophages in Mtb-infected human patients express active inducible nitric oxide synthase.[10] Under low oxygen conditions, 8HQ potency was slightly decreased compared with replicating conditions. A concentration of 3.3 μM resulted in a decrease in viability of only 2 $\log_{10}$ after a 4 day exposure (Figure 2b). This same trend had been observed for 8HQ against BCG under the oxygen-limiting conditions of the Wayne model, where activity was decreased compared with replicating conditions.[1] However, at pH 5.5, the bactericidal activity of 8HQ was enhanced, with ~1–1.5 $\log_{10}$ reduction in cfu at only 1 μM (Figure 2c and d). The presence of 0.5 mM sodium nitrite had no additional effect on viability in the presence of 8HQ. No toxicity of 8HQ towards mammalian cells was evident at the range of concentrations tested (Figure 2e).

With the exception of rifampicin, most clinically approved anti-tuberculosis agents kill either replicating or non-replicating Mtb *in vitro*. For example, isoniazid kills replicating bacteria, while pyrazinamide activity is restricted to acidic conditions in which Mtb replicates poorly if at all. Rifampicin is more active against replicating than non-replicating Mtb. Killing of Mtb under both replicating and non-replicating conditions, with better killing under the non-replicating conditions, as seen with 8HQ, has not been reported for clinically approved anti-tuberculosis agents. However, this pattern of anti-mycobacterial activity was recently described for nitazoxanide, an anti-infective in clinical use for other infections.[11]

Using standard methods for evaluation of FOR,[11] we determined the FOR for rifampicin (1 mg/L) to be ~7.1×10^{-8}, in agreement with previous estimations (10^{-7}–10^{-8}). In contrast, we were unable to isolate mutants resistant to 8HQ at 5 μM (2× MIC) following exposure of 5.5×10^9 cfu of Mtb in each of two experiments and 7.5×10^9 cfu of Mtb in each of two additional experiments. Because no resistant colonies were detected, the FOR for 8HQ could not be estimated, but based on our inoculum it has to be $\leq1\times10^{-10}$.

The bidentate metal-chelating property of 8HQ would support a non-specific mechanism of action. However, addition of 50 μM iron, copper, magnesium, zinc or nickel had no impact on the MIC of 8HQ, suggesting that scavenging of essential metals from Mtb is probably not the primary mechanism (data not shown).

The ability of 8HQ to evade resistance suggests interactions with multiple molecular targets. As long as this does not reflect a propensity for host toxicity, it may be a desirable property for anti-infectives.[12] 8HQ showed no toxic effects on Vero green monkey kidney cells. Little information on human safety of 8HQ is available. While high doses of clioquinol can be neurotoxic in experimental animals and arguably in people, the non-halogenated 8HQ is expected to be less disruptive to membrane potential and could prove safer. As noted for nitazoxanide,[11] compounds with an ultra-low FOR active against both replicating and non-replicating Mtb via inhibition of new target(s) may be suitable for monotherapy of latent tuberculosis, and, if only by necessity, for treatment of active tuberculosis resistant to all other available drugs.

Acknowledgements

We thank Nyoun-Soo Kwon for initial screening results that identified related compounds, Steven Brickner for advice, Luiz Pedro S. de Carvalho for advice and review of the manuscript, and Ben Gold for discussions.

Funding

This work was supported by TB Drug Accelerator grant 42672 from the Bill and Melinda Gates Foundation and by the Milstein Program in Chemical Biology of Infectious Disease. The Department of Microbiology and Immunology is supported by the William Randolph Hearst Foundation.

Transparency declarations

None to declare.

References

1 Murugasu-Oei B, Dick T. *In vitro* activity of the chelating agents nitroxoline and oxine against *Mycobacterium bovis* BCG. *Int J Antimicrob Agents* 2001; **18**: 579–82.

2 Hongmanee P, Rukseree K, Buabut B *et al*. *In vitro* activities of cloxyquin (5-chloroquinolin-8-ol) against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2007; **51**: 1105–6.

3 Tison F. The remarkable effect of a combination of iodochloroxyquinoline with a subactive dose of streptomycin on experimental tuberculosis in guinea pigs. *Ann Inst Pasteur* 1952; **83**: 275–6.

4 Ritchie CW, Bush AI, Mackinnon A *et al*. Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Aβ amyloid deposition and

toxicity in Alzheimer disease: a pilot phase 2 clinical trial. *Arch Neurol* 2003; **60**: 1685–91.

5 Ananthan S, Faaleolea ER, Goldman RC *et al*. High-throughput screening for inhibitors of *Mycobacterium tuberculosis* H37Rv. *Tuberculosis* 2009; **89**: 334–53.

6 Duman N, Cevikbas A, Johansson C. The effects of rifampicin and fluoroquinolones on tubercle bacilli within human macrophages. *Int J Antimicrob Agents* 2004; **23**: 84–7.

7 Via LE, Lin PL, Ray SM *et al*. Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infect Immun* 2008; **76**: 2333–40.

8 Aly S, Wagner K, Keller C *et al*. Oxygen status of lung granulomas in *Mycobacterium tuberculosis*-infected mice. *J Pathol* 2006; **210**: 298–305.

9 MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science* 2003; **302**: 654–9.

10 Nathan C. Role of iNOS in human host defense. *Science* 2006; **312**: 1874–5.

11 de Carvalho LP, Lin G, Jiang X *et al*. Nitazoxanide kills replicating and nonreplicating *Mycobacterium tuberculosis* and evades resistance. *J Med Chem* 2009; **52**: 5789–92.

12 Silver LL. Multi-targeting by monotherapeutic antibacterials. *Nat Rev Drug Discov* 2007; **6**: 41–55.