AAC Journals.ASM.org

# Mechanism of Action of 5-Nitrothiophenes against *Mycobacterium tuberculosis*

**Ruben C. Hartkoorn,[a] Olga B. Ryabova,[b] Laurent R. Chiarelli,[c] Giovanna Riccardi,[c] Vadim Makarov,[b] Stewart T. Cole[a]**

Ecole Polytechnique Fédérale de Lausanne, Global Health Institute, Lausanne, Switzerland[a]; A. N. Bakh Institute of Biochemistry, Russian Academy of Science, Moscow, Russia[b]; Università degli Studi di Pavia, Dipartimento di Biologia e Biotecnologie, Pavia, Italy[c]

**On using the streptomycin-starved 18b strain as a model for nonreplicating *Mycobacterium tuberculosis*, we identified a 5-nitrothiophene compound as highly active but not cytotoxic. Mutants resistant to 5-nitrothiophenes were found be cross-resistant to the nitroimidazole PA-824 and unable to produce the $F_{420}$ cofactor. Furthermore, 5-nitrothiophenes were shown to be activated by the $F_{420}$-dependent nitroreductase Ddn and to release nitric oxide, a mechanism of action identical to that described for nitroimidazoles.**

It is widely considered that compounds that effectively target and kill persistent or nonreplicating *Mycobacterium tuberculosis* should have a major impact on antituberculosis chemotherapy. Current compounds with this so-called sterilizing activity include the frontline drugs rifampin and pyrazinamide as well as a number of compounds in clinical development, bedaquiline (TMC207), Q203, and the nitroimidazoles PA-824 and delamanid (OPC-67683) (1–4). While the development of these new drug candidates remains promising, the search for novel chemical entities effective against nonreplicating bacteria is vital in order to complete and sustain the tuberculosis drug pipeline.

Several *in vitro* models have been developed that enable mycobacteria to remain in nonreplicating states, which are thought to reproduce some of the characteristics of persistent *M. tuberculosis in vivo* (5). Characteristically, such bacteria are phenotypically resistant to drugs targeting the bacterial cell well (such as isoniazid and ethambutol), while also becoming less susceptible to sterilizing drugs like rifampin. Of the models described, the streptomycin-starved *M. tuberculosis* strain 18b (ss18b) model is arguably the most amenable for screening compounds against nonreplicating bacteria, as it requires minimal manipulation and can be used both *in vitro* and *in vivo* (5–7). Strain 18b is an *rrs* mutant of *M. tuberculosis* that depends on streptomycin for its growth; removal of streptomycin results in the bacterium being unable to replicate further, while maintaining viability (6, 7).

We used the resazurin reduction assay (6, 7) to screen for activity against ss18b in 519 compounds previously demonstrated to be effective against the actively growing H37Rv strain of *M. tuberculosis* (8, 9). Hits active on ss18b were subsequently tested to confirm activity against growing H37Rv and for cytotoxicity on the human liver carcinoma cell line HepG2 and the human lung epithelial cell line A549. One compound in particular, the nitrothiophene 2-(3-methylpiperidin-1-yl)-5-nitrothiophene (PubChem substance identifier [SID] 24814045), was found to be equipotent against replicating H37Rv and nonreplicating ss18b (MIC of 6.25 μg/ml for both). While compounds with such a profile are frequently cytotoxic, this compound displayed no cytotoxicity against HepG2 and A549 cells at 20 μg/ml (Table 1). 5-Nitrothiophenes were therefore deemed interesting for further investigation to determine their killing mechanisms against both growing and nonreplicating mycobacteria.

Six close analogues of the 5-nitrothiophene hit were synthesized (see the supplemental material) to confirm activity and to determine the role of the nitro group. Data (Table 1) revealed that activity is associated with the nitro group on C-5 of the thiophene ring (compounds 1 and 2). Exchange of the nitro by an acetyl and movement of the nitro group from C-5 to C-3 on the thiophene ring rendered the compound inactive (compounds 3 and 4). Introduction of a second nitro group on C-3 of the ring (compounds 5 and 6) did not improve activity and made the compounds mutagenic, as determined using the SOS chromotest (10). Compound 1 was the most active analogue and was used for further investigations into the mechanism of action.

To learn more about the mechanism of action of the 5-nitrothiophenes, we isolated H37Rv mutants on solid medium containing compound 1 (20 μg/ml). The frequency of isolation of resistant mutants was high, at $5 \times 10^6$, and of the 11 mutants selected, all displayed a phenotype highly resistant to compound 1 (MIC, >100 μg/ml), with no altered susceptibility to isoniazid, rifampin, or moxifloxacin.

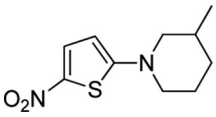
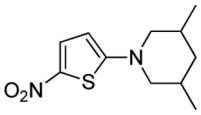
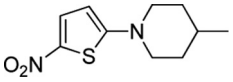
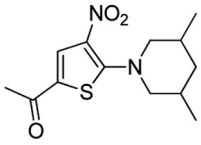
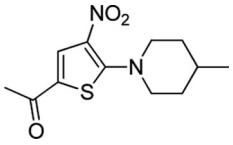
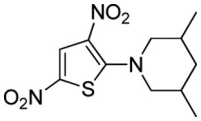
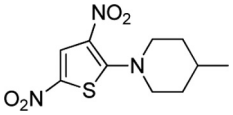
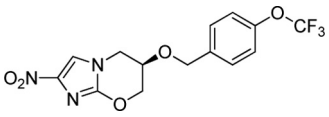
From a structural point of view, the nitrothiophene resembles the nitroimidazole part of PA-824 and delamanid. Interestingly, nitroimidazoles also have potent activity against nonreplicating bacteria, displaying comparable activity against strains H37Rv and ss18b. For these reasons we decided to investigate whether compound 1-resistant mutants were cross-resistant to PA-824. Data revealed that this was indeed the case as resistant mutants were fully cross-resistant to PA-824 (>100 μg/ml), suggesting that these two classes of compounds share a similar mechanism of activation or action (or both).

In *M. tuberculosis*, PA-824 has been elegantly shown to be a prodrug that is enzymatically reduced by the nonessential, deazaflavin ($F_{420}$)-dependent nitroreductase (Ddn or Rv3547) to re-

Received 11 December 2013 Returned for modification 20 January 2014
Accepted 11 February 2014
Published ahead of print 18 February 2014
Address correspondence to Stewart T. Cole, stewart.cole@epfl.ch.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.02693-13.

doi:10.1128/AAC.02693-13

**TABLE 1** Summary of activity of 5-nitrothiophenes

| Compound | Structure | Activity (MIC [μg/ml]) against: | | Cytotoxicity ($TD_{50}$[a] [μg/ml]) against: | | SOS chromotest |
|---|---|---|---|---|---|---|
| | | Strain H37Rv | ss18b | HepG2 cells | A549 cells | |
| PubChem SID 24814045 |  | 6.25 | 6.25 | >20 | >20 | Not done |
| 1 |  | 3.125 | 12.5 | 110 | 84 | Not mutagenic |
| 2 |  | 6.25 | 12.5 | 61 | 21 | Mutagenic |
| 3 |  | 100 | >100 | 41 | 29 | Not mutagenic |
| 4 |  | >100 | >100 | 61 | 65 | Not mutagenic |
| 5 |  | 6.25 | 6.25 | 72 | 75 | Mutagenic |
| 6 |  | 6.25 | 6.25 | 8.9 | 4 | Mutagenic |
| PA-824 |  | 0.078 | 0.156 | | | Not done |
| Rifampin | | 0.001 | 0.625 | >100 | >100 | Not mutagenic |

[a] $TD_{50}$, 50% toxic dose.

lease nitric oxide (NO) that nonspecifically damages and kills tubercle bacilli (4). While resistance to PA-824 through mutations in Ddn is infrequent, mutations preventing the proper biosynthesis of its cofactor $F_{420}$ are much more common (11, 12). The biosynthesis of $F_{420}$ in *M. tuberculosis* initially involves the formation of the intermediate, 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO), for which FbiC and other enzymes have been shown to be needed (11, 13). 2-Phospholactate is subsequently attached to FO, followed by the addition of predominantly 5 to 6 glutamate residues to form $F_{420}$-5 and $F_{420}$-6, a process involving FbiA and FbiB (11, 12, 14). $F_{420}$-5 and $F_{420}$-6 are the preferred cofactors for a number of $F_{420}$-dependent nitroreductases, and these cofactors are subsequently recycled to their reduced forms by an $F_{420}$-dependent glucose-6-phosphate dehydrogenase (FGD1) (11).

To determine the reason for the cross-resistance between PA-824 and the 5-nitrothiophenes, the *ddn* gene was sequenced in all 11 resistant mutants selected, but it was found to be wild type in all cases. Subsequently, the bacterial $F_{420}$ content was evaluated by fluorescence high-pressure liquid chromatography (HPLC) as described previously (12, 15). This analysis revealed that all the mutants were incapable of synthesizing $F_{420}$-5 and $F_{420}$-6. Of the 11 resistant mutants analyzed, six were possible *fbiC* mutants (being unable to produce FO), two mutants produced FO but no intermediates containing glutamate residues, and three mutants produced only short $F_{420}$ intermediates ($F_{420}$-2 to $F_{420}$-4), but no $F_{420}$-5 or $F_{420}$-6 (Fig. 1). Taken together, these results indicate that compound 1 is most probably converted by an $F_{420}$-5-dependent nitroreductase to release nitric oxide. Unlike results reported for PA-824, for which mutations in Ddn and FGD1 (20% of mutants) were found (11), all 11 mutants we analyzed were defective for $F_{420}$ production, although our sample size was smaller than that of the PA-824 study.

To determine if compound 1 exhibits its antituberculosis activity through the release of nitric oxide in a Ddn-dependent manner, this hypothesis was tested in *Mycobacterium smegmatis*. Intrinsically, *M. smegmatis* is resistant to compound 1, an

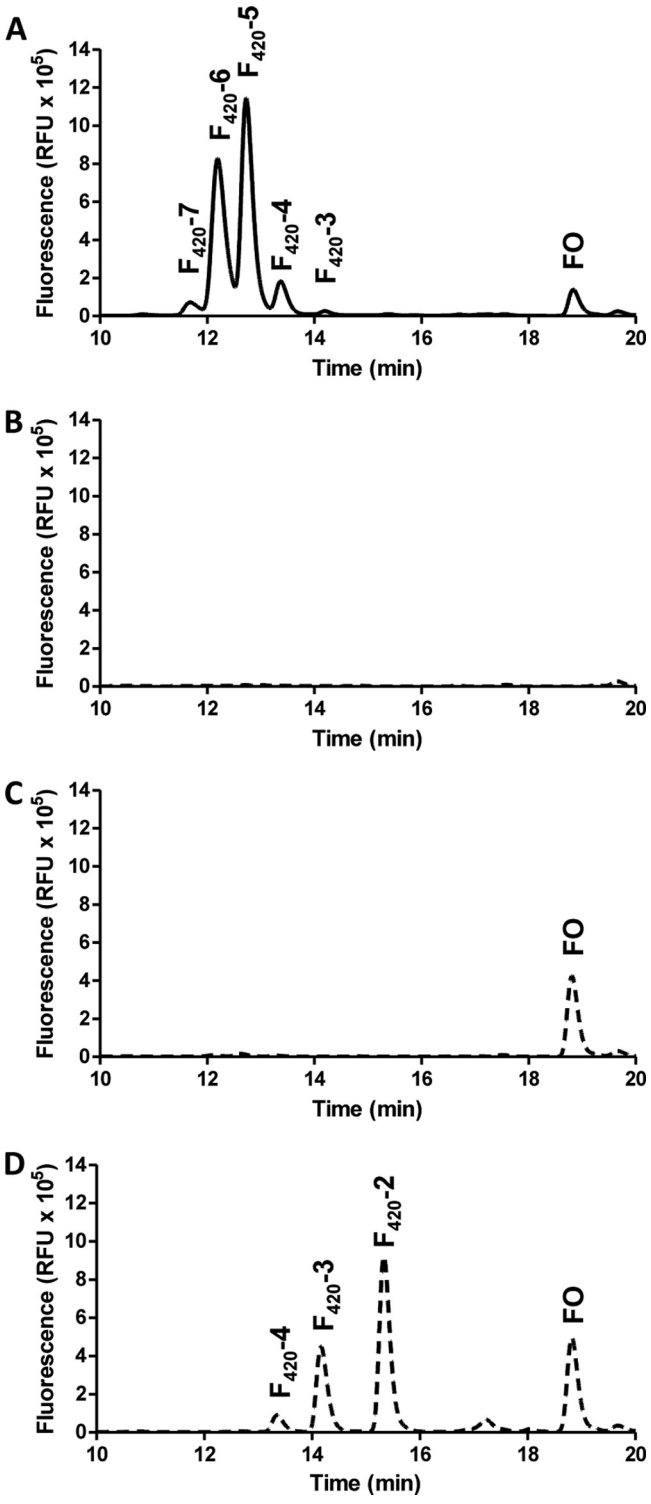



**FIG 1** Fluorescence chromatogram of the $F_{420}$ content of 5-nitrothiophene resistant H37Rv mutants. (A) H37Rv extract showing the elution of FO and $F_{420}$-3 to $F_{420}$-7. (B) One of six 5-nitrothiophene-resistant mutants that is unable to produce FO or any fluorescent $F_{420}$ intermediates. (C) One of two 5-nitrothiophene-resistant mutants that produced only FO. (D) One of three 5-nitrothiophene resistant mutants that produced short-chain $F_{420}$s but not $F_{420}$-5 or $F_{420}$-6 that are thought to be the active $F_{420}$ cofactors used in *M. tuberculosis*.

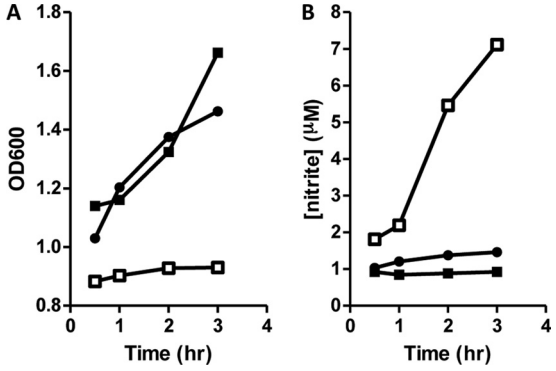



**FIG 2** 5-Nitrothiophenes are activated by Ddn to release nitric oxide. Bacterial growth curves (A) in the presence of 32 μg/ml of compound 1 show that wild-type *M. smegmatis* (solid circles) and *M. smegmatis* transformed with the control vector pSODIT (solid squares) grow in the presence of the compound. *M. smegmatis* transformed with pSODIT/*ddn* (open squares) is, however, sensitive to the action of compound 1, and unable to grow. Under the same conditions the concentration of nitrite (stable end product of NO) in the supernatant was measured (B). A time-dependent increase in nitrite levels, and therefore nitric oxide, was observed only in *M. smegmatis* transformed with pSODIT/*ddn* (and not in the controls lacking Ddn). OD600, optical density at 600 nm.

observation also reported for PA-824 (16). The recombinant expression of *ddn* (*Rv3547*) on a pSODIT plasmid in *M. smegmatis* was, however, found to make the bacterium susceptible to compound 1 (Fig. 2). Additionally, when measuring nitric oxide release following exposure of *M. smegmatis* to compound 1 (32 μg/ml) by the Griess reaction using nitrate reductase (17), we found that *M. smegmatis* expressing Ddn showed a time-dependent release of nitric oxide, while wild-type *M. smegmatis* and *M. smegmatis* containing the control vector released a basal amount of NO that did not increase over time (Fig. 2). These data confirm that compound 1 is a prodrug that gets enzymatically activated by *M. tuberculosis* Ddn to release nitric oxide, the active pharmacophore that nonspecifically kills both growing and nonreplicating bacteria. This does not, however, exclude the possibility that other $F_{420}$-dependent nitroreductases are able to bioactivate 5-nitrothiophenes.

Finally, to evaluate if compound 1 is a good scaffold for structure-based activity efforts, its metabolic stability was determined. Using both mouse and human liver microsomes, we found that the intrinsic clearance rates of compound 1 were very high, at 335 and 96 μl/min/mg protein, respectively. Similar results were obtained with compound 2. This high intrinsic clearance is a considerable obstacle for the development of 5-nitrothiophenes.

The data provided here demonstrate that 5-nitrothiophenes, such as compound 1, are active on both replicating and nonreplicating tubercle bacilli. The mechanism of action of these compounds was found to be similar to that of nitroimidazoles such as PA-824, most likely involving the reduction of the nitrothiophene by an $F_{420}$-dependent nitroreductase to release nitric oxide that nonspecifically kills *M. tuberculosis*, independently of growth rate. Indeed, loss of proper $F_{420}$ production by the bacterium renders the compounds inactive. The relative simplicity of the nitrothiophene compound makes it a potentially interesting scaffold for further development. However, since the compound has low metabolic stability and its activity is relatively weak compared to that of PA-824, and especially to that of delamanid (3), and resistant

mutants arise at a high frequency, we have decided not to pursue this series further.

## ACKNOWLEDGMENTS

The research leading to these results received funding from the European Community's Seventh Framework Programme (grant 260872).

We thank R. C. Goldman and the National Institute of Allergy and Infectious Diseases (NIAID) for making available the compounds of the Molecular Libraries Screening Center Network (MLSCN) for the initial screening performed here.

## REFERENCES

1. **Pethe K, Bifani P, Jang J, Kang S, Park S, Ahn S, Jiricek J, Jung J, Jeon HK, Cechetto J, Christophe T, Lee H, Kempf M, Jackson M, Lenaerts AJ, Pham H, Jones V, Seo MJ, Kim YM, Seo M, Seo JJ, Park D, Ko Y, Choi I, Kim R, Kim SY, Lim S, Yim S-A, Nam J, Kang H, Kwon H, Oh C-T, Cho Y, Jang Y, Kim J, Chua A, Tan BH, Nanjundappa MB, Rao SPS, Barnes WS, Wintjens R, Walker JR, Alonso S, Lee S, Kim J, Oh S, Oh T, Nehrbass U, Han S-J, No Z, Lee J, Brodin P, Cho SN, Nam K, Kim J.** 2013. Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. Nat. Med. **19:**1157–1160. http://dx.doi.org/10.1038/nm.3262.
2. **Andries K, Verhasselt P, Guillemont J, Gohlmann HWH, Neefs J-M, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V.** 2005. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science **307:**223–227. http://dx.doi.org/10.1126/science.1106753.
3. **Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, Sasaki H, Shimokawa Y, Komatsu M.** 2006. OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. PLoS Med. **3:**e466. http://dx.doi.org/10.1371/journal.pmed.0030466.
4. **Singh R, Manjunatha U, Boshoff HI, Ha YH, Niyomrattanakit P, Ledwidge R, Dowd CS, Lee IY, Kim P, Zhang L, Kang S, Keller TH, Jiricek J, Barry CE, 3rd.** 2008. PA-824 kills nonreplicating *Mycobacterium tuberculosis* by intracellular NO release. Science **322:**1392–1395. http://dx.doi.org/10.1126/science.1164571.
5. **Sala C, Hartkoorn RC.** 2011. Tuberculosis drugs: new candidates and how to find more. Future Microbiol. **6:**617–633. http://dx.doi.org/10.2217/fmb.11.46.
6. **Zhang M, Sala C, Hartkoorn RC, Dhar N, Mendoza-Losana A, Cole ST.** 2012. Streptomycin-starved *Mycobacterium tuberculosis* 18b, a drug discovery tool for latent tuberculosis. Antimicrob. Agents Chemother. **56:**5782–5789. http://dx.doi.org/10.1128/AAC.01125-12.
7. **Sala C, Dhar N, Hartkoorn RC, Zhang M, Ha YH, Schneider P, Cole ST.** 2010. Simple model for testing drugs against nonreplicating *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **54:**4150–4158. http://dx.doi.org/10.1128/AAC.00821-10.
8. **Maddry JA, Ananthan S, Goldman RC, Hobrath JV, Kwong CD, Maddox C, Rasmussen L, Reynolds RC, Secrist JA, 3rd, Sosa MI, White EL, Zhang W.** 2009. Antituberculosis activity of the molecular libraries screening center network library. Tuberculosis **89:**354–363. http://dx.doi.org/10.1016/j.tube.2009.07.006.
9. **Ananthan S, Faaleolea ER, Goldman RC, Hobrath JV, Kwong CD, Laughon BE, Maddry JA, Mehta A, Rasmussen L, Reynolds RC, Secrist JA, 3rd, Shindo N, Showe DN, Sosa MI, Suling WJ, White EL.** 2009. High-throughput screening for inhibitors of *Mycobacterium tuberculosis* H37Rv. Tuberculosis **89:**334–353. http://dx.doi.org/10.1016/j.tube.2009.05.008.
10. **Quillardet P, Huisman O, D'Ari R, Hofnung M.** 1982. SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K-12 to measure genotoxicity. Proc. Natl. Acad. Sci. U. S. A. **79:**5971–5975. http://dx.doi.org/10.1073/pnas.79.19.5971.
11. **Manjunatha UH, Boshoff H, Dowd CS, Zhang L, Albert TJ, Norton JE, Daniels L, Dick T, Pang SS, Barry CE, 3rd.** 2006. Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. U. S. A. **103:**431–436. http://dx.doi.org/10.1073/pnas.0508392103.
12. **Choi KP, Bair TB, Bae YM, Daniels L.** 2001. Use of transposon Tn5367 mutagenesis and a nitroimidazopyran-based selection system to demonstrate a requirement for fbiA and fbiB in coenzyme F(420) biosynthesis by *Mycobacterium bovis* BCG. J. Bacteriol. **183:**7058–7066. http://dx.doi.org/10.1128/JB.183.24.7058-7066.2001.
13. **Choi KP, Kendrick N, Daniels L.** 2002. Demonstration that fbiC is required by *Mycobacterium bovis* BCG for coenzyme F-420 and FO biosynthesis. J. Bacteriol. **184:**2420–2428. http://dx.doi.org/10.1128/JB.184.9.2420-2428.2002.
14. **Bair TB, Isabelle DW, Daniels L.** 2001. Structures of coenzyme F-420 in *Mycobacterium* species. Arch. Microbiol. **176:**37–43. http://dx.doi.org/10.1007/s002030100290.
15. **Gorris LGM, Vanderdrift C, Vogels GD.** 1988. Separation and quantification of cofactors from methanogenic bacteria by high-performance liquid-chromatography—optimum and routine analyses. J. Microbiol. Methods **8:**175–190. http://dx.doi.org/10.1016/0167-7012(88)90018-8.
16. **Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH, Anderson SW, Towell JA, Yuan Y, McMurray DN, Kreiswirth BN, Barry CE, Baker WR.** 2000. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature **405:**962–966. http://dx.doi.org/10.1038/35016103.
17. **Verdon CP, Burton BA, Prior RL.** 1995. Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by $NADP^+$ when the Griess reaction is used to assay for nitrite. Anal. Biochem. **224:**502–508. http://dx.doi.org/10.1006/abio.1995.1079.