ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2010, p. 4522–4525
0066-4804/10/$12.00 doi:10.1128/AAC.00422-10
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

Vol. 54, No. 10

Role of Mutations in Dihydrofolate Reductase DfrA (Rv2763c) and Thymidylate Synthase ThyA (Rv2764c) in *Mycobacterium tuberculosis* Drug Resistance

We would like to comment on a number of recent reports in this journal (6, 8, 12, 18) concerning *Mycobacterium tuberculosis* dihydrofolate reductase (DHFR), encoded by *dfrA* (*Rv2763c*). Around 36% of phenotypically *para*-aminosalicylic acid (PAS)-resistant *M. tuberculosis* strains harbor mutations in *thyA* (*Rv2764c*), which encodes a thymidylate synthase (20). In their effort to elucidate the remaining unknown resistance mechanism(s), Mathys et al. extended their sequence analysis to a number of additional genes, including *dfrA* (12). It was unclear whether the three *dfrA* mutations they identified in the PAS-resistant strains P-693 and P-3158 could contribute to PAS resistance on their own. Nonetheless, these findings are notable for two reasons.

First, isoniazid (INH) has been shown to inhibit *M. tuberculosis* DHFR *in vitro* (1). Whether the same holds true for ethionamide, which shares a number of common resistance mechanisms with INH, was not tested (J. Blanchard, personal communication). In any case, the clinical relevance of DHFR-mediated INH resistance remains enigmatic. To date, only Ho et al. have addressed this question, but they did not identify any *dfrA* mutations in a screen of 127 INH-resistant clinical isolates (8). Consequently, Mathys et al. remain the first to describe mutations in this target (12). However, given that isolates with mutated DHFR are members of a cluster with baseline INH resistance, the importance of these mutations with respect to INH resistance remains unclear.

Irrespective of their relevance in INH resistance, these *dfrA* mutations are noteworthy for a second reason. Contrary to previous wisdom, Forgacs et al. recently showed that *M. tuberculosis* is sensitive to the drug combination trimethoprim-sulfamethoxazole (TMP-SMX) (6, 18). DHFR is competitively inhibited by TMP, and consequently, mutations therein lead to resistance in a variety of organisms (9, 16, 19). The crystal structures of the wild-type *M. tuberculosis* DHFR in complex with NADPH alone and with NADPH combined with the inhibitor TMP, methotrexate, or an analogue of the antimalarial agent WR99210 have been solved (11). Interestingly, the wild-type [66]Ser, which is mutated to Cys in strain P-693 (12), interacts directly with NADPH via an H bond and a hydrophobic interaction. Similarly, the wild-type [54]Val, which is mutated to Ala in strain P-3158 (in addition to a second [110]Cys→Arg change), has a hydrophobic interaction with BR-WR99210 (11). Should these amino acid substitutions alter NADPH interactions or inhibitor binding, this raises the possibility that these PAS-resistant strains might also be TMP-SMX resistant. To investigate this possibility, phenotypic susceptibility testing of both isolates, as described by Forgacs et al., is warranted (6, 12). In addition, the treatment history of both patients should be reviewed for evidence of treatment with TMP-SMX.

We would now like to turn to the mutations in *M. tuberculosis thyA*, which were the focus of a number of studies (12, 13, 20). We note that the mutational loss of ThyA leads to TMP resistance in other organisms. Under these circumstances, tetrahydrofolate, the product of DHFR, is no longer required to regenerate N^5,N^{10}-methylenetetrahydrofolate, which would otherwise be oxidized by ThyA in the deoxyuridylate methylation process. Consequently, TMP-mediated competitive inhibition of DHFR can be tolerated by the cell (10, 13). In the case of *Staphylococcus aureus*, *thyA* mutations are also responsible for a small-colony-variant phenotype and are associated with hypermutability, which is thought to favor adaptation to long-term persistence and further antibiotic resistance (3–5, 14).

Taken together, this raises the possibility that *thyA* mutations that account for PAS resistance in *M. tuberculosis* might also lead to cross-resistance to TMP-SMX (6, 18). In fact, in their effort to show the importance of *thyA* mutations for PAS resistance in *M. tuberculosis*, Rengarajan et al. showed that transposon-disrupted *thyA* from *Mycobacterium bovis* BCG was not able to rescue a ThyA⁻ phenotype in *Escherichia coli* and restore TMP sensitivity (13). Moreover, they showed that these PAS-resistant *M. bovis* BCG strains harboring a transposon in *thyA* were resistant to the DHFR inhibitor 8710, which, unlike TMP on its own, is active against wild-type *M. bovis* BCG (13, 15). Should this hold true for TMP-SMX, this could have obvious implications for the utility of these antibiotics, comparable to the situation of isoniazid and ethionamide (17).

To assess the possible clinical impact and the degree of cross-resistance, we must quantify the proportion of laboratory and clinical TMP-SMX resistance which might be due to *thyA* mutations rather than mutation in DHFR or due to a third resistance mechanism involving the dihydropteroate synthase FolP1 (Rv3608c) (2, 7, 10). This also has practical implications for the experiment we suggested above. Mathys et al. should include P strains from their collection that carry the same double *thyA* mutations as P-693 and P-3158 but have a wild-type DHFR as the control (12).

We hope that these observations will spur a thorough preclinical examination of TMP-SMX, as called for by Young (18).

REFERENCES

1. **Argyrou, A., M. Vetting, B. Aladegbami, and J. Blanchard.** 2006. *Mycobacterium tuberculosis* dihydrofolate reductase is a target for isoniazid. Nat. Struct. Mol. Biol. **13**:408–413.
2. **Baca, A., R. Sirawaraporn, S. Turley, W. Sirawaraporn, and W. Hol.** 2000. Crystal structure of Mycobacterium tuberculosis 7,8-dihydropteroate synthase in complex with pterin monophosphate: new insight into the enzymatic mechanism and sulfa-drug action. J. Mol. Biol. **302**:1193–1212.
3. **Besier, S., A. Ludwig, K. Ohlsen, V. Brade, and T. Wichelhaus.** 2007. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of Staphylococcus aureus. Int. J. Med. Microbiol. **297**:217–225.
4. **Besier, S., J. Zander, B. C. Kahl, P. Kraiczy, V. Brade, and T. A. Wichelhaus.** 2008. The thymidine-dependent small-colony-variant phenotype is associated with hypermutability and antibiotic resistance in clinical Staphylococcus aureus isolates. Antimicrob. Agents Chemother. **52**:2183–2189.
5. **Chatterjee, I., A. Kriegeskorte, A. Fischer, S. Deiwick, N. Theimann, R. Proctor, G. Peters, M. Herrmann, and B. Kahl.** 2008. In vivo mutations of thymidylate synthase (encoded by thyA) are responsible for thymidine dependency in clinical small-colony variants of Staphylococcus aureus. J. Bacteriol. **190**:834–842.
6. **Forgacs, P., N. Wengenack, L. Hall, S. Zimmerman, M. Silverman, and G. Roberts.** 2009. Tuberculosis and trimethoprim-sulfamethoxazole. Antimicrob. Agents Chemother. **53**:4789–4793.
7. **Gengenbacher, M., T. Xu, P. Niyomrattanakit, G. Spraggon, and T. Dick.** 2008. Biochemical and structural characterization of the putative dihydropteroate synthase ortholog Rv1207 of Mycobacterium tuberculosis. FEMS Microbiol. Lett. **287**:128–135.
8. **Ho, Y., Y. Sun, S. Wong, and A. Lee.** 2009. Contribution of dfrA and inhA mutations to the detection of isoniazid-resistant *Mycobacterium tuberculosis* isolates. Antimicrob. Agents Chemother. **53**:4010–4012.
9. **Huovinen, P.** 2001. Resistance to trimethoprim-sulfamethoxazole. Clin. Infect. Dis. **32**:1608–1614.
10. **Huovinen, P., L. Sundström, G. Swedberg, and O. Sköld.** 1995. Tri-

methoprim and sulfonamide resistance. Antimicrob. Agents Chemother. **39:** 279–289.

11. **Li, R., R. Sirawaraporn, P. Chitnumsub, W. Sirawaraporn, J. Wooden, F. Athappilly, S. Turley, and W. G. J. Hol.** 2000. Three-dimensional structure of *M. tuberculosis* dihydrofolate reductase reveals opportunities for the design of novel tuberculosis drugs. J. Mol. Biol. **295:**307–323.

12. **Mathys, V., R. Wintjens, P. Lefevre, J. Bertout, A. Singhal, M. Kiass, N. Kurepina, X. Wang, B. Mathema, A. Baulard, B. Kreiswirth, and P. Bifani.** 2009. Molecular genetics of para-aminosalicylic acid resistance in clinical isolates and spontaneous mutants of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **53:**2100–2109.

13. **Rengarajan, J., C. M. Sassetti, V. Naroditskaya, A. Sloutsky, B. R. Bloom, and E. J. Rubin.** 2004. The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. Mol. Microbiol. **53:** 275–282.

14. **Sendi, P., and R. Proctor.** 2009. Staphylococcus aureus as an intracellular pathogen: the role of small colony variants. Trends Microbiol. **17:**54–58.

15. **Suling, W., R. Reynolds, E. Barrow, L. Wilson, J. Piper, and W. Barrow.** 1998. Susceptibilities of Mycobacterium tuberculosis and Mycobacterium avium complex to lipophilic deazapteridine derivatives, inhibitors of dihydrofolate reductase. J. Antimicrob. Chemother. **42:**811–815.

16. **Vickers, A., N. Potter, C. Fishwick, I. Chopra, and A. O'Neill.** 2009. Analysis of mutational resistance to trimethoprim in Staphylococcus aureus by genetic and structural modelling techniques. J. Antimicrob. Chemother. **63:** 1112–1117.

17. **Vilchèze, C., and W. J. Jacobs.** 2007. The mechanism of isoniazid killing: clarity through the scope of genetics. Annu. Rev. Microbiol. **61:**35–50.

18. **Young, L.** 2009. Reconsidering some approved antimicrobial agents for tuberculosis. Antimicrob. Agents Chemother. **53:**4577–4579.

19. **Yuvaniyama, J., P. Chitnumsub, S. Kamchonwongpaisan, J. Vanichtanankul, W. Sirawaraporn, P. Taylor, M. Walkinshaw, and Y. Yuthavong.** 2003. Insights into antifolate resistance from malarial DHFR-TS structures. Nat. Struct. Biol. **10:**357–365.

20. **Zhang, Z., Y. Zhao, Z. Li, H. Jia, Y. Liu, X. Chen, Z. Liu, B. Du, A. Xing, and Y. Ma.** 2007. Mutations in the thymidylate synthase gene is a major mechanism in the para-aminosalicylic acid resistance of *M. tuberculosis*. Zhonghua Jie He He Hu Xi Za Zhi **30:**683–685. (In Chinese.)

Claudio U. Köser*
Richard N. Veerapen-Pierce
David K. Summers
Department of Genetics
University of Cambridge
Cambridge, United Kingdom

John A. C. Archer
Computational Bioscience Research Center
King Abdullah University of Science and Technology
Thuwal, Kingdom of Saudi Arabia

*Phone: 44 (0)1223 333999
Fax: 44 (0)1223 333992
E-mail: cuk21@cam.ac.uk

Authors' Reply 1

Köser and colleagues propose a possible correlation between mutations in enzymes of the folate pathway, notably thymidine synthase A (*thyA*) and dihydrofolate reductase (*dfrA*), and cross-resistance to *para*-aminosalicylic acid (PAS), isoniazid (INH), and trimethoprim-sulfamethoxazole (TMP-SMX) in *Mycobacterium tuberculosis* (tuberculosis [TB]).

Recently, Argyrou and coworkers reported on possible inhibition of the DfrA protein by INH or an INH-NAD(P) adduct (1). Although the X-ray structures coupled with the genetic overexpression of the DfrA protein strongly suggest a plausible interaction of these components, more extensive genetic substitutions and molecular epidemiology are required to confirm the relevance of this observation clinically. To our knowledge, no correlations between INH resistance and mutations in DfrA have yet been found in clinical TB isolates (5). In contrast, a direct correlation has been reported between TMP-SMX resistance and DfrA mutations in clinical isolates of *S. aureus* (4); however, *M. tuberculosis* and *S. aureus* DfrA

proteins share only 49% sequence similarity and none of the important amino acid residues discussed here (7).

We recently reported on two clinical isolates encoding alterations to the *dfrA* gene; however, we could not determine whether the *dfrA* mutations alone contributed to PAS or INH resistance, as they additionally encoded mutations in the *thyA* and *katG* genes already known to correlate with PAS and INH resistance, respectively (2, 7–9). To directly address the independent contribution of *dfrA* mutations to PAS and INH resistance, further genetic studies involving isogenic pairs are required. Nevertheless, X-ray structure analysis showed that two of the three identified mutations in residues Ser[66] and Val[54] in DfrA, described for isolates P-693 and P-3158 (7), are involved in interactions with NADPH and BR-WR99210, respectively (6). In contrast, no interactions were found for residue Cys[110], as proposed by Köser et al. in their comment letter.

A closer examination of protein-ligand interactions implicate various other residues, where Ser[66] and Val[54] do not appear as major contributors in binding (Fig. 1). Ser[66] forms a hydrogen bond with a phosphate group situated at one extremity of NADPH, and Val[54] forms hydrophobic contacts with the phenyl ring of BR-WR99210 (6). Consequently, the contributions of Ser[66] and Val[54] are limited when the overall interactions of the protein and ligand are taken into account, i.e., a total of 20 hydrogen bonds and 25 hydrophobic contacts. Moreover, other X-ray complex structures show that Val[54] is not involved in binding interactions with methotrexate and trimethoprim (6), suggesting that Val[54] plays a secondary role in protein-ligand association. Clearly, it is difficult to predict the effect of a single point mutation on ligand binding properties, particularly when several residues are involved in the binding. Undoubtedly, as suggested by Köser et al., this point requires experimental investigations.

Köser and colleagues further question whether isolates P-693 and P-3158 are resistant to TMP-SMX based on a recent report by Forgacs and coworkers (3). This has not been tested and merits exploration.

REFERENCES

1. **Argyrou, A., M. Vetting, B. Aladegbami, and J. Blanchard.** 2006. *Mycobacterium tuberculosis* dihydrofolate reductase is a target for isoniazid. Nat. Struct. Mol. Biol. **13:**408–413.

2. **Banerjee, A., E. Dubnau, A. Quemard, V. Balasubramanian, K. S. Um, T. Wilson, D. Collins, G. de Lisle, and W. R. Jacobs, Jr.** 1994. inhA, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science **1263:**227–230.

3. **Forgacs, P., N. Wengenack, L. Hall, S. Zimmerman, M. Silverman, and G. Roberts.** 2009. Tuberculosis and trimethoprim-sulfamethoxazole. Antimicrob. Agents Chemother. **53:**4789–4793.

4. **Frey, K. M., M. N. Lombardo, D. L. Wright, and A. C. Anderson.** 2010. Towards the understanding of resistance mechanisms in clinically isolated trimethoprim-resistant, methicillin-resistant Staphylococcus aureus dihydrofolate reductase. J. Struct. Biol. **170:**93–97.

5. **Ho, Y., Y. Sun, S. Wong, and A. Lee.** 2009. Contribution of dfrA and inhA mutations to the detection of isoniazid-resistant *Mycobacterium tuberculosis* isolates. Antimicrob. Agents Chemother. **53:**4010–4012.

6. **Li, R., R. Sirawaraporn, P. Chitnumsub, W. Sirawaraporn, J. Wooden, F. Athappilly, S. Turley, and W. G. J. Hol.** 2000. Three-dimensional structure of *M. tuberculosis* dihydrofolate reductase reveals opportunities for the design of novel tuberculosis drugs. J. Mol. Biol. **295:**307–323.

7. **Mathys, V., R. Wintjens, P. Lefevre, J. Bertout, A. Singhal, M. Kiass, N. Kurepina, X. Wang, B. Mathema, A. Baulard, B. Kreiswirth, and P. Bifani.** 2009. Molecular genetics of para-aminosalicylic acid resistance in clinical isolates and spontaneous mutants of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **53:**2100–2109.

8. **Rengarajan, J., C. M. Sassetti, V. Naroditskaya, A. Sloutsky, B. R. Bloom, and E. J. Rubin.** 2004. The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. Mol. Microbiol. **53:**275–282.

9. **Zhang, Z., Y. Zhao, Z. Li, H. Jia, Y. Liu, X. Chen, Z. Liu, B. Du, A. Xing, and Y. Ma.** 2007. Mutations in the thymidylate synthase gene is a major mecha-

Key

- Ligand bond
- Non-ligand bond
- Hydrogen bond and its length

His 53 — Non-ligand residues involved in hydrophobic contact(s)

— Corresponding atoms involved in hydrophobic contact(s)

1DG7

FIG. 1. Plot of protein-ligand interactions in *M. tuberculosis* DfrA. Ndp and Wrb correspond to NADPH and the antimalarial agent BR-WR99210, respectively. Ser[66] and Val[54] residues are surrounded by green circles. The plot was generated with the program LIGPLOT. The protein code of the X-ray structure is 1DG7.

nism in the para-aminosalicylic acid resistance of *M. tuberculosis*. Zhonghua Jie He He Hu Xi Za Zhi **30**:683–685. (In Chinese.)

Pablo Bifani
Novartis Institute for Tropical Diseases
Singapore 138670, Singapore

René Wintjens
Laboratoire de Chimie Générale
Université Libre de Bruxelles
Brussels, Belgium

Vanessa Mathys
Laboratory of Molecular Pathology of Tuberculosis
Scientific Institute of Public Health
Brussels, Belgium

Barun Mathema
Tuberculosis Center
Public Health Research Institute
University of Medicine & Dentistry
Newark, New Jersey 07103

Alain Baulard
INSERM U629
Pasteur Institute
Lille, France

Phone: 6567223526
Fax: 6567222917
E-mail: Pablo.bifani@novartis.com

Authors' Reply 2

We thank Köser et al. for their letter, which was stimulated by our study and their own interest in mutations and in the genetics of resistance in *Mycobacterium tuberculosis* (3). They summarize a number of interesting recent findings raising the possibility that dihydrofolate reductase inhibition may represent an additional mechanism of action for INH and PAS in *Mycobacterium tuberculosis*.

We requested for the editor to make V. Mathys aware of Köser et al.'s requests that Mathys et al. review the medical records of their two patients with *dfrA* mutations and perform susceptibility testing to TMP-SMX. It may be worthwhile to test the susceptibility to trimethoprim alone or to a known effective inhibitor of *M. tuberculosis* DHFR (5, 6) if one wishes to look for resistance to DHFR inhibitors.

Köser et al. also wonder whether patients with tuberculosis treated with TMP-SMX, as part of their antituberculous regimen, might develop problems with a small-colony variant (SCV) of *M. tuberculosis*. SCV bacteria, associated primarily with cystic fibrosis (CF) and infected prosthetic devices, are associated with a high rate of persistent and recurrent infections. One of the most extensively studied small-colony variants is the SCV *Staphylococcus aureus*, isolated primarily from CF patients on long-term TMP-SMX prophylaxis. These organisms have an absent or inactive thymidylate synthase A enzyme and grow *in vitro* only in the presence of thymidine supplementation. They are able to survive and grow *in vivo* under "slime-like" conditions in patients with cystic fibrosis (and near-infected prosthetic devices), where presumably there is increased thymidine or thymine concentration (4) and where antibiotic penetration can be poor. It should be noted that SCV *S. aureus* can develop in CF patients also receiving other long-term antibiotics (4). In patients with cystic fibrosis, there is also a frequent problem with small-colony-variant *Pseudomonas aeruginosa*; 38% of CF patients who grew *Pseudomonas aeruginosa* had small-colony-variant *P. aeruginosa*, usually associated with long-term inhaled tobramycin or colistin (4). In other words, the small-colony variants found in cystic fibrosis

patients do not represent a specific shortcoming of TMP-SMX as an antibiotic. This complication would not be expected to occur with the prolonged use of TMP-SMX in other diseases. Nor has it happened in the extensive experience with using TMP-SMX long term for other prophylactic indications (e.g., urinary infections and *Pneumocystis jirovecii* in HIV patients) or treatment (e.g., atypical mycobacterial infections, Ellman's 1941 randomized noncontrolled study of the treatment of tuberculosis with sulfapyridine versus no drug, etc. [1]).

We concur with Köser et al. that additional research on different aspects of tuberculosis and TMP-SMX would be worthwhile. Köser et al. mention some of the literature on the possible inhibition of the tetrahydrofolate pathway by INH. We presume that inhibition of the enzymes in the mycobacterial tetrahydrofolate pathway is the main mechanism of action of TMP-SMX in *M. tuberculosis*, as in other bacteria. However, superficial similarities in the chemical structures of sulfonamides and PAS have been noted (2); there are, to a lesser extent, similarities to those of the pyridine and pyrimidine mycolic acid inhibitors (INH, pyrazinamide, and ethionamide). The converse of the possible inhibition of the tetrahydrofolate pathway by INH would be the possibility of inhibition of mycolic acid synthesis by tetrahydrofolate pathway inhibitors.

REFERENCES

1. **Ellman, P.** 1941. Investigation in the value of sulfapyridine in the treatment of pulmonary tuberculosis. Tubercle **22**:296–302.
2. **Mathys, V., R. Wintjens, P. Lefevre, J. Bertout, A. Singhal, M. Kiass, N. Kurepina, X. Wang, B. Mathema, A. Baulard, B. Kreiswirth, and P. Bifani.** 2009. Molecular genetics of para-aminosalicylic acid resistance in clinical isolates and spontaneous mutants of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **53**:2100–2109.
3. **Niemann, S., C. U. Köser, S. Gagneux, C. Plinke, S. Homolka, H. Bignell, R. J. Carter, R. K. Cheetham, A. Cox, N. A. Gormley, P. Kokko-Gonzales, L. J. Murray, R. Rigatti, V. P. Smith, F. P. Arends, H. S. Cox, G. Smith, and J. A. Archer.** 2009. Genomic diversity among drug sensitive and multidrug resistant isolates of Mycobacterium tuberculosis with identical DNA fingerprints. PLoS One **4**:e7407.
4. **Proctor, R. A., C. von Eiff, B. C. Kahl, K. Becker, P. McNamara, M. Herrmann, and G. Peters.** 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat. Rev. Microbiol. **4**:295–305.
5. **Rengarajan, J., C. M. Sassetti, V. Naroditskaya, A. Sloutsky, B. R. Bloom, and E. J. Rubin.** 2004. The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. Mol. Microbiol. **53**:275–282.
6. **Sendi, P., and R. Proctor.** 2009. Staphylococcus aureus as an intracellular pathogen: the role of small colony variants. Trends Microbiol. **17**:54–58.

Pierre Forgacs
Department of Research

Sarah K. Zimmerman
Department of Clinical Microbiology

Mark L. Silverman
Department of Anatomic Pathology
Lahey Clinic Medical Center
Burlington, Massachusetts 01805

Nancy L. Wengenack
Leslie Hall
Glenn D. Roberts
Department of Clinical Microbiology
Mayo Clinic
Rochester, Minnesota 55901

Phone: (781) 744-3017
Fax: (781) 744-5486
E-mail: pltcf@verizon.net

Ed. Note: Ho et al. (reference 8 in the comment letter) declined to respond.