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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).

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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.

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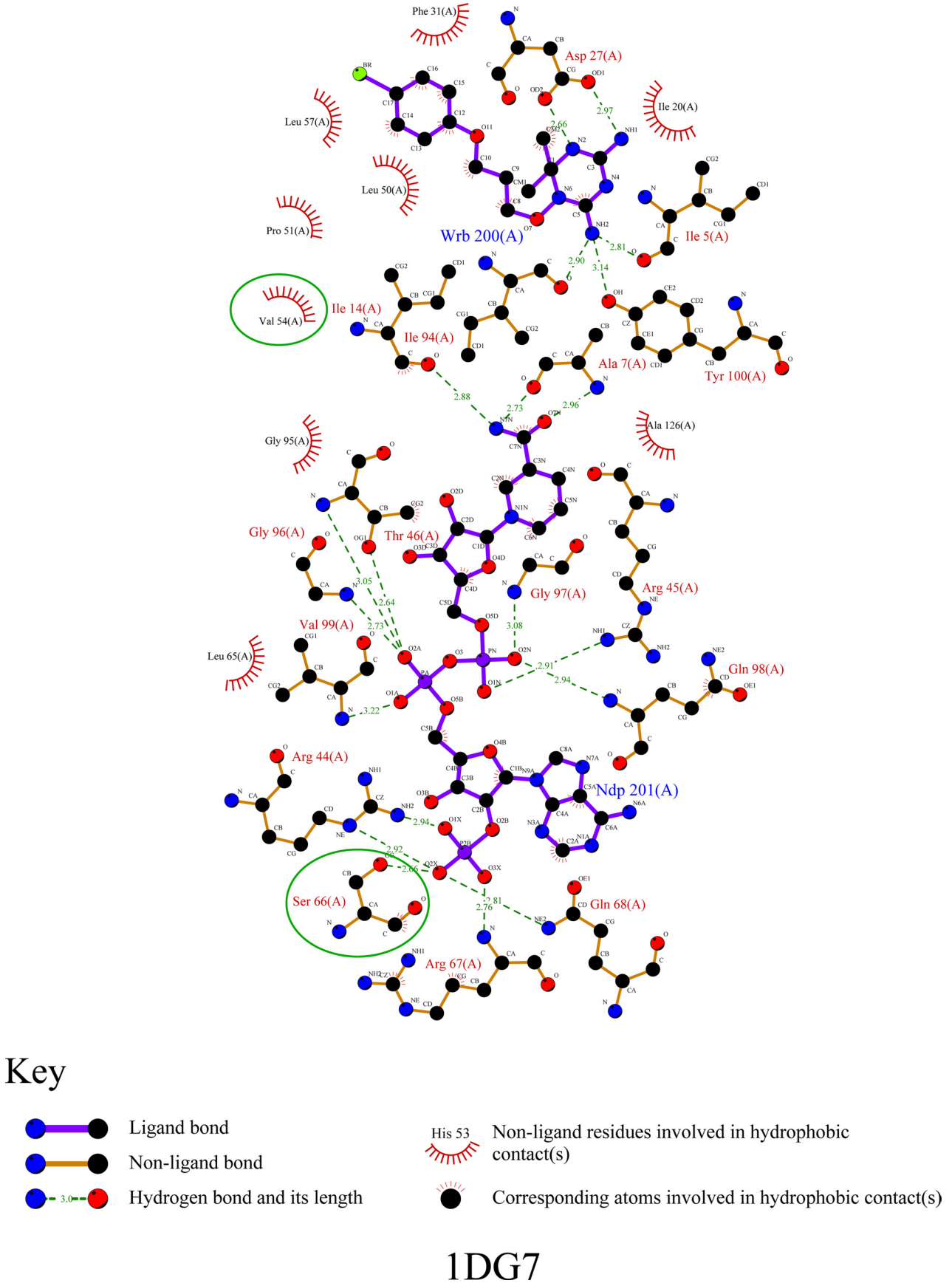

FIG. 1. Plot of protein-ligand interactions in *M. tuberculosis* DfrA. Ndp and Wrb correspond to NADPH and the antimalarial agent BR-WR99210, respectively. Ser^{66} and Val^{54} residues are surrounded by green circles. The plot was generated with the program LIGPLOT. The protein code of the X-ray structure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

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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.

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