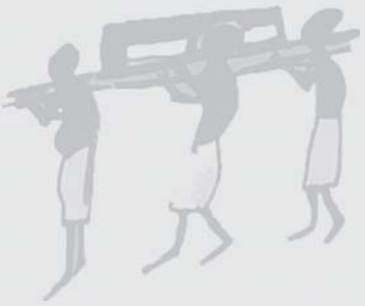

Chapter 6

In vitro synergy between linezolid and clarithromycin against *Mycobacterium tuberculosis*

M.S. Bolhuis, T. van der Laan, J.G.W. Kosterink,
T.S. van der Werf, D. van Soolingen, and J.W.C. Alffenaar

Eur Respir J. **2014** Sep; 44(3): 808–11

To the Editor:

Approximately 3% of new tuberculosis (TB) cases worldwide represent multidrug-resistant tuberculosis (MDR-TB) (1). In these MDR-TB cases, resistance of *Mycobacterium tuberculosis* against the otherwise effective rifampicin and isoniazid forces clinicians to diverge to other antimicrobial agents. Such treatment options include World Health Organization (WHO) group 5 drugs linezolid and clarithromycin (1). Linezolid shows excellent efficacy in the treatment of MDR-TB, but its use is often troubled by adverse events (2-4). Linezolid has shown *in vitro* bacteriostatic activity against *M. tuberculosis* and is also effective at achieving culture conversion in drug resistant patients (5). *In vitro* testing revealed that clarithromycin is not very active against *M. tuberculosis* since the minimal inhibitory concentrations (MIC) are relatively high. Clinical efficacy seems questionable, since MICs, as reported in literature, are significantly higher than achievable serum peak levels *in vivo* (6). On the contrary, clarithromycin reaches adequate local concentrations in alveolar cells and in epithelial lining fluid, where most mycobacteria reside (7). Besides that lower clarithromycin MICs were observed by the Dutch National Mycobacteria Reference Laboratory (Bilthoven, the Netherlands; unpublished data).

Due to the limited number of new treatment options, optimizing existing treatment regimens is a conceivable option. Exploring synergy between tuberculosis drugs might help in improving treatment regimens. A study that investigated several anti-TB drugs, such as isoniazide, rifampin, and/or ethambutol, but not linezolid, revealed *in vitro* synergistic activity with clarithromycin against *M. tuberculosis* (8). In this study, we aim to investigate the possible *in vitro* synergy between linezolid and clarithromycin in *Mycobacterium tuberculosis* isolates obtained from multidrug-resistant, resistant, and drug-susceptible TB patients.

We randomly selected a panel of 24 *M. tuberculosis* isolates from the strain collection of the Tuberculosis Reference Laboratory of the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands). The selected collection consisted of 13 multidrug-resistant, five drug-sensitive and six mono-resistant *M. tuberculosis* isolates. Drug susceptibility testing (DST) was performed using two methods: the absolute concentration method (ACM) and a Mycobacteria Growth indicator tube (MGIT) 960 system (9, 10).

For the ACM, we used a sterilized Middlebrook 7H10 agar of pH 6.6 supplemented with oleic-acid-dextrose-catalase (both Becton Dickinson and Company, Sparks, MD) and varying concentrations of drugs (9). The plates were checked for mycobacterial growth after 4, 8, 12, 14, 16, 19, and 21 days. The plates were analyzed when the growth in the control well

without anti-TB drugs was considered sufficient, i.e. when colonies were clearly visible and countable. At this point of time, all wells were checked for growth inhibition. Growth inhibition was defined as less than 90% of the colonies of the control well.

Next to the ACM, the MGIT 960 system with EpiCenter TB eXiST software was used (Becton Dickinson and Company, Sparks, MD) (10). Each tube contained 0.8 ml of Bactec MGIT drug susceptibility supplement and 100 µl of the appropriate drug solution. Growth was monitored hourly. The tubes were analyzed when growth unit (GU) value of the growth control tube, containing a 1:100 dilution of the inocula, reached 400. Growth inhibition was defined as GU value <100.

The checkerboard method was used to study *in vitro* synergy between linezolid and clarithromycin (both Sigma Aldrich, St.Louis, MO, USA). Linezolid was added in concentrations between 0 – 0.5 µg/mL and clarithromycin with a range of 0 – 8 µg/mL as is shown in Figure 1. We calculated the lowest fractional inhibitory concentration (FIC) to determine synergy as follows: $(MIC_{linezolid,\ combination} / MIC_{linezolid,\ alone}) + (MIC_{clarithromycin,\ combination} / MIC_{clarithromycin,\ alone})$. Synergy was defined as a FIC ≤0.5, indifference as FIC >0.5 to 4, and antagonism as FIC >4 (11).

Of the selected *M. tuberculosis* isolates (n=24), synergy between clarithromycin and linezolid was determined for 74% by using the MGIT method and in 59% by using the ACM. The median (interquartile range, IQR) FIC was 0.37 (0.31 – 0.47) using the MGIT and 0.50 (0.38 – 0.75) using the ACM. The combination of drugs did not display antagonism in any of the isolates. A median checkerboard composed from all selected *M. tuberculosis* strains with clarithromycin and linezolid is shown in Figure 1. Synergy was observed in 77% of the MDR-TB isolates (n=13) using MGIT and in 46% in the ACM method. Combining clarithromycin and linezolid resulted in a median (IQR) FIC of 0.37 (0.32 – 0.37) using MGIT and 0.62 (0.375 – 1.0) using ACM in the MDR-TB isolates.

In conclusion, we observed synergy between clarithromycin and linezolid both with the MGIT and with the ACM method. This finding is in line with a previous observation that clarithromycin displayed synergy with isoniazide, rifampin, and/or ethambutol in *M. tuberculosis* (8).

Although the underlying mechanism is yet to be elucidated, it has been suggested that disorganization or disruption of the outer cell wall layer and the cytoplasmic membrane of the cell envelope by either clarithromycin or linezolid may play a role (8). This disruption

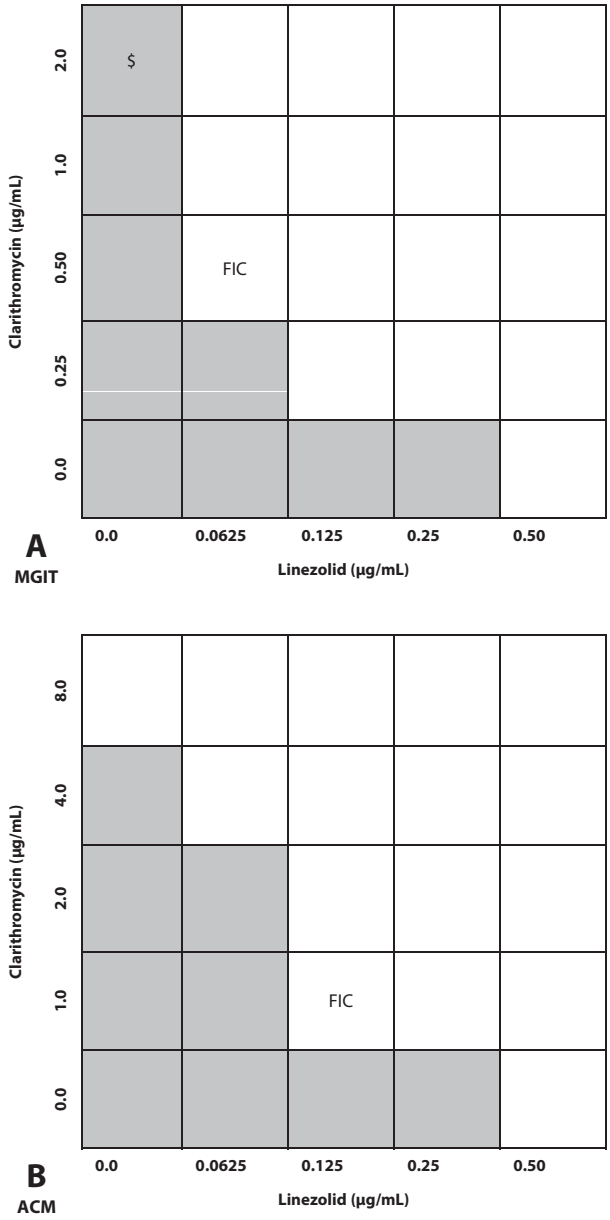

Figure 1 Schematic median checkerboard of *Mycobacteria tuberculosis* growth inhibition with varying concentrations of clarithromycin and/or linezolid using A. Mycobacteria Growth Indicator Tubes (MGIT, n=23) and B. Absolute Concentration Method (ACM, n=23).

Each cell represents the median result, i.e. growth or no-growth, of all tested isolates. Grey cells indicate growth, white cells indicate >90% less growth than controls. $ in order to calculate the fractional inhibitory concentrations (FIC) 4 µg/mL is used as the $MIC_{clarithromycin,\ alone}$. The lowest FIC cell is marked by 'FIC'. For example: the FIC of Figure 1B is ($MIC_{linezolid,\ combination}$ / $MIC_{linezolid,\ alone}$) + ($MIC_{clarithromycin,\ combination}$ / $MIC_{clarithromycin,\ alone}$) = (0.125 / 0.50) + (1.0 / 8.0) = 0.375.

might allow easier penetration by the other drug, resulting in the observed *in vitro* synergy. However, this hypothesis assumes that permeability is normally a limiting factor. Further research studying the underlying mechanism is needed and might also explain the fact that we observed *in vitro* synergy in the majority, but not all of the isolates. Although the majority of the isolates in this research displayed *in vitro* synergy when clarithromycin and linezolid were combined, it would be more interesting to determine or predict which isolates display synergy before applying these drugs in treatment regimens. Indeed, checkerboard experiments have not been validated or widely accepted for tailoring treatment in individual cases, and therefore cut-off values for FIC to deviate from the theoretical cut-off value of 1.0 have been employed (11). Consequently, the number of isolates displaying synergy might be under- or overestimated.

Previously, we showed that clarithromycin increases linezolid exposure by 44% in MDR-TB patients (12). The implications were summarized as follows: clarithromycin might be used as a booster to increase linezolid exposure, comparable to low-dose ritonavir and protease inhibitors; and the relatively cheap clarithromycin might reduce costs of treatment of the relatively expensive linezolid (13). The *in vitro* synergy we observed in this study further strenghtens the case for adding clarithromycin as a secondary drug to MDR-TB treatment regimens. The increased drug susceptibility of linezolid and clarithromycin in combination with the increased linezolid exposure might allow for further reduction of linezolid dosage, further reducing costs and adverse events. A prospective evaluation of MDR-TB patients receiving both drugs as a part of their treatment regimen is warranted to investigate efficacy and tolerability in real life. Furthemore, synergy testing should be performed with both other second-line TB drugs, and new TB drugs in the pipeline. Especially since the number of new MDR-TB drugs emerging from the pipeline in the next years is expected to be limited, drug resistance should be avoided at all costs. Optimizing treatment regimens through use of combinations that show synergy could be one strategy to avoid over exended use of new drugs. This is particularly important when considering the new World Health Organization post-2015 Strategy, which is based on the concept of TB elimination (14, 15).

To conclude, clarithromycin and linezolid display *in vitro* synergy in multidrug-resistant *M. tuberculosis* isolates. Due to the boosting effect with linezolid, low incidence of adverse effects, low costs, observed higher concentrations in lung tissue, and the *in vitro* synergy with linezolid and other antimicrobial drugs, the role of clarithromycin might become more important in future MDR-TB treatment.

ACKNOWLEDGMENTS

Reproduced with permission of the European Respiratory Society © *Eur Respir J September 2014 44:808-811; published ahead of print May 2, 2014, doi:10.1183/09031936.00041314.*

REFERENCES

1. **Falzon, D., E. Jaramillo, H. J. Schunemann, M. Arentz, M. Bauer, J. Bayona, L. Blanc, J. A. Caminero, C. L. Daley, C. Duncombe, C. Fitzpatrick, A. Gebhard, H. Getahun, M. Henkens, T. H. Holtz, J. Keravec, S. Keshavjee, A. J. Khan, R. Kulier, V. Leimane, C. Lienhardt, C. Lu, A. Mariandyshev, G. B. Migliori, F. Mirzayev, C. D. Mitnick, P. Nunn, G. Nwagboniwe, O. Oxlade, D. Palmero, P. Pavlinac, M. I. Quelapio, M. C. Raviglione, M. L. Rich, S. Royce, S. Rusch-Gerdes, A. Salakaia, R. Sarin, D. Sculier, F. Varaine, M. Vitoria, J. L. Walson, F. Wares, K. Weyer, R. A. White, and M. Zignol.** 2011. WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update. Eur. Respir. J. **38:**516-528.

2. **De Lorenzo, S., J. W. Alffenaar, G. Sotgiu, R. Centis, L. D'Ambrosio, S. Tiberi, M. S. Bolhuis, R. van Altena, P. Viggiani, A. Piana, A. Spanevello, and G. B. Migliori.** 2012. Efficacy and safety of meropenem/clavunate added to linezolid containing regimens in the treatment of M/XDR-TB. Eur. Respir. J. **41:**1386-1392.

3. **Migliori, G. B., B. Eker, M. D. Richardson, G. Sotgiu, J. P. Zellweger, A. Skrahina, J. Ortmann, E. Girardi, H. Hoffmann, G. Besozzi, N. Bevilacqua, D. Kirsten, R. Centis, C. Lange, and TBNET Study Group.** 2009. A retrospective TBNET assessment of linezolid safety, tolerability and efficacy in multidrug-resistant tuberculosis. Eur. Respir. J. **34:**387-393.

4. **Sotgiu, G., R. Centis, L. D'Ambrosio, J. Alffenaar, H. Anger, J. Caminero, P. Castiglia, S. De Lorenzo, G. Ferrara, W. Koh, G. Schecter, T. Shim, R. Singla, A. Skrahina, A. Spanevello, Z. Udwadia, M. Villar, E. Zampogna, J. Zellweger, A. Zumla, and G. B. Migliori.** 2012. Efficacy, safety and tolerability of linezolid containing regimens in treating MDR-TB and XDR-TB: systematic review and meta-analysis. Eur. Respir. J. **40:**1430-1442.

5. **Lee, M., J. Lee, M. W. Carroll, H. Choi, S. Min, T. Song, L. E. Via, L. C. Goldfeder, E. Kang, B. Jin, H. Park, H. Kwak, H. Kim, H. Jeon, I. Jeong, J. S. Joh, R. Y. Chen, K. N. Olivier, P. A. Shaw, D. Follmann, S. D. Song, J. Lee, D. Lee, C. T. Kim, V. Dartois, S. Park, S. Cho, and C. E. Barry.** 2012. Linezolid for Treatment of Chronic Extensively Drug-Resistant Tuberculosis. N. Engl. J. Med. **367:**1508-1518.

6. **Truffot-Pernot, C., N. Lounis, J. H. Grosset, and B. Ji.** 1995. Clarithromycin is inactive against Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **39:**2827-2828.

7. **Honeybourne, D., F. Kees, J. M. Andrews, D. Baldwin, and R. Wise.** 1994. The levels of clarithromycin and its 14-hydroxy metabolite in the lung. Eur. Respir. J. **7:**1275-1280.

8. **Cavalieri, S. J., J. R. Biehle, and W. E. Sanders Jr.** 1995. Synergistic activities of clarithromycin and antituberculous drugs against multidrug-resistant Mycobacterium tuberculosis. Antimicrob. Agents Chemother. **39:**1542-1545.

9. **van Klingeren, B., M. Dessens-Kroon, T. van der Laan, K. Kremer, and D. van Soolingen.** 2007. Drug susceptibility testing of Mycobacterium tuberculosis complex by use of a high-throughput, reproducible, absolute concentration method. J. Clin. Microbiol. **45:**2662-2668.

10. **Alffenaar, J. W., T. van der Laan, S. Simons, T. S. van der Werf, P. J. van de Kasteele, H. de Neeling, and D. van Soolingen.** 2011. Susceptibility of clinical Mycobacterium tuberculosis isolates to a potentially less toxic derivate of linezolid, PNU-100480. Antimicrob. Agents Chemother. **55:**1287-1289.

11. **Odds, F. C.** 2003. Synergy, antagonism, and what the chequerboard puts between them. J. Antimicrob. Chemother. **52:**1.

12. **Bolhuis, M. S., R. V. Altena, D. V. Soolingen, W. C. Lange, D. R. Uges, T. S. Werf, J. G. Kosterink, and J. W. Alffenaar.** 2013. Clarithromycin increases linezolid exposure in multidrug-resistant tuberculosis patients. Eur. Respir. J. **42:**1614-1621.

13. **Srivastava, S., C. A. Peloquin, G. Sotgiu, and G. B. Migliori.** 2013. Therapeutic drug management: is it the future of multidrug-resistant tuberculosis treatment? Eur. Respir. J. **42:**1449-1453.

14. **Diel, R., R. Loddenkemper, J. P. Zellweger, G. Sotgiu, L. D'Ambrosio, R. Centis, M. J. van der Werf, M. Dara, A. Detjen, P. Gondrie, L. Reichman, F. Blasi, G. B. Migliori, and European Forum for TB Innovation.** 2013. Old ideas to innovate tuberculosis control: preventive treatment to achieve elimination. Eur. Respir. J. **42:**785-801.

15. **D'Ambrosio, L., M. Dara, M. Tadolini, R. Centis, G. Sotgiu, M. J. van der Werf, M. Gaga, D. Cirillo, A. Spanevello, M. Raviglione, F. Blasi, G. B. Migliori, and on behalf of the European national programme representatives.** 2014. Tuberculosis elimination: theory and practice in Europe. Eur. Respir. J. **43:** 1410-1420.