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# *In Vitro* Synergy between Clofazimine and Amikacin in Treatment of Nontuberculous Mycobacterial Disease

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**Disease caused by nontuberculous mycobacteria (NTM) is increasing in frequency. The outcome of treatment for NTM lung disease is poor, particularly lung disease caused by *Mycobacterium simiae* and *M. abscessus*. Exploring synergy between active available drugs is a sensible way forward given the lack of new active drugs. We tested for synergy between amikacin and clofazimine, using standardized methods, in 564 consecutive clinical isolates identified as 21 species of rapidly growing mycobacteria, 16 clinical *M. avium* complex isolates, and 10 *M. simiae* isolates. Clofazimine and amikacin are each active *in vitro* against NTM; 97% ($n = 548$) of the rapid growers revealed MICs of clofazimine of ≤1 μg/ml, and 93% ($n = 524$) proved susceptible to amikacin. The combination showed significant synergistic activity in 56 of 68 (82%) eligible *M. abscessus* isolates, 4 of 5 *M. chelonae* isolates, and 1 *M. fortuitum* and 1 *M. cosmeticum* isolate, with 4- to 8-fold decreases in MICs to both drugs. Significant synergy could also be demonstrated against all *M. avium* complex and *M. simiae* isolates, with fractional inhibitory concentrations of <0.5. Clofazimine and amikacin show significant synergistic activity against both rapidly and slowly growing nontuberculous mycobacteria. The safety and tolerability of adding clofazimine to amikacin-containing regimens should be tested in clinical trials, and the results of susceptibility tests for these two compounds and their combination merit clinical validation. Synergy between clofazimine and other antibiotics with intracellular targets should be explored.**

Disease caused by nontuberculous mycobacteria (NTM) is increasing in frequency in many parts of the world, especially those where the incidence of tuberculosis is in decline (23). NTM are divided into slow growers (e.g., *Mycobacterium avium* complex, *M. kansasii*, and *M. simiae*) and rapid growers (e.g., *M. abscessus*, *M. chelonae*, and *M. fortuitum*). Within these groups, treatment regimens and treatment outcomes differ by species (8). In general, treatment outcomes are poor, but they are particularly so in lung disease caused by *M. simiae* and *M. abscessus* (8, 9, 10, 19). Unfortunately, there are few new active drugs available, so exploration of synergistic activity may provide means to develop better treatment regimens utilizing the most active combinations of existing drugs.

Clofazimine, a drug designed for tuberculosis treatment though mainly used in the treatment of leprosy (14), is among the active existing drugs that could be of use in itself and as an adjunctive drug. Clofazimine has been used as a replacement for rifampin, with similar outcomes, in lung disease caused by *M. avium* complex (MAC) (6). Recently, *in vitro* evidence for synergy between clofazimine and amikacin was recorded in rapidly growing NTM species, including *M. abscessus*, *M. chelonae*, and *M. fortuitum* (17). The potential of clofazimine combined with amikacin warrants investigation in both slow and rapid growers, as amikacin is an important cornerstone of therapy for disease caused by both groups of NTM (8). To further explore clofazimine-amikacin synergy, we tested a large series of NTM, both slow and rapid growers, using validated methods.

## MATERIALS AND METHODS

We tested for synergy between amikacin and clofazimine in 564 consecutive clinical isolates identified as rapidly growing mycobacteria (342 *M. abscessus* subsp. *abscessus*, 48 *M. abscessus* subsp. *bolletii*, 57 *M. chelonae*, 44 *M. fortuitum*, 14 *M. immunogenum*, 14 *M. mucogenicum*, 9 *M. porcinum*, 8 *M. peregrinum*, 5 *M. mageritense*, 4 *M. smegmatis*, 4 *M. conceptionense*, 3 *M. goodii*, 3 *M. senegalense*, 2 *M. septicum*, 2 *M. wolinskyi*, and 1 each of *M. cosmeticum*, *M. duvalii*, *M. phocaicum*, *M. neoaurum*, and *M. llatzerense*). This synergy was also assessed in 26 slowly growing NTM (16 clinical *M. avium* complex and 10 *M. simiae*). One strain per species per patient was eligible for analysis.

All NTM were identified by sequencing of the partial 16S rRNA and *rpoB* genes (1, 2). We applied the updated taxonomy of *M. abscessus* published by Leao and colleagues (12).

We performed drug susceptibility testing (DST) as advised by the Clinical and Laboratory Standards Institute (CLSI) (5), i.e., by broth microdilution in cation-adjusted Mueller-Hinton broth for the rapid growers (5) and by the broth macrodilution method, using the BacTec460 platform (5, 18), for the slow growers. For the rapid growers, synergy was determined by testing susceptibility to amikacin concentrations of 2, 8, 16, 32, and 64 μg/ml, clofazimine concentration of 0.5, 1, 2, and 4 μg/ml, and clofazimine concentrations of 2, 1, and 0.5 μg/ml combined with 2 μg/ml of amikacin. For amikacin, MICs of ≤16 were interpreted as susceptible, 32 μg/ml as intermediate, and ≥64 μg/ml as resistant (5). For clofazimine, no interpretative criteria exist. Synergy was defined as susceptibility to the combination of clofazimine and amikacin at less than half of the MICs to the individual compounds.

For the slow growers, MICs to the individual drugs were determined first. For clofazimine, concentrations of 0.06, 0.12, and 0.25 μg/ml were tested. For amikacin, concentrations of 2, 4, 8, and 16 μg/ml were tested. Subsequently, 2-, 4-, 8-, and 16-fold dilutions of the MICs were prepared and tested in combination. To assess synergy, we calculated fractional

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**TABLE 1** Clofazimine and amikacin susceptibility in rapidly growing mycobacteria

| Species | No. of isolates[a] | Clofazimine MIC (μg/ml) | | Amikacin MIC (μg/ml) | | No. eligible for synergy testing[b] | % of tests revealing synergy (no. of isolates with synergistic result/total no. eligible for testing) | Clofazimine-amikacin combination MICs (μg/ml) |
|---|---|---|---|---|---|---|---|---|
| | | $MIC_{50}$ | $MIC_{90}$ | $MIC_{50}$ | $MIC_{90}$ | | | |
| *M. abscessus* subsp. *abscessus* | 342 | ≤0.5 | 1.0 | 16.0 | 32.0 | 68 | 82 (56/68) | ≤0.5/2.0 ($MIC_{50}$), 1.0/2.0 ($MIC_{90}$) |
| *M. abscessus* subsp. *bolletii* | 48 | ≤0.5 | 1.0 | 8.0 | 32.0 | 9 | 67 (6/9) | ≤0.5/2.0 |
| *M. chelonae* | 57 | ≤0.5 | 1.0 | 8.0 | 16.0 | 5 | 80 (4/5) | ≤0.5/2.0 |
| *M. fortuitum* | 44 | ≤0.5 | ≤0.5 | ≤2.0 | ≤2.0 | 1 | 100 (1/1) | ≤0.5/2.0 |

[a] Only results for 491 isolates of the 3 most frequent species are given; see text for results for the remaining 73 isolates of 16 species.
[b] Isolates were eligible for synergy testing if the clofazimine MIC was >0.5 μg/ml and the amikacin MIC was >2.0 μg/ml (see the text).

inhibitory concentrations (FICs) using the formula FIC = ($MIC_a$ combination/$MIC_a$ alone) + ($MIC_b$ combination/$MIC_b$ alone), with clofazimine as drug *a* and amikacin as drug *b*. Synergy was defined as an FIC of ≤0.5 (13).

## RESULTS

**Rapid growers.** For the three major species *M. abscessus*, *M. chelonae*, and *M. fortuitum*, the $MIC_{50}$ and $MIC_{90}$ of clofazimine are given in Table 1. The single *M. cosmeticum* isolate had a MIC of clofazimine of 1 μg/ml. All isolates of the other species had MICs of ≤0.5 μg/ml; as a result, clofazimine-amikacin synergy could not be assessed for these species.

Amikacin susceptibility (MIC of ≤16 μg/ml) was measured in 93% (524/564) of the isolates tested (92% [314/342] for *M. abscessus* subsp. *abscessus*, 90% [43/48] for *M. abscessus* subsp. *bolletii*, 98% [56/57] for *M. chelonae*, 98% [43/44] for *M. fortuitum*, and 93% [68/73] for isolates of remaining species). There was no concordance between amikacin and clofazimine susceptibility results. The $MIC_{50}$s and $MIC_{90}$s for amikacin of *M. abscessus*, *M. chelonae*, and *M. fortuitum* are recorded in Table 1.

Eighty-four isolates (68 *M. abscessus* subsp. *abscessus*, 9 *M. abscessus* subsp. *bolletii*, 5 *M. chelonae*, 1 *M. fortuitum*, and 1 *M. cosmeticum*) demonstrated baseline clofazimine MICs of >0.5 μg/ml and amikacin MICs of >2 μg/ml and were eligible to assess clofazimine-amikacin synergy. Of these 84, 68 (81%) showed synergy. Synergy was noted for 56 of 68 *M. abscessus* subsp. *abscessus* isolates (85%), 6 of 9 *M. abscessus* subsp. *bolletii* isolates (67%) (all belonged to the former "*M. massiliense*"), 4 of 5 *M. chelonae* isolates, and the single *M. fortuitum* and *M. cosmeticum* isolates, with 4- to 8-fold decreases in MICs (see Table 1).

A total of 14 (10 *M. abscessus* subsp. *abscessus*, 2 *M. abscessus* subsp. *bolletii*, 1 *M. chelonae*, and 1 *M. fortuitum*) isolates that tested intermediate or resistant to amikacin alone (MIC of >16 mg/liter) proved susceptible (MIC of <16 mg/liter) after the addition of clofazimine.

**Slow growers.** The results for susceptibility to clofazimine, amikacin, and the clofazimine-amikacin combination in the 16 selected *M. avium* complex strains (6 *M. avium*, 7 *M. intracellulare*, and 3 *M. chimaera*) and 10 *M. simiae* strains are recorded in Table 2. The MICs did not differ between *M. avium*, *M. intracellulare*, and *M. chimaera* strains. Clofazimine and amikacin showed synergistic activity against all isolates, with 4- to 16-fold decreases in MICs to both drugs for individual isolates; we measured FICs of ≤0.5 for all isolates, with mean FICs of 0.22 for the *M. simiae* and 0.38 for the MAC isolates.

## DISCUSSION

Significant synergy between clofazimine and amikacin is observed across a range of nontuberculous *Mycobacterium* species, including both slow and rapid growers. This *in vitro* observation is most prominent for *M. abscessus*, MAC, and *M. simiae*, which are well-known causative agents of human disease. Disease caused by *M. abscessus* and *M. simiae* is notorious for the poor outcomes of drug treatment (8, 9, 10, 19). Amikacin plays a central role in the treatment of *M. abscessus* disease (8), and the combination with clofazimine may improve its efficacy. In contrast to a previous study in Taiwan (17), which applied similar methods to a smaller number of isolates, we did encounter *M. abscessus* isolates with MICs of ≥1 μg/ml to clofazimine and intermediate susceptibility or resistance to amikacin in which no synergy could be demonstrated. Hence, synergy should be tested, rather than assumed, prior to treatment.

In MAC disease, amikacin is mainly used in the first 2 to 3 months of treatment for moderate or severe disease (8); here, too, a combination with clofazimine may increase the antimycobacterial activity of the intensive phase of treatment. In *M. simiae* disease, the role of amikacin used to be limited, due to its limited *in vitro* activity (20). However, if that activity can be as enhanced by clofazimine, as our *in vitro* evidence suggests, there may be a future role for the amikacin-clofazimine combination in *M. simiae* disease. The outcome of treatment in *M. simiae* has been very poor, especially with the rifampin-ethambutol-macrolide regimen (8, 19). This has, in part, been related to the lack of synergistic activity between rifampin and ethambutol against *M. simiae* (20). Amikacin-clofazimine-based regimens may provide interesting

**TABLE 2** Clofazimine and amikacin susceptibilities in *M. avium* complex and *M. simiae* isolates

| Species | No. of isolates | Clofazimine MIC (μg/ml) | | Amikacin MIC (μg/ml) | | MIC in combination (μg/ml) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Clofazimine | | Amikacin | |
| | | $MIC_{50}$ | $MIC_{90}$ | $MIC_{50}$ | $MIC_{90}$ | $MIC_{50}$ | $MIC_{90}$ | $MIC_{50}$ | $MIC_{90}$ |
| MAC[a] | 16 | 0.12 | >0.25 | 8.0 | >16.0 | 0.03 | 0.06 | 2.0 | 4.0 |
| *M. simiae* | 10 | 0.12 | 0.25 | 16.0 | >16.0 | 0.015 | 0.03 | 2.0 | 4.0 |

[a] MAC, *Mycobacterium avium* complex (6 *M. avium*, 7 *M. intracellulare*, and 3 *M. chimaera*).

new leads and should be tested in clinical trials. However, the MICs and their interpretation for amikacin and, particularly, clofazimine still await clinical validation (21), and the relationship of MICs to achievable drug serum concentrations in patients and outcome of treatment remains to be established (22); this, too, could be part of the trial design. Despite the use of the synergistic combination of rifampin and ethambutol in MAC lung disease, the treatment outcome of current regimens is suboptimal (8, 22), and the *in vitro* synergy between clofazimine and amikacin is thus no guarantee for synergy and good outcome of treatment *in vivo*. It is also important to realize that in one trial of HIV-related disseminated *M. avium* disease, the use of clofazimine was associated with excess mortality (16). Hence, any new trial of clofazimine therapy should proceed with caution.

Clofazimine is currently registered for use in leprosy only, although it is also used to treat multidrug-resistant tuberculosis and, in some cases, NTM. The drug is not registered for NTM disease, and the production and dissemination of clofazimine are limited. In many settings, specific approval to acquire clofazimine for an individual patient has to be sought. Previously, more active analogs of clofazimine have been produced (14), and the synthesis of novel compounds in this class may help circumvent the limited availability of clofazimine and offer more-active alternatives in the long term.

A biological explanation for this synergistic activity is lacking. Clofazimine is thought to have cell wall-destabilizing properties (3, 4). This may allow increased influx of amikacin, possibly by attaching to clofazimine and using it as a "Trojan horse" (14). The synergistic activity of clofazimine may extend to other drugs with intracellular targets, including macrolides, fluoroquinolones, and rifamycins.

The synergistic activity of clofazimine and amikacin, as well as kanamycin, has been previously noted in mouse models of MAC disease (7, 15). Subsequent researchers suggested that the synergy *in vivo* may be overestimated and result partly from carryover of clofazimine from tissue samples to culture media, thereby inhibiting growth and, thus, bacterial load assessments (11). The carryover hypothesis was later refuted (14).

This study has three important limitations. The first is that interpretation of clofazimine MICs as susceptible or resistant has not been clinically validated (21). In fact, the clinical efficacy of clofazimine against rapid growers has never been evaluated and its efficacy against MAC has only been demonstrated in an uncontrolled trial (6). The second is the low number of *M. abscessus* subsp. *bolletii* isolates. As we were able to demonstrate clofazimine-amikacin synergy in a range of rapidly growing mycobacteria, as well as slowly growing mycobacteria, it is likely that this synergy is relevant to all *M. abscessus* subspecies and all MAC members. For the rapid growers, the test method probably resulted in an underestimation of the synergistic activity. As most isolates were already susceptible to either 0.5 μg/ml of clofazimine or 2 μg/ml of amikacin, synergy could not be detected; the combination of 0.5 μg/ml of clofazimine and 2 μg/ml of amikacin was the lowest concentration tested. And yet, it is likely that synergy is present at equal levels in those isolates; this, as well as its potential clinical relevance, warrants separate study.

In summary, clofazimine and amikacin show significant synergistic activity against a variety of NTM, including rapid and slow growers; it could be observed in all MAC, *M. simiae*, and *M. chelonae* isolates, 84% of *M. abscessus* isolates, and 1 of 2 *M. fortuitum* isolates. Therefore, the addition of clofazimine to amikacin-containing regimens should be tested in clinical trials. The activity of clofazimine, in itself, against NTM and the synergy with amikacin may warrant a "renaissance" of clofazimine, or of riminophenazines in the broader sense.

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