Title: The Non-Specific Effect of Mycograb on Amphotericin B Minimum Inhibitory
Concentration

Running Title: Non-Specific Effect of Mycograb on Amphotericin B MIC

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Abstract

Background: Mycograb C28Y is a recombinant human antibody fragment thought to target HSP-90 and potentiate amphotericin B. Absence of in vivo efficacy led us to reevaluate its in vitro activity. Methods: Interactions between AMB and Mycograb were investigated using a checkerboard design. Results: Addition of Mycograb or various unrelated proteins including human serum resulted in similar decreases in MIC of AMB. Conclusion: Potentiation of AMB by Mycograb appears to be a non-specific protein effect.
Continued high mortality, drug-drug interactions, and the narrow armamentarium available to combat fungal infections have led to an increasing interest in the development of therapeutic antibodies for the treatment of systemic fungal infections (9). Mycograb, a 28kd human recombinant antibody fragment thought to target cell wall-associated fungal heat-shock protein-90 (epitope - KILKVIRK) has been reported to synergize with amphotericin B (AMB) in vitro against a broad range of pathogenic yeast species (4, 6). Subsequent in vivo studies in an immune-normal murine model of systemic candidiasis demonstrated that a single dose of Mycograb (2 mg/kg) in combination with AMB improved both mean organ colony counts, and the number of negative biopsies observed (4). Furthermore, in a multinational Phase II clinical trial, Mycograb in combination with lipid-associated AMB was reported to improve the overall clinical response from 48 to 84% as compared to AMB monotherapy in patients with invasive candidiasis (7).

Manufacturing quality control concerns of Mycograb included structural inconsistencies between batches believed to be caused by autoaggregation, and it was hypothesized that the heterogeneity of the molecule was due to the presence of an unpaired cysteine at position 28 (3). Consequently, a modified form of Mycograb (Mycograb C28Y variant) was developed in which the cysteine at position 28 was changed to a tyrosine (3). Manufacturing quality test of Mycograb C28Y variant indicated that the heterogeneity of the compound was markedly reduced and antimicrobial evaluation demonstrated synergy with AMB similar to the original Mycograb. However, Mycograb C28Y lacked in vivo efficacy in a neutropenic murine candidiasis model (3). While evaluating the antifungal properties of unrelated proprietary antibodies, we unexpectedly observed in vitro potentiation of AMB similar to that previously reported for
Mycograb. In light of these findings we set out to re-evaluate the *in vitro* activity of Mycograb in comparison with a range of other proteins.

Antifungal susceptibility testing was performed by broth microdilution according to CLSI guidelines against two strains of *C. albicans* (ATCC 90028 and ATCC 24433) and *Aspergillus fumigatus* strain ATCC MYA3637 (1, 2). Combinations of AMB (USP, 1032007) and Mycograb C28Y (Lot UV 0012), were done in a checkerboard design with unrelated proteins including 2 murine IgG antibodies (courtesy of Marc Nasoff, The Genomics Institute of the Novartis Research Foundation, La Jolla, CA), human γ-globulin (Calbiochem, 345886), bovine serum albumin (Fisher, BP671-10), and human serum (Sigma, S7023, 115K89091) run in parallel to serve as protein controls. Minimum inhibitory concentration (MIC) was measured at 24 hours by visual inspection. Selected assays were repeated using a resazurin-based redox dye (R&D Systems, AR002) for a quantitative readout to eliminate scoring bias (data not shown).

Mycograb C28Y variant alone, up to 128 µg/ml, had no antifungal activity against either *C. albicans* or *A. fumigatus*. MICs of AMB alone ranged from 0.25 – 0.5 for *C. albicans* and 1 µg/ml for *A. fumigatus*. The addition of Mycograb C28Y variant (up to 128 µg/ml) caused a dose-dependent decrease of 2 to 3 dilution steps in the MIC of AMB in both organisms, in agreement with previous reports (3 - 5). However, the addition of unrelated proteins at 5 µg/ml, including gamma-globulin, bovine serum albumin, and non-related IgG murine antibodies also produced a 4-dilution step reduction in the MIC of AMB against *C. albicans* (ATCC 24433). Human serum displayed a paradoxical effect with a 3 - 5 step dilution reduction at concentrations up to 5%, and a 1 dilution step reduction at concentrations above 10%. The nonspecific protein
potentiation of AMB was also observed in the mould pathogen *A. fumigatus* using non-specific antibodies and human serum as protein controls.

We then asked whether Mycograb C28Y could decrease the MIC beyond the observed non-specific protein effect. As described earlier, the introduction of serum to RPMI 1640 displayed a paradoxical effect where human serum supplemented at 1% (16-fold) displayed a greater decrease in the MIC as compared to the 25% serum supplemented media (2-fold). In the presence of human serum at 1%, which caused a maximal 4 step decrease in the MIC of AMB, the addition of Mycograb C28Y variant up to 100 µg/ml had no additive effect on the activity of AMB against *C. albicans*, nor was an additive effect seen at 25% human serum. Our results demonstrate that small amounts of serum present in RPMI can potentiate the activity of AMB which is attenuated at higher concentrations of serum. This result was not predicted as the effect of serum on the activity of AMB reported in the literature is varied dependent upon the formulation of AMB, the fungal strains used, the percent serum tested, and type of media (5, 10, 11).

To independently validate our findings, similar experiments were performed at the Center for Medical Mycology, Case Western Reserve University. A modification of the CLSI microdilution assay was performed in checkerboard fashion against two strains of *C. albicans* (ATCC 90028, ATCC 24433) to quantify the antimicrobial activity of AMB in combination with various protein sources. This included human gamma globulin and serum albumin, the original Mycograb, Mycograb C28Y, and Aurograb, a similar recombinant antibody fragment designed to bind to an unrelated bacterial target (8). All protein sources tested in combination with AMB recorded a fractional inhibitory concentration index (FICI) of ≤ 0.5 indicating an additive to
synergistic relationship (Table 1). The non-specific, synergistic protein effect was not observed in combination studies with fluconazole or caspofungin (Table 1).

In conclusion, the antifungal potentiation of AMB by Mycograb in vitro appears to be a non-specific effect that can be reproduced by a wide range of unrelated proteins; however, the mechanism of the observed MIC shift of AMB is not currently understood. Our data do not explain the discrepancy in reported in vivo and clinical activity of the original Mycograb, while the C28Y variant lacked efficacy in a murine candidiasis model, but our results clearly demonstrate that in vitro testing of these constructs does not correlate with or predict in vivo efficacy (3, 7). These findings underline the requirement for rigorous controls when testing biological agents for direct antimicrobial activity in vitro.

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References


TABLE 1. Fraction inhibitory concentration index (FICI) of amphotericin B, caspofungin, and fluconazole in combination with Mycograb C28Y and various protein sources

<table>
<thead>
<tr>
<th>Tested article added</th>
<th>Fractional Inhibitory Concentration Index</th>
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<tbody>
<tr>
<td></td>
<td>AMB</td>
</tr>
<tr>
<td>Mycograb C28Y</td>
<td>0.27 ± 0.18</td>
</tr>
<tr>
<td>“Original” Mycograb</td>
<td>0.23 ± 0.12</td>
</tr>
<tr>
<td>Aurograb</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Human gamma-globulin</td>
<td>0.18 ± 0.07</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>0.15 ± 0.05</td>
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Results are shown as mean ± SD of 6 determinations (2 strains each assayed 3 times). A FICI of ≤0.5 is indicative of a synergistic interaction.