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Preclinical Assessment of the Efficacy of Mycograb, a Human Recombinant Antibody against Fungal HSP90

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Mycograb (*Neu*Tec Pharma plc) is a human genetically recombinant antibody against fungal heat shock protein 90 (HSP90). Antibody to HSP90 is closely associated with recovery in patients with invasive candidiasis who are receiving amphotericin B (AMB). Using in vitro assays developed for efficacy assessment of chemotherapeutic antifungal drugs, Mycograb showed activity against a wide range of yeast species (MICs against *Candida albicans* [fluconazole {FLC}-sensitive and FLC-resistant strains], *Candida krusei*, *Candida tropicalis*, *Candida glabrata*, and *Candida parapsilosis*, 128 to 256 μg/ml). Mycograb (4 or 8 μg/ml) showed synergy with AMB, the fractional inhibitory index being 0.09 to 0.31. Synergy was not evident with FLC, except for FLC-sensitive *C. albicans*. Murine kinetics showed that Mycograb at 2 mg/kg produced a maximum concentration of drug in serum of 4.7 μg/ml, a half-life at alpha phase of 3.75 min, a half-life at beta phase of 2.34 h, and an area under the concentration-time curve from 0 to *t* h of 155 μg · min/ml. Mycograb (2 mg/kg) alone produced significant improvement in murine candidiasis caused by each species: (i) a reduction (Scheffe's test, $P < 0.05$) in the mean organ colony count for the FLC-resistant strain of *C. albicans* (kidney, liver, and spleen), *C. krusei* (liver and spleen), *C. glabrata* (liver and spleen), *C. tropicalis* (kidney), and *C. parapsilosis* (kidney, liver, and spleen) and (ii) a statistically significant increase in the number of negative biopsy specimens (Fisher's exact test, $P < 0.05$) for *C. glabrata* (kidney), *C. tropicalis* (liver and spleen), and *C. parapsilosis* (liver). AMB (0.6 mg/kg) alone cleared the *C. tropicalis* infection but failed to clear infections caused by *C. albicans*, *C. krusei*, *C. glabrata*, or *C. parapsilosis*. Synergy with AMB, defined as an increase (Fisher's exact test, $P < 0.05$) in the number of negative biopsy specimens compared with those obtained using AMB alone, occurred with the FLC-resistant strain of *C. albicans* (kidney), *C. krusei* (spleen), *C. glabrata* (spleen), and *C. parapsilosis* (liver and spleen). Only by combining Mycograb with AMB was complete resolution of infection achieved for *C. albicans*, *C. krusei*, and *C. glabrata*.

Mortality from bloodstream infections due to *Candida* species remains as high as 35% (49), despite improvements in the range of antifungal drugs available (20, 55). A contributory factor has been the rising proportion of infections due to non-*Candida albicans Candida* species (22, 25). The intrinsic resistance of some of these species to drugs such as fluconazole (FLC), combined with acquired resistance in the commonest species, *C. albicans*, has resulted in increasing reports of clinically significant antifungal drug resistance (16, 20, 22, 24, 25). Combination therapy is therefore being suggested as a means of combating resistance and improving clinical outcome (24). Potential problems with this approach include antagonism between fungistatic and fungicidal drugs, indifference between drugs that have the same target, increased risk of side effects when several potentially toxic antifungal drugs are used in combination, and greater risk of undesirable interactions with other drugs such as immunosuppressive agents. Characteristics of an ideal partner in combination therapy include the ability to enhance the efficacy of another class of antifungal drug, a broad spectrum of activity against all clinically important *Candida* species, and intrinsic lack of toxicity and undesirable interactions with other drugs. Mycograb (*Neu*Tec Pharma plc) is a new class of antifungal drug, a human recombinant antibody directed against a novel target, which has been designed to work in combination with the best current antifungal therapeutics.

Numerous studies examining the antibody response to *C. albicans* in infected patients and animal models of the infection have demonstrated immunodominant antigens in the size range 45 to 52 kDa (2, 17, 39, 47, 48, 54, 60, 62, 71, 78). One of these antigens, at 48 kDa, was identified as enolase (34) and exploited as the basis of a diagnostic test (76). An antigen at 47 kDa has been identified as the carboxy fragment of *C. albicans* heat shock protein 90 (HSP90) (42, 58, 72). It is more abundant than, and clearly distinct from, enolase, as shown by the pattern of reactivity of an enolase-specific monoclonal antibody (18). This 47-kDa antigen is immunodominant in patients with AIDS (39) and chronic mucocutaneous candidiasis (39, 60). An antibody response to this antigen is significantly more common in patients with deep-seated candidiasis than those with superficial candidiasis ($P < 0.05$) (62) and has been associated with abdominal sepsis in intensive care patients (78). In patients with deep-seated, invasive candidiasis, a sustained antibody response to this antigen correlated closely with a good prognosis, whereas lack of or falling levels of antibody were associated with fatality (47, 48). Dissecting this poten-

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tially protective antibody response to the level of individual epitopes showed that it was primarily directed against the epitope NKILKVIRKNIVKK (1, 41, 45). Patient sera containing such an antibody, and both a mouse monoclonal antibody and human recombinant antibodies against this epitope, were therapeutically active in murine models of invasive candidiasis (40, 43). Homologous epitopes have been identified in a range of fungal species: *Candida parapsilosis*, *Torulopsis glabrata*, *Candida tropicalis*, *Candida krusei*, and *Aspergillus fumigatus* (9, 12, 26, 38, 44, 66). HSP90 plays a critical role in chaperoning cellular molecules and is essential for yeast viability (46, 58). The importance of HSP90 to the viability of *C. albicans* is shown by the failure of repeated attempts to generate a homozygous *C. albicans* Δhsp90/Δhsp90 null mutant (72). Total soluble protein extracts from exponentially growing *C. albicans* or *Saccharomyces cerevisiae* yield not only full-length HSP90 but also subfragments of 72 to 76 kDa and 47 kDa, which are the result of partial degradation within viable yeast cells (58). Examination of the antibody response in mice challenged with candidal protoplasts showed no evidence of an antibody response to HSP90 or its subfragments (61), indicating loss of this antigen family during removal of the yeast cell wall (75). This is consistent with immunoelectronmicroscopy studies showing partial localization in the cell wall (37). Likewise, surface-expressed HSP90 serves as an antigen in Chagas' disease, ascariasis, leishmaniasis, toxoplasmosis, and infection due to *Schistosoma mansoni* (13, 23, 27, 64, 70). HSP90 secretion has been demonstrated when vascular smooth muscle cells were exposed to oxidative stress (30). It has been postulated that antibodies to HSP90 convey protection against *Plasmodium falciparum* malaria (8). Immunization with a membrane fraction from *C. albicans* likely to contain HSP90 in combination with FLC protected mice against systemic infection (50).

These features make an antibody-based HSP90 inhibitor, replicating a naturally occurring antibody response to the NKILKVIRKNIVKK epitope of candidal HSP90, an obvious candidate for combination antifungal therapy. This work describes the preclinical assessment of the efficacy of such a drug, Mycograb, which has now entered a multinational, double-blind, placebo-controlled trial in patients with invasive candidiasis. Mycograb is a human recombinant antibody derived from the anti-HSP90 antibody cDNA of patients recently recovered from invasive candidiasis (41). It consists of the antigen-binding variable domains of antibody heavy and light chains linked together to create a recombinant protein which is expressed in *Escherichia coli* (7). It does not have an Fc component. Its synergy with anticandidal drugs was assessed in vitro by the fractional inhibitory concentration (FIC) and the FIC index (FICI) (15, 29, 79). In the case of amphotericin B (AMB) this was assessment was extended to a murine model of invasive candidiasis.

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MATERIALS AND METHODS

Yeasts. The yeasts examined were an FLC-sensitive strain of *C. albicans* (10), an FLC-resistant (MIC = 64 μg/ml) strain of *C. albicans* (36), an FLC-resistant (MIC = 100 μg/ml) strain of *C. krusei* (FA/157) (35), *C. tropicalis* National Collection of Pathogenic Fungi (NCPF) *C. parapsilosis* (NCPF 3104), a clinical isolate of *C. parapsilosis* from a case of candidal peritonitis, and *C. glabrata* (NCPF 3240). For short-term storage, isolates were grown on Sabouraud dextrose agar slopes (Oxoid, Basingstoke, United Kingdom) and stored at room temperature. NCPF strains were obtained from the NCPF and held by the Public Health Laboratory Service, London, United Kingdom. For long-term storage yeasts were stored at −70°C in a suspension of Sabouraud broth with glycerol (9:1, vol/vol).

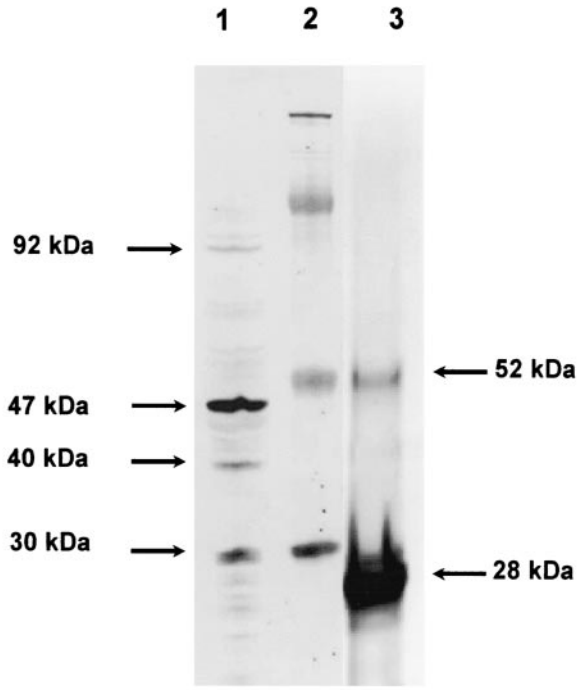

FIG. 1. Lane 1, *C. albicans* extract immunoblotted against Mycograb; lane 2, molecular mass markers (31, 52, 98, and 185 kDa; Invitrogen, Paisley, United Kingdom); lane 3, silver stain of purified Mycograb showing monomer (28 kDa) and dimer (52 kDa).

Antifungal agents. Mycograb is a polyhistidine-tagged purified antibody fragment produced, to current good manufacturing practice standards, by 1,000-liter batch fermentation of recombinant *E. coli*, purified by three-step chromatography, filter sterilized, and lyophilized. The resulting recombinant protein >99% pure and reconstituted by the addition of sterile water to a final concentration of 2 mg/ml. A silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 5 μg (Fig. 1, lane 3) demonstrated that it was 86.2% monomer (28 kDa) and 10.5% dimer (52 kDa), and the remaining 3.3% protein ran as a 30.5-kDa isoform which was indistinguishable from the monomer by mass spectrometry and reacted with the monoclonal specific to the histidine tag. Endotoxin levels, at 0.21 IU/mg, are 10-fold below the cutoff level for human use. Mycograb functionality was assessed by a capture enzyme-linked immunosorbent assay. A 96-well high-bind StreptaWell plate (Roche Diagnostics GmbH, Mannheim, Germany) was coated overnight at 4°C with 200 μl of 4-μg/ml biotin-NKILKVIRKNIVKK peptide in 0.1% (wt/vol) bovine serum albumin (BSA)–0.1% Tween-PBS (vol/vol). Plate wells were washed three times with vigorous shaking for 5 min with 200 μl of 0.1% (wt/vol) BSA–PBS (vol/vol). Two hundred microliters of Mycograb at 20 μg/ml in 10 mM ammonium acetate buffer (pH 9.0)–0.1% BSA was serially double diluted in 100 μl of 10 mM ammonium acetate buffer (pH 9.0)–0.1% BSA. Control sets of blank wells containing 10 mM ammonium acetate buffer (pH 9.0)–0.1% BSA were included. Plates were incubated for 1 h at room temperature, washed as above, overlaid with 200 μl of mouse monoclonal anti-His horseradish peroxidase conjugate (Sigma Aldrich, Gillingham, Dorset, United Kingdom) at 1:1,000 in 0.1% (wt/vol) BSA–0.1% PBS (vol/vol) Tween, incubated for 1 h at room temperature, and washed as above. The bound Mycograb was detected by addition of 200 μl of ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)]reagent (Roche Diagnostics, GmbH, Mannheim, Germany). The colorimetric development was read at 405

nM after 30 min of incubation. The activity of the product was specified in terms of its enzyme-linked immunosorbent assay activity, so that 1 μg of Mycograb always gave within 20% of an optical density of 1.75 and 0.0625 μg of Mycograb gave an optical density within 20%of 1.0.

AMB was purchased from Sigma Aldrich, and FLC was supplied by Pfizer (Sandwich, Kent, United Kingdom).

Gel electrophoresis. *C. albicans* (FLC-sensitive) cells were grown overnight at 37°C in Sabouraud dextrose broth, harvested, washed in saline, frozen, and subjected to pressure-induced cell disruption by an Xpress (LKB, Bromma, Sweden), as previously described (48). Immunoblots were prepared from the candidal extract run on the NuPAGE one-dimensional gel electrophoresis system (Invitrogen, Carlsbad, Calif.) and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia, Amersham, United Kingdom). Membranes were blocked with 5% (wt/vol) milk in PBS–0.1% Tween 20 (PBS-T) overnight, prior to application of either Mycograb (histidine tagged) at 5, 25, or 50 μg/ml or a rat anti-human HSP90 monoclonal antibody at a 1 in 1,000 dilution (Stress Gen Biotechnologies, Victoria, Canada), both in 0.1% milk PBS-T for 1 h. The Mycograb blots were developed with a rabbit anti-His polyclonal antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), used at a 1:300 dilution, having been preabsorbed with *C. albicans* (extract diluted 1:10,000 in PBS-T), followed by anti-rabbit immunoglobulin horseradish-peroxidase conjugate (Sigma Aldrich), 1:1,000 in PBS-T. The anti-human HSP90 blots were probed with anti-rat immunoglobulin horseradish peroxidase conjugate (Sigma Aldrich). Blots were developed with 3,3′-diaminobenzidine (Sigma Aldrich).

For two-dimensional gel electrophoresis, the candidal extract was added to 200 μl of a solution containing 9.8.M urea, 2% pharmalytes (pH 3 to 10; Amersham Pharmacia), 2% Triton X-100, and 10 mM dithiothreitol (DTT); mixed vigorously for 2 min; and used to hydrate an immobilized pH gradient (IPG; pH 4 to 7) 18-cm Immoboline gel strip (Amersham Pharmacia). Rehydrated strips were added to a MultiPhor II flatbed cooling plate (Amersham Pharmacia) and focused for 75,250 V/h. Strips were incubated for 10 min in 6 M urea–2% SDS–10 mM DTT–30% (vol/vol) glycerol followed by 6 M urea–2% SDS–30% glycerol–135 mM iodoacetamide and applied to an Excel gradient gel (8 to 18%). Separated proteins were visualized by standard silver stain procedure (Owl Silver Stain Kit, NBS Biological, United Kingdom). A duplicate gel was immunoblotted onto polyvinylidene difluoride blotting membrane. Membranes were blocked with 5% milk–PBS-T prior to application of Mycograb (10 μg/ml), which was followed by the rabbit anti-His polyclonal and anti-rabbit immunoglobulin conjugate as above.

Antifungal susceptibility testing. MIC endpoints and checkerboard titrations were determined using broth microdilution tests according to NCCLS document M27-A (53) with AMB stock solutions prepared as a 100× series in 100% dimethyl sulfoxide and diluted in medium to a concentration range from 0.016 to 8 μg/ml (74). FLC was tested from 0.1 to 100 μg/ml, and Mycograb was tested from 1 to 256 μg/ml. Yeast suspensions were prepared from individual colonies (diameter ≥ 1 mm) in RPMI 1640 with glutamine broth medium (Sigma Aldrich R7880), buffered to pH 7.0 with morpholinepropanesulfuric acid (MOPS) at 0.165 mmol/liter. The suspension was diluted 1 in 100 in sterile saline, and cells were counted with a hemacytometer. The yeast inoculum size was 10^3 CFU/ml. Trays were incubated at 37°C for 48 h. For AMB and Mycograb, either alone or in combination, the MIC endpoints were the lowest concentrations to produce no growth or a cell count of ≤5% of the control well, as determined by plating out the wells onto Columbia blood agar plates (Oxoid) and performing a colony count. For FLC, and for Mycograb in combination with FLC, the MIC endpoints were the lowest concentration producing no growth or a cell count of ≤20% of that in the control well as determined by plating out the wells onto Columbia blood agar plates (Oxoid) and performing a colony count (3, 29, 63, 74). The FIC was calculated for each drug by dividing the MIC in the presence of the second drug by the MIC in its absence. For each combination this produced two fractions, which were summated to produce the FICI: synergy was defined by a value of ≤0.5, indifference was defined by a value of >0.5 to <4.0, and antagonism was defined by a value of ≥4.0 (3, 29, 73).

Pharmacokinetics. Adult female, outbred, CD-1 mice (25 g; Charles River, Maidstone, United Kingdom) were injected intravenously with either 2 or 10 mg of Mycograb/kg of body weight. Duplicate mice were sacrificed at 2, 15, 30, 60, 120, 240, and 360 min and 24 h. Mycograb levels were determined in sera (obtained by cardiac puncture) and urine (obtained by suprapubic aspiration). Organs (kidneys, liver, spleen, brain, heart, and lungs) from the high-dose group were weighed, flash-frozen, mechanically macerated in the presence of a protease inhibitor cocktail, and centrifuged to remove debris, and the supernatant was used for analysis. All samples were assayed by SDS-PAGE against Mycograb reference standards of 0.156, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 ng, prepared in the same loading buffer. For samples, 5 μl was added to 12.5 μl of NuPAGE 4× SDS-PAGE loading buffer and 5 μl of 100 mM DTT, made up to 50 μl with water, and after boiling, 10 μl was applied to a NuPAGE 4-to-12% Bis-Tris 1.0-mm gradient gel (Invitrogen), alongside 5 μl of broad-range MultiMark marker, thereby equating to 1 μl of sample per track. The separated proteins were transferred onto nitrocellulose membrane, blocked in 5% milk–PBS-T, washed and incubated with rabbit polyclonal anti-His horseradish peroxidase conjugate (Santa Cruz) at a 1:500 dilution in 5% milk–PBS-T, rewashed, and finally visualized by ECL developing reagents (Amersham Pharmacia). The film was developed at 5 min, and the bands quantified by densitometry using Total Lab (Non linear Dynamic Ltd., California). Levels were expressed as micrograms per gram of tissue and micrograms per milliliter of serum.

Data were interpreted by noncompartmental pharmokinetic analysis (PK Solutions, version 2.0) for maximum drug concentration in serum (C_{max}) (in micrograms per milliliter), area under the concentration-time curve from 0 to 24 h (AUC_{0-24}) and $AUC_{0-\infty}$ (in microgram-minutes per milliliter), half-life at alpha phase ($t_{1/2\alpha}$) (in minutes), $t_{1/2\beta}$ (in hours), and mean residence time (MRT) (in hours).

Assessment in murine candidiasis model. Yeasts were grown overnight in Sabouraud dextrose broth at 37°C and washed in saline, and concentrations were determined by hemacytometer count and confirmed by plating dilutions on Sabouraud dextrose agar. After preliminary dose-ranging experiments, the activities of Mycograb and AMB, alone and in combination, were assessed against the following yeast infections (intravenous inoculum size in parentheses): (i) FLC-resistant *C. albicans* (4×10^6 CFU), (ii) *C. krusei* (4×10^7 CFU), (iii) *C. glabrata* (9×10^6 CFU), (iv) *C. tropicalis* (3×10^6 CFU), (v) *C. parapsilosis* NCPF 3104 (10^7 CFU), and (vi) the clinical isolate of *C. parapsilosis* (10^7 CFU). These yeasts were injected as a 0.1-ml bolus into the lateral tail vein of 25-g female CD1 mice (Charles River). Two hours after infection, randomized groups of 10 animals were given intravenously one of the following: (i) 100 μl of Mycograb at a dose of 0.2 or 2.0 mg/kg plus 100 μl of placebo (dextrose saline); (ii) 100 μl of AMB at a dose of 0.6 mg/kg plus 100 μl of placebo (Mycograb formulation buffer); (iii) combination therapy with both 100 μl of AMB (0.6 mg/kg) and 100 μl of Mycograb (0.2 or 2.0 mg/kg); or (iv) 200 μl of placebo (100 μl dextrose saline plus 100 μl Mycograb formulation buffer). Mice were culled at 48 h, and yeast cell counts determined for kidney, liver, and spleen. If ≥60% of the organ biopsy specimens were negative (defined as counts <1 CFU/mg of tissue), statistical analysis was performed by Fisher's exact test, comparing the number of negative biopsy specimens between test and control groups. If organ biopsy specimens were culture positive, groups were compared by analysis of variance followed by Scheffe's test for multiple comparisons. The results were expressed as means ± standard deviations. A P of <0.05 was considered significant for all statistical determinations. All of the above analyses were performed on SPSS (version 10.1) software, supplied by the University of Manchester (Manchester, United Kingdom) Computation Department.

All animals were housed and handled according to nationally recommended guidelines and all procedures carried out under an approved British Home Office License.

RESULTS

Electrophoresis. Immunoblots of one-dimensional electrophoresis of *C. albicans* showed Mycograb reacted with antigen bands at 30, 40, 47, and 92 kDa (Fig. 1, lane 1). The same bands cross-reacted with the anti-human HSP90 antibody, with the strongest reactivity in each case being against the bands at 40 and 47 kDa. Two-dimensional gel electrophoresis of *C. albicans* showed Mycograb binding to the spots at 40 and 47 kDa (Fig. 2).

Susceptibility assessment. Mycograb showed intrinsic antifungal activity against all five species of *Candida* examined, including both FLC-sensitive and FLC-resistant strains of *C. albicans.* The MICs ranged from 128 to 256 μg/ml (Tables 1 and 2).

With the exception of the FLC-sensitive strain of *C. albicans*, Mycograb demonstrated indifference when combined with FLC against all yeasts examined, even at relatively high (100 μg/ml) levels of Mycograb (Table 1). Mycograb did show synergy with FLC against the FLC-sensitive *C. albicans.* In con-

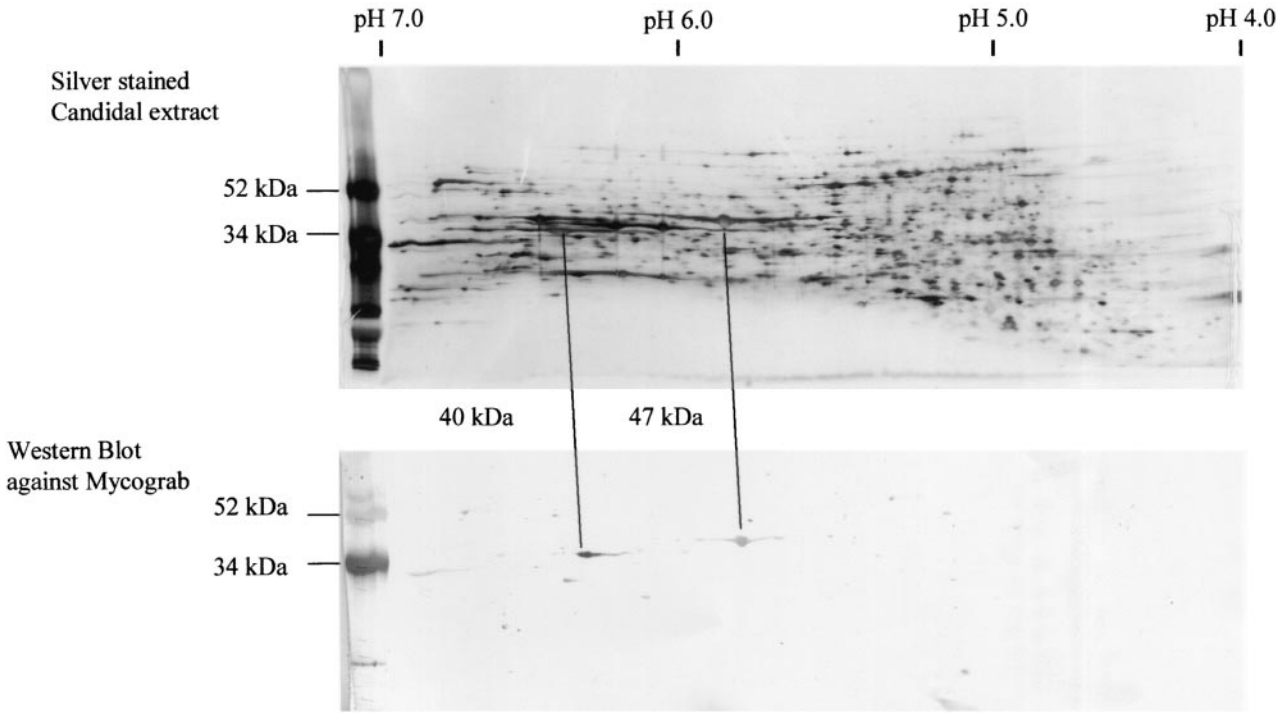

FIG. 2. Two-dimensional electrophoresis and Western blot of *C. albicans* extract versus Mycograb.

trast, there was evidence of pronounced synergy between AMB and Mycograb against all yeasts tested. At levels of Mycograb of 4 or 8 μg/ml, the FICI values varied from 0.09 to 0.31 (Table 2). Mycograb at 2 μg/ml produced no synergy for *C. albicans* (FLC-R), *C. krusei*, *C. tropicalis*, *C. parapsilosis* (NCPF 3104), and *C. parapsilosis* (clinical isolate). For *C. albicans* (FLC-S) this level of Mycograb altered the MIC for AMB to 0.25 μg/ml, and for *C. glabrata* it became 0.5 μg/ml.

Pharmacokinetics. Mycograb was detectable as a 28-kDa band on immunoblot, the sensitivity level of the assay being 0.2 μg/ml. The low dose (2 mg/kg) produced a C_{max} of 4.7 μg/ml, while the high dose (10 mg/kg) produced a C_{max} of 54.9 μg/ml (Table 3). In the mice given the high dose, samples from the spleen and liver demonstrated trace levels at the limit of the assay, varying from 0.2 to 1.5 μg/g in all the biopsy samples examined, including those at 24 h.

TABLE 1. Checkerboard assay of FLC and Mycograb versus yeast isolates examined

Species	Agent	MIC (μg/ml) of each agent		FIC (μg/ml)	FICI	Outcome
		Alone	In combination			
C. albicans (FLC-S)	FLC	1.56	0.4	0.26	0.34	Synergy
	Mycograb	128	10	0.08		
C. albicans (FLC-R)	FLC	50	12.5	0.25	0.64	Indifference
	Mycograb	256	100	0.39		
C. krusei	FLC	100	50	0.5	1.28	Indifference
	Mycograb	128	100	0.78		
C. tropicalis	FLC	3.12	3.12	1.0	1.78	Indifference
	Mycograb	128	100	0.78		
C. glabrata	FLC	6.25	1.56	0.25	0.75	Indifference
	Mycograb	128	64	0.5		
C. parapsilosis (NCPF3104)	FLC	1.56	1.56	1	1.39	Indifference
	Mycograb	256	100	0.39		
C. parapsilosis (clinical isolate)	FLC	1.56	1.56	1	1.39	Indifference
	Mycograb	256	100	0.39		

TABLE 2. Checkerboard assay of AMB and Mycograb versus yeast isolates examined

Species	Agent	MIC (μg/ml) of each agent		FIC (μg/ml)	FICI	Outcome
		Alone	In combination			
C. albicans (FLC-S)	AMB	1	0.03	0.03	0.09	Synergy
	Mycograb	128	8	0.06		
C. albicans (FLC-R)	AMB	0.5	0.125	0.25	0.27	Synergy
	Mycograb	256	4	0.02		
C. krusei	AMB	1	0.25	0.25	0.28	Synergy
	Mycograb	128	4	0.03		
C. tropicalis	AMB	0.125	0.016	0.13	0.16	Synergy
	Mycograb	128	4	0.03		
C. glabrata	AMB	1	0.25	0.25	0.31	Synergy
	Mycograb	128	8	0.062		
C. parapsilosis (NCPF3104)	AMB	1	0.25	0.25	0.27	Synergy
	Mycograb	256	4	0.016		
C. parapsilosis (clinical isolate)	AMB	1	0.25	0.25	0.27	Synergy
	Mycograb	256	4	0.016		

The renal biopsy specimens from the high-dose group showed a C_{max} of 21.7 μg/ml. In the urine a 20-kDa breakdown product was detected which produced a diffuse banding pattern and occurred in urine between 45 min and 6 h. Its presence matched the $t_{1/2\beta}$ of 10.94 h and an MRT of 9.3 h in the renal biopsy specimens.

Assessment in murine candidiasis model. The intrinsic antifungal activity of Mycograb alone at a dose of 2 mg/kg (Table 4) was shown by a statistically significant reduction in the mean organ colony count (Scheffe's test, $P < 0.05$) for the FLC-resistant strain of *C. albicans* (kidney, liver, and spleen), *C. krusei* (liver and spleen), *C. glabrata* (liver and spleen), *C. tropicalis* (kidney), and *C. parapsilosis* clinical isolate (kidney, liver, and spleen). This was also seen at the 0.2-mg/kg dose with *C. krusei* (liver and spleen), *C. glabrata* (liver), *C. tropicalis* (kidney), and *C. parapsilosis* clinical isolate (kidney, liver, and spleen). A statistically significant increase in the number of negative biopsy specimens (Fisher's exact test $P < 0.05$) was demonstrated at a Mycograb dose of 2 mg/kg by *C. glabrata* (kidney $P = 0.0003$), *C. tropicalis* (liver $P = 0.00006$ and spleen $P = 0.0015$), and *C. parapsilosis* (liver $P = 0.0098$) and at 0.2 mg/kg by *C. tropicalis* (liver $P = 0.00006$ and spleen $P = 0.0015$) and *C. parapsilosis* (liver $P = 0.0098$). AMB alone failed to clear the FLC-resistant strain of *C. albicans* (kidney), *C. krusei* (spleen), *C. glabrata* (liver and spleen), and *C. parapsilosis* (kidney, liver, and spleen).

Synergy was measured by a statistically significant increase (Fisher's exact test, $P < 0.05$) in the number of negative biopsy specimens on combination therapy compared to AMB alone (group 3). With Mycograb at 2 mg/kg, this was seen with the FLC-resistant strain of *C. albicans* (kidney, $P = 0.028$), *C. krusei* (spleen, $P = 0.011$), *C. glabrata* (spleen, $P = 0.034$), and *C. parapsilosis* (liver, $P = 0.016$; spleen, $P = 0.0000054$). At 0.2 mg/kg of Mycograb synergy was still evident with the FLC-resistant strain of *C. albicans* (kidney, $P = 0.0054$) and *C. glabrata* (spleen, $P = 0.034$). The liver counts from *C. glabrata* showed an increase in the number of negative organs but this did not achieve statistical significance compared to AMB alone (group 3). The renal counts from *C. parapsilosis* were unaltered by either drug alone or in combination. The *C. parapsilosis* clinical isolate showed indifference, with either drug being equally active on its own.

DISCUSSION

This work describes a human genetically recombinant antibody with antifungal activity demonstrated in vitro using assays developed for the assessment of chemotherapeutic antifungal drugs. Mycograb does not have an Fc component, and its antifungal activity was not dependent on Fc-mediated recruitment of white blood cells or complement. It was due to its ability to bind to and inhibit HSP90.

Recently FIC and FICI have been applied to the examination of antifungal drug combinations. There has been debate as to whether synergism should be defined as an FICI of <1 or ≤ 0.5 and whether antagonism should be defined as >1, >2, or

TABLE 3. Pharmacokinetic parameters of Mycograb in mice given a single dose

Mycograb dose (mg/kg) (sample studied)	C_{max} (μg/ml)	AUC_{0-t} (μg · min/ml)	$AUC_{0-\infty}$ (μg · min/ml)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (h)	MRT (h)
2 (serum)	4.7	155	157	3.75	2.34	3.17
10 (serum)	54.9	2,857.4	2,859.6	7.05	2.51	2.35
10 (renal biopsy specimen)	21.7	9,108	9,120	10.2	10.94	9.3

TABLE 4. Organ colony counts from a series of experiments

Expt no.[b]	Group	Dose (mg/kg)		Colony count (mean $\log_{10}$ CFU/g ± SD)[a]		
		AMB	Mycograb	Kidney	Liver	Spleen
1	1	0.6	2	(9)[e]	(9)[d]	(9)[d]
	2	0.6	0.2	(10)[e]	(10)[d]	(9)[d]
	3	0.6		[b]3.66 ± 0.82 (4)	(9)[d]	(10)[d]
	4		2	[b]4.58 ± 0.76 (2)	[b]3.50 ± 0.47 (3)	[b]3.12 ± 0.11 (4)
	5		0.2	5.1 ± 0.11 (0)	3.85 ± 0.81 (3)	3.35 ± 0.33 (3)
	6			5.5 ± 0.5 (0)	4.42 ± 0.59 (2)	3.65 ± 0.69 (2)
2	1	0.6	2	(9)[d]	(9)[d]	(8)[d,e]
	2	0.6	0.2	(9)[d]	(8)[d]	3.71 ± 0.64 (4)
	3	0.6		(9)[d]	(7)	[b]4.18 ± 0.22 (2)
	4		2	(6)	[b]4.32 ± 0.82 (4)	[b]3.87 ± 0.93 (1)
	5		0.2	(6)	[b]4.35 ± 0.26 (4)	[b]3.81 ± 0.78 (1)
	6			4.05 ± 0.1 (0)	5.15 ± 0.18 (0)	4.81 ± 0.33 (0)
3	1	0.6	2	(9)[d]	(6)	(8)[d,e]
	2	0.6	0.2	(10)[d]	(6)	(8)[d,e]
	3	0.6		(9)[d]	[b]4.74 ± 1.0 (2)	[b]4.24 ± 0.25 (3)
	4		2	(8)[d]	[b]5.54 ± 0.18 (0)	[b]5.19 ± 0.31 (0)
	5		0.2	5.17 ± 0.19 (2)	[b]6.01 ± 0.25 (0)	5.30 ± 0.39 (0)
	6			5.19 ± 0.90 (0)	6.57 ± 0.34 (0)	5.86 ± 0.76 (0)
4	1	0.6	2	(8)[d]	(10)[d]	(10)[d]
	2	0.6	0.2	(8)[d]	(10)[d]	(10)[d]
	3	0.6		(8)[d]	(10)[d]	(10)[d]
	4		2	[b]4.24 ± 0.95 (1)	(9)[d]	(10)[d]
	5		0.2	[b]4.41 ± 0.72 (1)	(10)[d]	(10)[d]
	6			5.30 ± 0.13 (0)	3.82 ± 0.69 (1)	4.41 ± 0.93 (3)
5	1	0.6	2	3.82 ± 0.81 (4)	(10)[d,e]	(10)[d,e]
	2	0.6	0.2	3.84 ± 0.78 (1)	(9)[d]	3.89 ± 0.94 (2)
	3	0.6		4.01 ± 0.93 (1)	3.08 ± 0.12 (5)	4.10 ± 0.98 (0)
	4		2	4.01 ± 0.92 (1)	(9)[d]	4.03 ± 0.33 (2)
	5		0.2	4.01 ± 0.09 (3)	(9)[d]	4.07 ± 0.31 (3)
	6			4.00 ± 0.04 (0)	3.54 ± 0.65 (3)	4.05 ± 0.08 (0)
6	1	0.6	2	[b]4.56 ± 0.81 (0)	[b]3.66 ± 0.25 (4)	[b]4.08 ± 0.02 (2)
	2	0.6	0.2	[b]4.48 ± 0.34 (0)	[b]4.09 ± 0.27 (2)	[b]4.11 ± 0.29 (1)
	3	0.6		[b]4.62 ± 0.94 (0)	[b]3.98 ± 0.06 (3)	[b]4.24 ± 0.29 (2)
	4		2	[b]4.60 ± 0.65 (0)	[b]4.07 ± 0.26 (3)	[b]4.32 ± 0.31 (1)
	5		0.2	[b]4.77 ± 0.79 (0)	[b]4.28 ± 0.28 (0)	[b]4.48 ± 0.38 (2)
	6			5.66 ± 0.64 (0)	5.31 ± 0.44 (0)	5.24 ± 0.16 (0)

[a] Number of negative biopsy specimen in parentheses.
[b] Experiments: 1, FLC-resistant *C. albicans;* 2, *C. krusei;* 3, *C. glabrata* 4, *C. tropicalis;* 5, *C. parapsilosis;* 6, *C. parapsilosis* (clinical isolate).
[c] Statistically significant (Scheffe's test, $P < 0.05$) versus the control group 6.
[d] Statistically significant number of negative biopsy specimens (Fisher's exact test, $P < 0.05$) versus the control group 6.
[e] Statistically significant numbers of negative biopsy specimens (Fisher's exact test, $P < 0.05$) versus the AMB-treated group 3.

>4 (5, 15, 57, 67, 73, 77, 79). The present data showed synergy between AMB and Mycograb regardless of definition. This enhanced activity might reflect the effect of combining two drugs directed against different targets within the fungus or it may occur because at sublethal doses of AMB the yeast cells became leakier, allowing Mycograb greater access to cytoplasmic HSP90 (20). Studies of the pharmacokinetics of AMB in patients have demonstrated peak serum levels of 1 to 2 μg/ml (4, 6, 20), compatible with a therapeutic response being achieved when the MIC of AMB for an isolate is ≤0.5 μg/ml (63). It is also consistent with reports of clinical failure on AMB, in patients with candidemia, when the MIC for the isolate was ≥1 μg/ml (56). The MIC data (Table 2) suggest that Mycograb, at levels achieved in mice, reduces the MIC to AMB to 0.5 μg/ml or less, levels of AMB readily obtainable in patients' sera. This is even more important with the lipid-associated formulations of AMB since these increase total drug concentration while decreasing the unbound drug concentration in plasma due to AMB sequestration (14).

Antifungal activity was further demonstrated in a murine model of invasive candidiasis (Table 4). Mycograb alone produced a statistically significant improvement in the infections caused by each species. AMB alone cleared the *C. tropicalis* infection but failed to clear infections caused by *C. albicans*, *C. krusei*, *C. glabrata*, or *C. parapsilosis*, there still being foci of infection in one or more organs. Only by combining Mycograb with AMB was complete resolution of the infection achieved for *C. albicans*, *C. krusei*, and *C. glabrata*; clearing of the liver and spleen of *C. parapsilosis* NCPF 3104 was achieved by giving the drugs in combination, but renal counts were unaltered by either drug alone or in combination. For *C. parapsilosis* (clinical isolate) the two drugs showed comparable activity alone,

with no significant improvement in combination. It should be borne in mind that in this particular model, the infection was sublethal and did not examine delayed or repeated therapy

The determination of whether synergy is occurring between two antifungal drugs in vivo is complicated by three problems (32, 33, 65). First, individual isolates vary in virulence, this being both species and strain dependent (24, 25). Pilot studies (data not shown) were used to determine the minimum dose of each of *Candida* species required to produce a sustained infection. Second, a single agent may be so active that synergy due to the second drug is obscured. For example, giving either Mycograb alone or AMB alone was sufficient to clear infections from the organs cited: *C. krusei* (kidney), *C. glabrata* (kidney), *C. tropicalis* (liver and spleen), and *C. parapsilosis* NCPF 3104 (liver). Third, there is the issue of the statistical definition of synergy. This has historically been based on a decrease in the mean organ colony count (32, 33, 65). The combination of Mycograb and AMB produced indifference against infection caused by the clinical isolate of *C. parapsilosis*, both drugs being equally active. In all other cases, the combination of the two drugs enhanced activity. Only by combining the two drugs was there complete resolution of the infections caused by *C. albicans*, *C. krusei*, and *C. glabrata* from all organs tested. This is clinically relevant as organ sterilization is desirable in any therapeutic combination.

The serum levels of Mycograb obtained from the mouse kinetic studies were lower than the levels required to achieve demonstrable antifungal activity in vitro with Mycograb alone, but compatible with the Mycograb levels required to achieve synergy with AMB. A peak serum level of 4.7 μg/ml followed a single dose of 2 mg/kg, with a level of ≥1 μg/ml for 20 min. After a dose of 10 mg/kg, a level of ≥4 μg/ml was detected for 2.4 h in serum and for 16.4 h in renal biopsies. Trace amounts of Mycograb were also seen in the liver and spleen biopsies throughout the 24 h period.

Mycograb was discovered by examining the antibody response to *Candida* in patients recovering from invasive infections, being treated with AMB, and comparing it with those in patients with fatal cases. Initially the association between recovery and antibody to HSP90 was identified in terms of antibody reactivity to the 47-kDa antigen, on immunoblots of *C. albicans* (47, 48). This was usually associated with reactivity to bands at 40 and 92 kDa. The predicted molecular mass from the amino acid sequence of candidal HSP90 is 81 kDa (11), but when cloned into *S. cerevisiae*, it ran, on gel electrophoresis, at approximately 90 kDa, and both Mycograb and a mouse monoclonal against human HSP90 reacted with a band at 92 kDa (58, 72, 81). The pattern of cross-reactivity of rabbit and mouse monoclonal antibodies against specific HSP epitopes confirmed the relatedness of the three antigens: the parent molecule at 92 kDa was heat inducible while the 40- and 47-kDa proteins were expressed irrespective of whether the yeast was grown at 23 or 37°C or in the yeast or mycelial phase (44). Similarly, HtpG, the HSP 90 homologue from *Porphyromonas gingivalis*, produces a stress-inducible 68-kDa band on immunoblots, as well as a constitutively expressed major band at 44 kDa and a minor band at 40 kDa (31).

The pattern of cross-reactivity of Mycograb on two dimensional electrophoresis immunoblots demonstrated that the epitope targeted (NKILKVIRKNIVKK) was more accessible in the truncated forms of HSP90 represented by the 40- and 47-kDa spots. Two ATP binding sites have been described within HSP90. The C-terminal ATP binding site has been shown to be the first example of a cryptic chaperone nucleotide-binding site, opened by the occupancy of the N-terminal site. Communication between these two sites involves a phosphate-binding motif, analogous to members of the GHKL family (14) involving QQSKILKVI, which overlaps the N-terminal end of the peptide recognized by Mycograb (69). This region is responsible for the conformational change in molecular shape that occurs when HSP90 binds ATP, suggesting Mycograb might inhibit HSP90 by blocking this change. Candidal HSP90 has been shown to be up-regulated in response to the mammalian steroid hormone 17-β-estradiol. This has been linked to increased growth, toxin production, and germination rates in clinical isolates of *C. albicans* (80). But the antifungal activity of Mycograb in vitro occurred in the absence of this hormone suggesting its mechanism of action involved inhibition of HSP90 in a more fundamental role in yeast cell physiology.

The mechanisms by which Mycograb achieves efficacy against candidiasis in vivo may not depend solely on inhibition of fungal HSP90 alone. The epitope that Mycograb targets is conserved between yeast, murine, and human homologues of HSP90 (21, 52). HSP90 has been shown to bind to endothelial nitric oxide synthase leading to the release of nitric oxide (19). Nitric oxide derived from the endothelium is important in regulating cardiovascular hemodynamics. When administered, it causes large vessel vasodilation, the proliferation of vascular smooth muscle cells, leukocyte adhesion, platelet aggregation, and, in a human volunteer study, activation of the microcirculation (51, 68). HSP90 has also been shown to catalyze the activation of the prekallikrein-kininogen complex, in the absence of factor XII, this pathway leading to release of bradykinin. This zinc dependent activation was inhibited by a polyclonal antibody against HSP90. Bradykinin is a major mediator of swelling in C1 inhibitor deficiency and responsible for the angioedema seen with ACE inhibitors. It is released from mast cells during asthma attacks and it is a gastrointestinal vasodilator (28). Inhibition of pathways such as these by an HSP90 inhibitor would occur at physiological rather than pharmacological levels, and could contribute towards the benefit of Mycograb in murine candidiasis at relatively low serum levels.

Antibody to human HSP90 is part of the natural human antibody repertoire (59). The lack of detectable antibody to HSP90 in patients succumbing to invasive candidiasis could reflect the patient's inability to produce the antibody (because they are immunosuppressed, for example) or that the antibody is being produced but rapidly forms complexes with an excess of HSP90 antigen. This imbalance between HSP90 and the natural antibody can be corrected by giving Mycograb. Based on the data presented here, Mycograb is now being assessed in a multinational placebo-controlled trial. The combination of Mycograb and AMB is being compared with AMB alone in patients with culture-confirmed invasive candidiasis.

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