Recombinant Single-Chain Anti-idiotypic Antibody: An Effective Fungal β-1,3-Glucan Synthase Inhibitor

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Recombinant single-chain fragment variable anti-idiotypic antibodies were produced to represent the internal image of HM-1 killer toxin and were used as novel and effective antifungal agents to inhibit *in vitro* β-1,3-glucan synthase and cell growth. The mechanism of cytocidal activity of anti-idiotypic antibodies was investigated and was compared with the actions of aculeacin A and papulacandin B, the most common antibiotics acting as β-1,3-glucan synthase inhibitors. The degree of inhibition of β-1,3-glucan synthase by both antibodies and antibiotics were examined for yeasts *Saccharomyces cerevisiae* A451, *Cryptococcus albidus* NBRC 0612 and *Candida albicans* IFM 40215. Although the mechanism of actions of the anti-idiotypic antibodies and antibiotics seems identical, the IC_{50} values for the various yeasts used in this study confirmed that anti-idiotypic antibodies could be used as more effective fungal β-1,3-glucan synthase inhibitors than those of antibiotics.

Key words recombinant anti-idiotypic antibody; β-1,3-glucan synthase; *Candida albicans*; *Cryptococcus albidus*; *Saccharomyces cerevisiae*; anti-fungal antibiotic

Enzymes that synthesize fungal cell walls are hypothesized to be effective targets for anti-fungal drugs because of the absence of similar mammalian enzymes.[1,2] β-1,3-Glucan synthase is an enzyme localized in the plasma membrane that catalyzes the synthesis of β-1,3-glucan, a major polymer component of the cell wall of yeasts and fungi.[3] Inhibition of cell wall glucan synthesis provides a possibility to protect against fungal infections.

Many yeast strains secrete proteins called "killer toxins" to inhibit the growth of other yeast strains. HM-1 killer toxin (HM-1), a small protein comprising 88 amino acids and five disulfide bridges, is produced by *Williopsis saturnus* var. *mrakii* IFO 0895 (previously known as *Hansenula mrakii*) and is strongly cytocidal against *Saccharomyces cerevisiae*.[4,5] It affects sensitive yeast cells primarily in the growing stage, but it is not toxic to yeast cells in the resting stage or to mammalian cells.[6] The mechanism of cytocidal activity of HM-1 has been studied extensively, and the accumulated data indicate that HM-1 kills yeast cells by extracellularly inhibiting β-1,3-glucan synthase.[4,6—8] This inhibition by HM-1 forms a pore at the distal tip of the developing bud and the protruding conjugation tube where cell wall synthesis is active; cells treated with HM-1 die by discharging cellular materials from pores because of osmotic pressure.[6]

Opportunistic infections are becoming increasingly common due to the growing number of individuals immunocompromised by chemotherapy or immunosuppressants.[9] The incidence of fungal infections is increasing worldwide because of increasing numbers of immunocompromised patients at advanced age, or with AIDS or cancer, or are undergoing organ transplantation.[10] Nowadays, fungi have also been implicated to be causal agents for chronic rhinosinusitis, infecting tens of millions of people around the world.[11] Candidiasis and cryptococcosis are the most important fungal infections in humans; followed by aspergillosis.[12,13]

The development, not only of new conventional antibiotics but also of novel compounds and alternative strategies for the battle against fungal infections, is becoming a topical and widely recognized need. The excellent biochemical properties of HM-1 can be used as a useful source to develop antifungal drugs. One creative approach in this field is the development of recombinant single-chain fragment variable (scFv) anti-idiotypic antibodies, which have the internal image of the HM-1 active site responsible for β-1,3-glucan synthase inhibition and anti-fungal activity (Selvakumar *et al.*, unpublished results).

Aculeacin A (MW 1036.2), consisting of cyclic peptide and 1 mol of fatty acid, and papulacandin B (MW 901.0), consisting of a disaccharide, 1 mol of resorcinol and 2 mol of unsaturated fatty acid, are antifungal antibiotics.[14—16] Evident is that the killing mechanism of these two amphiphilic antibiotics a similar to HM-1 killer toxin due to the inhibition of cell wall glucan synthesis through inhibiting β-1,3-glucan synthase.[6,17]

In this study, the mechanism of inhibiting cell growth by scFv anti-idiotypic antibodies is explained, and their potential to inhibit β-1,3-glucan synthase in the membrane fractions of pathogenic strains *Candida albicans* and *Cryptococcus albidus* and the non-pathogenic yeast *S. cerevisiae* are compared with the potential of aculeacin A and papulacandin B. This study highlights the possibility of using anti-idiotypic antibodies having biological activities similar to HM-1 as a novel, effective and alternative strategy to inhibit β-1,3-glucan synthase. The usefulness and effectiveness of anti-idiotypic antibodies is discussed, and their action is compared with that of antibiotics, aculeacin A and papulacandin B.

MATERIALS AND METHODS

Fungal Strains and Antibiotics *S. cerevisiae* A451 was a kind gift from Nippon Roche Research Center. *C. albidus* NBRC 0612 was purchased from NITE Biological Resource Center and *C. albicans* IFM 40213 was a generous gift from Dr. Koji Yokoyama, Research Center for Pathogenic Fungi

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and Microbial Toxicoses, Chiba University. Aculeacin A was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and papulacandin B was donated by Ciba-Geigy (Basel, Switzerland).

Preparation of Anti-idiotypic Antibodies The scFv anti-idiotypic antibodies used in this study were produced according to a procedure established previously (Selvakumar *et al.*, unpublished results) by using the Recombinant Phage Antibody System (Amersham Biosciences, U.S.A.). Briefly, 50 μg of purified neutralizing monoclonal antibody of HM-1 (nmAb-KT) that was prepared at the Technology Incubation & Transfer Ltd. (Saitama, Japan) was used to immunize female Balb/c mice (3 weeks old, 10—12 g) subcutaneously and intraperitoneally (booster injection). Three days after the final injections the mice were killed and their spleens were removed. mRNA was isolated from the splenic lymphocytes of mice immunized with nmAb-KT. The purified mRNA primed with random hexamers was reverse transcribed. The genes encoding antibody variable regions of heavy and light chains were amplified and assembled into a single gene using a linker fragment and were cloned into a specific phagemid vector pCANTAB 5E. Recombinant phages produced in transformed *Escherichia coli* TG1 were repeatedly panned against nmAb-KT and were screened by using conventional enzyme-linked immunosorbent assay against the nmAb-KT. The selected recombinant phages were used to infect the nonsuppressor *E. coli* HB2151 strain to produce soluble recombinant scFv antibodies that were purified by using affinity chromatography with an anti-E tag Sepharose column (Amersham Biosciences). The protein concentration of the purified scFv antibodies was measured by using the bicinchoninic acid method.[18] The yield of the purified scFv antibodies varied between 1.0 and 3.0 mg/l. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done to check the purity. Four selected antibodies gave a single band with SDS-PAGE corresponding to the molecular mass of around 30 kDa, which agreed well with the calculated masses based on the amino acid sequences of antibodies. The nucleic acids of selected clones were sequenced by using a CEQ 2000 Dye Terminator Cycle Sequencing kit (BECKMAN COULTER), and the products were analyzed by using a CEQ^{TM} 2000XL DNA Analysis System. Sequencing primers R1, S3, S4 and S6 for the pCANTAB 5E vectors were used. The sequences were aligned and manipulated by using DNASIS and Genetyx software.

***In Vitro* Anti-yeast Activity by scFv Anti-idiotypic Antibody** A qualitative anti-yeast assay of purified scFv antibodies against *S. cerevisiae* A451 was done by using conventional colony forming unit (CFU) assay, as described previously.[19] Briefly, approximately 5×10^2 cells of *S. cerevisiae* were suspended in 10 μl of phosphate buffered saline (PBS) and was incubated with 90 μl of scFv antibodies for 16 h at 37 °C. PBS was used as a control; as a further control, cells were prepared by the adding scFv antibodies pre-incubated overnight at 4 °C with 20 μl of nmAb-KT to give final concentration 200 μg/ml. Yeast cells incubated with the scFv antibodies were spread on the surface of Sabouraud dextrose agar plates and were incubated at 30 °C. The yeast cells were incubated for 48 h, and their CFU was enumerated. To measure the IC_{50} values, the concentration requiring 50% inhibition, different concentrations of each of the four scFv antibodies were added to inhibit the cell growth. IC_{50} values were evaluated from semi-logarithmic graphs. Each experiment was done in triplicate.

Pore Formation by Anti-idiotypic Antibodies and Antibiotics *S. cerevisiae* A451 (4×10^6 cells/ml) was incubated with 10 μg/ml of antibiotic or scFv anti-idiotypic antibody in YPD medium containing 0.8 M sorbitol and the mixture was shaken at 175 rpm at 30 °C for 3 h. Cells without treatment with scFv antibodies and antibiotics were examined as a control. All cells were stained with 0.1% methylene blue in 0.8 M sorbitol and were photographed under a phase-contrast microscope using immersion oil.

Preparation of Membrane Fraction A membrane fraction containing β-1,3-glucan synthase was prepared by using the method described previously[20] with some modifications. Cells of *S. cerevisiae* A451, *C. albidus* NBRC 0612 and *C. albicans* IFM 40213 in the mid-exponential phase were collected by centrifugation and were washed with 1 mM ethylenediaminetetraacetic acid (EDTA). They were collected and suspended in breaking buffer consisting of 50 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA and 1 mM phenylmethanesulfonyl fluoride and then they were disrupted with glass beads by vortexing and were centrifuged for 5 min at $1000\times g$ at 4 °C. The supernatant was then centrifuged for 30 min at $100000\times g$ at 4 °C. The membrane fraction obtained was homogenized in membrane buffer consisting of 50 mM Tris–HCl (pH 7.5), 10 mM EDTA, 1 mM 2-mercaptoethanol and 33% glycerol, and stored at −80 °C.

Measurement of β-1,3-Glucan Synthase Activity and IC_{50} The assay of β-1,3-glucan synthase was done by using the method described previously.[20] The reaction mixture consisted of 5 mM UDP-D-[U-^{14}C]glucose, 75 mM Tris–chloride (pH 7.5), 0.75% bovine serum albumin, 25 mM KF, 0.75 mM EDTA, 20 μM guanosine 5′-[γ-thio]triphosphate and 20 μl membrane fraction, in a total volume of 40 μl. The reaction was started by adding membrane fraction and the mixture was incubated at 30 °C for 60 min. The reaction was stopped by adding 250 μl 10% trichloroacetic acid, and the mixture stood for 10 min and then was filtered through a glass microfibre filter (Whatman GF/B). The filter was washed four times with 250 μl 10% trichloroacetic acid and was further washed twice with 250 μl 95% ethyl alcohol. The radioactivities retained on the filter were counted by using a liquid scintillation counter. To measure the IC_{50} values, each scFv antibody or antibiotic was added to the reaction mixture. The IC_{50} values were read from semi-logarithmic graphs. Each experiment was done in triplicate.

RESULTS

Recombinant Anti-idiotypic Antibodies Figure 1 shows the primary structures of four different scFv anti-idiotypic antibodies that were deduced by nucleotide sequence analysis. The clones of these four scFv antibodies were confirmed from their specific binding to nmAb-KT and their nucleotide sequences encoding the V_H and V_L chains were compared. One noticeable observation was that all these clones shared the same V_H genes, but the amino acid sequences of their V_L genes differed. These four different scFv antibodies that had different V_L domains sequences were designated as scFv-A1, scFv-A2, scFv-A3 and scFv-A4.

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Heavy chain:
scFv          10         20         30         40         50         60         70
A1    MAQVKLQQSG AELMKPGASV KISCKATGYT FSSYWIEWVK QRPGHGLEWI GEILPGSGST NYNEKFKGKA
A2    MAQVKLQQSG AELMKPGASV KISCKATGYT FSSYWIEWVK QRPGHGLEWI GEILPGSGST NYNEKFKGKA
A3    MAQVKLQQSG AELMKPGASV KISCKATGYT FSSYWIEWVK QRPGHGLEWI GEILPGSGST NYNEKFKGKA
A4    MAQVKLQQSG AELMKPGASV KISCKATGYT FSSYWIEWVK QRPGHGLEWI GEILPGSGST NYNEKFKGKA
            80         90        100        110        120
      TFTADTSSNT AYMQLSSLTS EDSAVYYCAR YYGNYYAMDY WGQGTTVTVS S
      TFTADTSSNT AYMQLSSLTS EDSAVYYCAR YYGNYYAMDY WGQGTTVTVS S
      TFTADTSSNT AYMQLSSLTS EDSAVYYCAR YYGNYYAMDY WGQGTTVTVS S
      TFTADTSSNT AYMQLSSLTS EDSAVYYCAR YYGNYYAMDY WGQGTTVTVS S


Light chain:
scFv          10         20         30         40         50         60         70
A1    AIMSASPGEK VTITCSVSSS ISSSNLHWYQ QKSETSPKPW IYGTSNLASG VPGRFSGSGS GNSYSLTISS
A2    AIMSASPGEK SPSPAVSAQV YVPATCTGTS RSQKPPPNPG FMAHPTWLLE SQVASVAVGL ENSYSLTISS
A3    AIMSASPGEK VTITCSVSSS RSSSNLHWYQ QKSETSPKPW IYGTSNLASG VPGRFSGSGS GKLLLSHDQQ
A4    AIMSASPGEK SPSPAVSAQV YVPATCTGTS RSQKPPPNPG FMAHPTWLLE SQVASVAVGL ENSYSLTISS
            80         90        100
      MEAEDVATYY CFQGSGYPLT FGSGTKLEIK
      MEAARCCHLL LFSGEWVPTH VGSGTKLEIK
      HEAEDVATYY CFQGSGYPLT FGSGTKLEIK
      MEAEDVATYY CFQGSGYPLT FGSGTKLEIK
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Fig. 1. Amino Acid Sequences of scFv Anti-idiotypic Antibodies

Amino acid sequences were deduced from the nucleotide sequence. To compare with scFv-A1, amino acid differences in V_L domain of scFv clones are in bold.

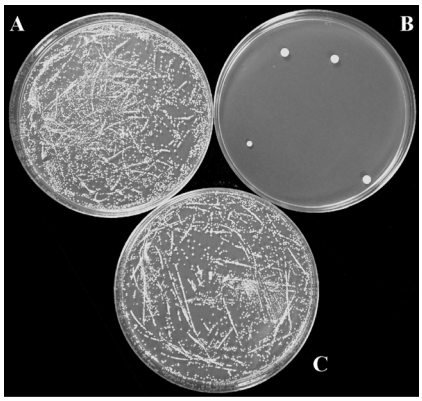

Fig. 2. Anti-yeast Activity of Anti-idiotypic Antibody

Effect of scFv-A4 anti-idiotypic antibody on the growth of *S. cerevisiae* A451 cells in a CFU assay. A, standardized yeast inocula treated with PBS; B, yeast cells treated with 8 μg/ml of scFv-A4; C, yeast cells treated with scFv-A4 neutralized with nmAb-KT. Each experiment was in triplicate.

In Vitro* Anti-yeast Activity of scFv Anti-idiotypic Antibodies against *S. cerevisiae Figure 2 shows the anti-yeast activity of scFv-A4 anti-idiotypic antibody against *S. cerevisiae* A451. Our preliminary study shows the growth of *S. cerevisiae* was strongly inhibited at concentration 8 μg/ml scFv-A4 (Fig. 2B) compared with the control (Fig. 2A). The anti-yeast activity of scFv-A4 antibody was markedly neutralized by nmAb-KT (Fig. 2C), and suggested the specificity of nmAb-KT to scFv-A4 anti-yeast activity. Other three scFv antibodies also showed similar results. Table 1 shows the IC_{50} values of scFv anti-idiotypic antibodies against *S. cerevisiae* A451 cell growth *in vitro*. Extensive studies by our assay system showed that 2.15 to 3.10 μg/ml of scFv anti-idiotypic antibodies, corresponding to values from 7.2×10^{-8} to 10.3×10^{-8} M, were sufficient to produce 50% inhibition of *S. cerevisiae* A451.

Mechanism of Anti-yeast Effect of scFv Anti-idiotypic Antibodies and Antibiotics The inhibition of the growth of yeast cells by HM-1 is characterized by formation of a pore at the growing tip of daughter cells, resulting in the formation of a protruding structure and eventual cell death.[6] Although the inhibitory mechanism of aculeacin A and papulacandin B was studied previously for *Saccharomyces bayanus* and was compared with that of HYI killer toxin, which is highly homologous to HM-1,[17] in this study the mechanism of the action of both anti-idiotypic antibody and antibiotics was examined for yeast *S. cerevisiae*. To know if scFv anti-idiotypic antibodies and antibiotics use the same mechanism of forming a pore in growing cells, we added purified scFv antibody and antibiotic aculeacin A to a *S. cerevisiae* A451 cell culture and analyzed the change in morphology of yeast cells when scFv antibody and aculeacin A inhibited the growth of yeast cells effectively. Figure 3 shows photographs of phase-contrast microscopy of cells treated with scFv-A4 and aculeacin A. Most culture cells treated with anti-idiotypic antibody had a pear-like structure characteristic of pore formation (Figs. 3C—F), similar to the morphology change after treatment with aculeacin A (Figs. 3G, H). This morphological change was caused by scFv antibody and aculeacin A and was clearly distinguished from the smooth and round cells of untreated control cells (Figs. 3A, B). The extent of damaged cells with a pear-like structure characteristic of pore formation caused by anti-idioypic antibody and antibiotic was estimated as about 65% and 52%, respectively.

Table 1. IC_{50} Values of Recombinant scFv Anti-idiotypic Antibodies against *S. cerevisiae* A451 Cell Growth

scFv antibodies	IC_{50} of cell growth (M)
scFv-A1	7.3×10^{-8}
scFv-A2	8.3×10^{-8}
scFv-A3	10.3×10^{-8}
scFv-A4	7.2×10^{-8}

Inhibition of Fungal β-1,3-Glucan Synthase by Anti-idiotypic Antibodies and Antibiotics The potential of the four scFv anti-idiotypic antibodies and antibiotics aculeacin A and papulacandin B as anti-fungal β-1,3-glucan synthase inhibitors was evaluated and compared. The activity of β-1,3-glucan synthase was measured for the membrane fractions of *S. cerevisiae*, *C. albidus* and *C. albicans* using UDP-D-[U-^{14}C]glucose as the substrate. Figure 4 shows the inhibitory effect of each of the four antibodies and two antibiotics on β-1,3-glucan synthase. Different concentrations of scFv antibodies and antibiotic were used to monitor the percentage of β-1,3-glucan synthase activity; each activity for *S. cerevisiae*, *C. albidus* and *C. albicans* was plotted (Figs.

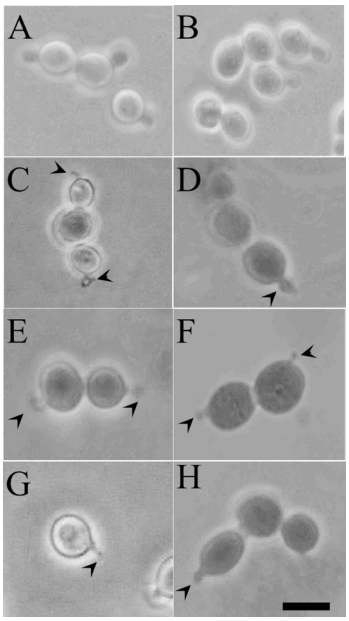

Fig. 3. Pore Formation in Yeast Cells by scFv-A4

Phase-contrast microscopy of *S. cerevisiae* A451 treated with scFv-A4 and aculeacin A. Sample preparation is described in Materials and Methods. (A, B) Control yeast cells; (C—F) cells treated with scFv-A4; (G, H) cells treated with aculeacin A. The bar indicates a length of 5 μm. The arrowhead shows the cell burst.

4A—C). Both scFv antibodies and antibiotics inhibited β-1,3-glucan synthase activities of the three different yeast strains dose dependently.

IC_{50} values of all scFv antibodies and antibiotics were evaluated for each yeast to compare the effectiveness of the two types of β-1,3-glucan synthase inhibitors (antibody and antibiotic). Table 2 summarizes the IC_{50} values of scFv antibodies and antibiotics against the yeasts *S. cerevisiae*, *C. albidus* and *C. albicans*. For these three yeasts the range of IC_{50} values of scFv antibodies was $0.43\times10^{-7}-2.33\times10^{-7}$ M. Whereas IC_{50} of antibiotics were ranging from $3.66\times10^{-5}-11.4\times10^{-5}$ M for three yeasts. The lower concentration of scFv antibodies and antibiotics showed no marked effect. However, scFv-A4 (IC_{50} value at 1.02×10^{-7} M) was the most effective inhibitor of *S. cerevisiae* and scFv-A2 was most effective in inhibiting *C. albidus* and *C. albicans* (IC_{50} values at 0.43×10^{-7} M and 0.44×10^{-7} M, respectively). When the IC_{50} values of aculeacin A and papulacandin B inhibitory actions on the three different yeasts were compared, aculeacin A was more effective in inhibiting fungal β-1,3-glucan synthase. The overall results clearly showed a more effective inhibition by anti-idiotypic antibodies than antibiotics, and both types of inhibitors showed differences in their activities between themselves.

DISCUSSION

HM-1 has a wide-spectrum of anti-fungal activity by inhibiting β-1,3-glucan synthase,[21] and so HM-1 derived scFv anti-idiotypic antibodies with an internal image of HM-1 have been presumed to have the same effect on the fungal cells. Because the mechanism of the action of HM-1 and HYI killer toxin is identical with that of aculeacin A and papulacandin B,[6,8,17] a comparative study between scFv antibodies and the two antibiotics should provide us with a

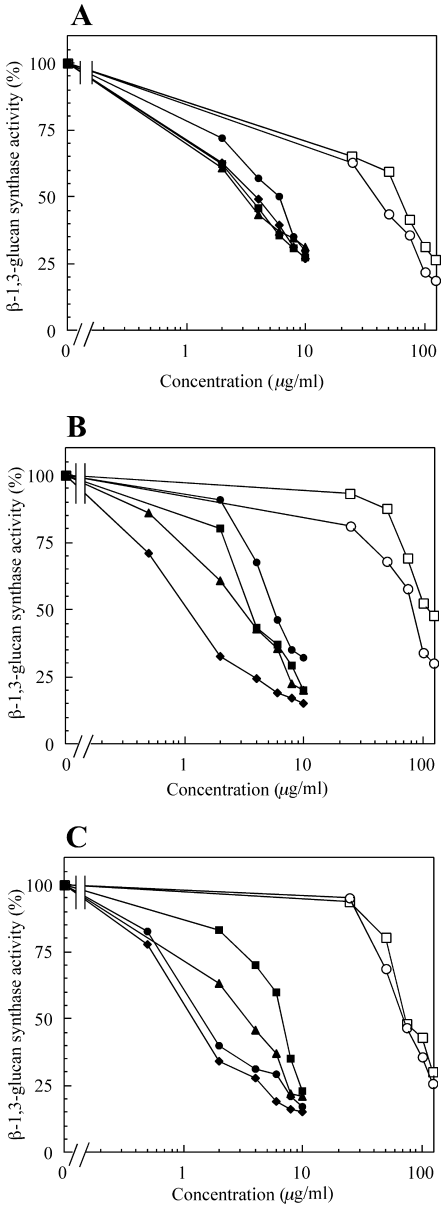

Fig. 4. Inhibition of β-1,3-Glucan Synthase by scFv Anti-idiotypic Antibodies and Antibiotics

The membrane fraction prepared from the cells of A, *S. cerevisiae* A451; B, *C. albidus* NBRC 0612; C, *C. albicans* IFM 40213 and the method of measuring the activity are described in Materials and Methods. The activities were expressed as a percentage of controls without scFv antibodies and antibiotics. scFv anti-idiotypic antibodies or aculeacin A or papulacandin B was added to the reaction mixture. ■, scFv-A1; ◆, scFv-A2; ●, scFv-A3; ▲ scFv-A4; ○, aculeacin A and □, papulacandin B.

Table 2. IC_{50} Values of scFv Anti-idiotypic Antibodies and Antibiotics against β-1,3-Glucan Synthase Activity of *S. cerevisiae* A451, *C. albidus* NBRC 0612 and *C. albicans* IFM 40213

Glucan synthase inhibitors	IC_{50} (M)		
	S. cerevisiae	*C. albidus*	*C. albicans*
scFv-A1	1.07×10^{-7}	1.23×10^{-7}	2.33×10^{-7}
scFv-A2	1.30×10^{-7}	0.43×10^{-7}	0.44×10^{-7}
scFv-A3	1.73×10^{-7}	1.80×10^{-7}	0.53×10^{-7}
scFv-A4	1.02×10^{-7}	1.00×10^{-7}	1.13×10^{-7}
Aculeacin A	3.66×10^{-5}	8.01×10^{-5}	6.75×10^{-5}
Papulacandin B	6.99×10^{-5}	11.4×10^{-5}	7.99×10^{-5}

promising way to select the best possible candidate to inhibit fungal β-1,3-glucan synthase and thus to protect against fungal infections.

Human fungal pathogens are a highly divergent group of fungal species. To prove their wide spectrum of activities, the sensitivity of scFv antibodies was tested for three different yeast species, which differ by their degree of pathogencity. *S. cerevisiae* is a non-pathogenic yeast, *C. albidus* is an opportunistic, pathogenic yeast that causes human infections mainly in immunosuppressed patients[22—24] and *C. albicans* is a most dangerous pathogenic yeast causing severe systematic infections in immune-compromised populations.[25] However, one important features thought to be common to all these pathogenic and non-pathogenic fungi are the participation of β-1,3-glucan synthase in cell wall biosynthesis.[26—28] To inhibit fungal growth, various efficacious antibiotics has been developed to interfere with cell wall synthesis targeting β-1,3-glucan synthase.[16,29—36] We believe, however, that no anti-fungal antibody that can inhibit β-1,3-glucan synthase activity has ever been reported.

In this study, the effective inhibitory action of scFv anti-idiotypic antibodies on β-1,3-glucan synthase was not hampered due to the difference in light chain amino acid sequences (Fig. 1). The potential inhibition may depend on heavy chain amino acid sequences, because four scFv antibodies were selected solely because of their high binding affinity to the idiotype of nmAb-KT. We expect that the functional mimicking of HM-1 (*i.e.*, β-1,3-glucan synthase inhibition, pore formation in the growing yeast and the consequential anti-fungal activity) may reflect amino acid sequences similar to HM-1. However, the selected scFv antibodies share no apparent homology to HM-1 in their amino acid sequences. Perhaps, a three-dimensional structure, particularly formed by the V_H domain shared by all four scFv antibodies, may be most responsible for functional mimicry of scFv antibodies. The results clearly indicate the almost identical function of the four scFv antibodies (Table 1).

We used *S. cerevisiae* as the model organism to show the cytocidal effect of scFv anti-idiotypic antibodies. All scFv antibodies strongly inhibit the growth of medically important opportunistic pathogens, for example, the species of *Cryptococcus* (40) and the species of *Candida* (Selvakumar *et al.*, unpublished results). The comparative analysis of the mechanism of cytocidal activity of antibody scFv-A4 and aculeacin A on *S. cerevisiae* in this study clearly indicated the importance of pore formation in the growing end of the cell wall because of defective cell wall glucan synthesis caused by the direct inhibition of β-1,3-glucan synthase (Fig. 3). Aculeacin A and papulacandin B have identical mechanisms of action to kill sensitive yeasts.[6,17] Therefore, the four scFv anti-idiotypic antibodies and two antibiotics are obviously cytocidal against a wide range of yeasts that have cell walls containing glucan.

However, why and how cells treated with scFv anti-idiotypic antibodies and antibiotics form pores in the growing bud might be explainable if we consider the cytocidal effect by HM-1.[6,17] Cell wall synthesis of sensitive yeast cells inhibited by HM-1 is very active in budding cells.[6] HM-1 kills yeast cells by extracellularly inhibiting β-glucan synthase.[4,6,7] With the internal image of HM-1, scFv antibodies kills yeast and fungal cells by same mechanism of inhibiting cell wall β-1,3-glucan synthase (Table 2). The antibiotics used in this study are well-known β-1,3-glucan synthase inhibitors and their mechanism of killing activities is the same mechanism as the mechanism of scFv antibodies (Fig. 3), indicating a strong possibility of using scFv antibodies as novel anti-fungal β-1,3-glucan synthase inhibitors.

The inhibition by each scFv antibody and antibiotic was compared against each of the three different yeasts (Fig. 4). The comparison of the IC_{50} values for each yeast allowed us to identify the most potential inhibitor of individual yeasts and to compare the potential of our newly prepared scFv anti-idiotypic antibodies with the two antibiotics. All antibodies and antibiotics clearly inhibited β-1,3-glucan synthase dose dependently: β-1,3-glucan synthase activity sharply decreased in the membrane fractions of *S. cerevisiae*, *C. albidus* and *C. albicans* with increasing concentrations of scFv antibodies. The IC_{50} values of all scFv antibodies were much lower than for the two antibiotics (Table 2). Compared with antibiotics, the antibodies were more than hundred times more effective in inhibiting β-1,3-glucan synthase. The potential effect of one single inhibitor on β-1,3-glucan synthase was somewhat dependent on the type of yeast used in this study.

Besides aculeacin A and papulacandin B, the echinocandins (caspofungin, micafungin or anidulafungin) antifungal are clinically useful antibiotics that inhibit the fungal growth thorough the inhibition of β-1,3-glucan synthase.[36] Although echinocandins are reported to be active against several medically important fungi, these are relatively ineffective against *Cryptococcus* species.[37—39] However, our previous[40] and present study strongly indicate that HM-1 derived scFv antibodies are able to inhibit potentially β-1,3-glucan synthase and cell growth of *Cryptococcus* species.

In summary, the results of this study showed the capability of recombinant scFv anti-idiotypic antibodies to inhibit β-1,3-glucan synthase, which is the specific target of wide-spectrum anti-fungal drugs. The need for safe and effective antifungal agents increases in parallel with the increasing number of immunocompromised patients at risk from invasive fungal infections. However, currently, an effective drug therapy to treat fungal infections is very limited and its widespread use has led to an increasing incidence of drug resistance. To address this problem, scFv anti-idiotypic antibodies derived from HM-1 have been successfully developed and applied *in vitro* as novel β-1,3-glucan synthase inhibitors. The effectiveness of these anti-idiotypic antibodies is in their characteristics identical with HM-1, which is the anti-fungal activity against wide spectrum of fungi. Our comparative study with two antibiotics has increased the feasibility of using scFv antibodies as potential β-1,3-glucan synthase inhibitory agents to tackle the emergence of microbial infections.

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