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Toward Developing a Universal Treatment for Fungal Disease Using Radioimmunotherapy Targeting Common Fungal Antigens

R. A. Bryan,

Albert Einstein College of Medicine, 1695A Eastchester Rd., Bronx, NY 10461, USA

A. J. Guimaraes,

Albert Einstein College of Medicine, 1695A Eastchester Rd., Bronx, NY 10461, USA

S. Hopcraft,

Albert Einstein College of Medicine, 1695A Eastchester Rd., Bronx, NY 10461, USA

Z. Jiang,

Albert Einstein College of Medicine, 1695A Eastchester Rd., Bronx, NY 10461, USA

K. Bonilla,

Albert Einstein College of Medicine, 1695A Eastchester Rd., Bronx, NY 10461, USA

A. Morgenstern,

Institute for Transuranium Elements, Karlsruhe, Germany

F. Bruchertseifer,

Institute for Transuranium Elements, Karlsruhe, Germany

M. Del Poeta,

Medical University of South Carolina, Charleston, SC, USA

A. Torosantucci,

Istituto Superiore di Sanita, Rome, Italy

A. Cassone,

Istituto Superiore di Sanita, Rome, Italy

J. D. Nosanchuk,

Albert Einstein College of Medicine, 1695A Eastchester Rd., Bronx, NY 10461, USA

A. Casadevall, and

Albert Einstein College of Medicine, 1695A Eastchester Rd., Bronx, NY 10461, USA

E. Dadachova

Albert Einstein College of Medicine, 1695A Eastchester Rd., Bronx, NY 10461, USA

E. Dadachova: ekaterina.dadachova@einstein.yu.edu

Abstract

Background—Previously, we demonstrated the ability of radiolabeled antibodies recognizing the cryptococcal polysaccharide capsule to kill *Cryptococcus neoformans* both in vitro and in infected mice. This approach, known as radioimmunotherapy (RIT), uses the exquisite ability of antibodies to bind antigens to deliver microbicidal radiation. To create RIT reagents which would be efficacious against all major medically important fungi, we have selected monoclonal antibodies (mAbs) to common surface fungal antigens such as heat shock protein 60 (HSP60), which is found on the surface of diverse fungi; beta (1,3)-glucan, which is a major constituent of fungal cell walls; ceramide which is found at the cell surface, and melanin, a polymer present in the fungal cell wall.

Methods—MAbs 4E12, an IgG2a to fungal HSP60; 2G8, an IgG2b to beta-(1,3)-glucan; and 6D2, an IgM to melanin, were labeled with the alpha particle emitting radionuclide ^{213}Bi using the chelator CHXA[®]. B11, an IgM antibody to glucosylceramide, was labeled with the beta emitter ^{188}Re . Model organisms *Cryptococcus neoformans* and *Candida albicans* were used to assess the cytotoxicity of these compounds after exposure to either radiolabeled mAbs or controls.

Results— ^{213}Bi -mAbs to HSP60 and to the beta-(1,3)-glucan each reduced the viability of both fungi by 80–100%. The ^{213}Bi -6D2mAb to melanin killed 22% of *C. neoformans*, but did not kill *C. albicans*. B11 mAb against fungal ceramide was effective against wild-type *C. neoformans*, but was unable to kill a mutant lacking the ceramide target. Unlabeled mAbs and radiolabeled irrelevant control mAbs caused no killing.

Conclusion—Our results suggest that it is feasible to develop RIT against fungal pathogens by targeting common antigens and such an approach could be developed against fungal diseases for which existing therapy is unsatisfactory.

Keywords

C. albicans; *C. neoformans*; Glucan; Hsp60; Melanin; Ceramide; Radioimmunotherapy

Introduction

Fungal diseases are a major threat to individuals with cancer, transplants, HIV, and with other immune compromised conditions. Invasive fungal infections (IFI) cause devastating morbidity and mortality in these at risk patients [1] with the number of cases more than tripling since 1979. There are three main categories of medications used for IFI: polyenes (primarily formulations of amphotericin B), azoles (fluconazole, itraconazole, voriconazole, posaconazole), and echinocandins (caspofungin, micafungin, anidulafungin). Additionally, flucytosine is utilized in combination with amphotericin B for the treatment of cryptococcosis. The number of available medications for mycoses is significantly less than for bacterial diseases and the number of targets of these antifungal medications far fewer (polyenes and azoles: cell membrane sterols; echinocandins: cell wall beta-1,3-glucans; flucytosine: interferes with RNA and DNA synthesis). Furthermore, the echinocandins, the last new class approved by the FDA, have by now been available for nearly 10 years and no

subsequent classes are expected in the foreseeable future. Consequently, new approaches are needed to combat IFI.

Radioimmunotherapy (RIT) utilizes the specificity of the antigen–antibody interaction to deliver lethal doses of ionizing radiation to cancer cells and is currently utilized for the treatment of primary, refractory and recurrent non-Hodgkin lymphoma. RIT has distinct advantages over other drugs including: (1) its cytotoxic nature, which means that RIT not merely abrogates a certain cellular pathway but physically destroys targeted cells or cellular machinery; (2) invulnerability to drug resistance mechanisms; and (3) its independence of the immune status of a host. Our laboratory was the first to demonstrate the potency of RIT for an infectious disease using a radiolabeled mAb specific for the polysaccharide capsule for treating cryptococcal disease in a mouse model [2]. The RIT significantly increased survival times and decreased the colony forming units (CFUs) in infected organs. Moreover, RIT decreased the amount of inflammatory immune cells present in the infected organs [3] and had minimal toxicity to the host [3, 4]. Most recently, we found RIT to be more effective than amphotericin B in reducing cryptococcal fungal burden in mice [5].

At the same time, the major options for fungal therapy are suboptimal as the diagnosis of a particular fungus in clinical setting may be time-consuming and difficult. Thus, an ideal therapy would target multiple fungi without the need for prior diagnosis at species or strain level. Additionally, a therapy that does not lead to the emergence of drug resistance is highly desirable. Fortunately, IFI-causing fungi do share common antigens which also constitute major virulence factors for these fungi, such as beta-glucans, melanin, heat shock protein 60 (Hsp60), and ceramide. β 1,3 glucan (linear β 1,3 linked glucose polymer) is a major component of the cell wall of fungi that is also found on cell wall proteins at the surface of fungi [6]. mAb 2G8, an IgG2b recognizing β 1,3 glucan, binds to the yeast and hyphal forms of *C. albicans* as well as the cell wall of *C. neoformans*, as shown by immunostaining [7]. Treatment with 2G8 mAb increased clearance of *C. neoformans* from the brain and liver of infected mice [7], and prolonged the life of mice infected with *C. albicans* [8]. Heat shock protein 60 (Hsp60) is also found on the surface of diverse fungi. Antibody 4E12, raised against *Histoplasma capsulatum* Hsp60, inhibited the growth of the *H. capsulatum* and did not cross-react with human Hsp60 [9]. Several pathogenic fungi display melanin on their cell surface [10, 11]. Melanin from *C. neoformans* melanin ghosts was used to immunize mice [12]. The resulting IgM 6D2 [13] recognizes melanin from diverse fungi as well as melanin from human melanoma cells [14]. mAb 6D2 has an inhibitory effect on the growth of melanized *C. neoformans* but the antibody is not cytotoxic [13]. mAb B11 binds to glucosylceramide in *C. neoformans* and *C. albicans* but not mammalian glucosylceramide [15]. Here, we present the results of the study of the ability of the radiolabeled mAbs to a variety of highly conserved fungal antigens to kill fungal cells in vitro, using *C. neoformans* and *C. albicans* as model organisms.

Materials and Methods

Microorganisms and mAbs

The mAbs used in the study and their sources are listed in Table 1. 24067 strain of *C. neoformans* was obtained from the ATCC (Manassas, VA). For those experiments using

mAbs 6D2, 4E12, and 2G8, the cells were grown overnight in Sabouraud (SAB) broth at 30°C. To obtain melanized *C. neoformans*, the cells were subcultured from SAB into minimal media containing 1 mM L-DOPA for 4–6 days at 30°C [13]. For experiments using the mAb B11 to GlcCer, the strains used were H99 and an H99-derived deletion mutant missing a gene required for glucosylceramide synthesis (*Cn gcs1*) [16].

C. albicans SC5314 strain was obtained from ATCC. Cells were grown overnight in SAB at 30°C, subcultured 1:200 in fresh SAB, and regrown for another 24 h. A modification of the method of Walker et al. was used to decrease the prevalence of the hyphal forms [17]. Growing cells were pelleted and layered on top of a sucrose gradient composed of 15 ml 2% sucrose, 8 ml of 6% sucrose, and 8 ml of 15% sucrose. The cells went immediately to the 2–6% interface, and were allowed to percolate through the sucrose gradient for 3 h, then the cells above the 15% sucrose cushion were collected and washed twice with PBS. The percentage of multicellular forms (4 or more cells), determined by counting in a hemocytometer, decreased from about 50% of the potential CFU's in the starting culture to 25% following passage over a sucrose gradient. To obtain melanized cells, *C. albicans* cultures were grown for several days in minimal medium containing 5 mM N-acetyl glucosamine and 1 mM L-DOPA [17].

Western Blot and Immunofluorescence for Hsp60 in *C. neoformans* and *C. albicans* Using mAb 4E12

To produce the mitochondrial (M) extracts [18] from *H. capsulatum*, *B. dermatitidis*, *A. niger*, *C. albicans* and *C. neoformans*, cells were lysed using mechanical disruption with liquid nitrogen in a mortar until a white powder was formed. The powder was subsequently thawed in a buffer containing 0.6 M sorbitol, 20 mM HEPES, pH 7.4, and then sonicated for 10 min alternating 30 s pulses and incubations on ice. Cell debris were pelleted down by centrifugation at 1,500×g for 5 min and the collected supernatant was again centrifuged at 1,200×g for 10 min to precipitate the mitochondrial fractions. The brownish pellet was washed with the same buffer and centrifuged at 1,500×g for 5 min. This step was repeated twice and the pellets were resuspended in the same buffer containing 0.6% SDS. Mitochondrial protein preparations were kept at –80°C until use.

Two cell wall (CW) extracts were also evaluated. The first was obtained by β -mercaptoethanol extraction as described in [19]. Briefly, yeasts were pelleted by centrifugation at 1,500×g for 15 min and rinsed three times with phosphate-buffered saline (PBS). Cells were suspended in 20 mM ammonium carbonate (pH 8.6) with 1% β -mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride and vortexed for 1 h at 36°C. Soluble components were separated from the cells by centrifugation at 1,500×g for 15 min. The second cell wall extract was produced as described in [20]. Briefly, yeast cells were centrifuged at 1,100×g for 10 min and pellet washed three times with PBS. Yeasts were killed by treatment for 1 h at 25°C with thimerosal (1:10,000) and suspended in PBS containing protease inhibitor cocktail (Complete Mini–Roche, Mannheim, Germany). Cell disruption was conducted in an ultrasonicator with 10 cycles of 1 min followed by 1 min incubations on ice. The yeast homogenate was centrifuged at 1,100×g for 10 min. Pellet and supernatant were collected and the supernatant centrifuged at 11,000×g for 20 min at 4°C

and isolated. The two pellets were pooled and washed three times using PBS. Then, the particulate material was boiled in 125 mM Tris pH 6.9, containing 6 M urea, 20 mM β -mercaptoethanol and 1% Tween 20 for 10 min. The samples were incubated at 4°C overnight to optimize the solubilization of the material. Supernatant was isolated after centrifugation and the soluble material dialyzed against PBS for 36 h to remove detergent. Immunoblots using 1 μ g/mm of CW/M preparation and mAbs against HSP60 were done as described below. Protein contents were estimated using BSA as a standard [19].

Binding of 4E12 mAb to mitochondrial extracts was assessed by immunoblotting, as described previously [9]. After transfer, membranes containing the different extracts were blocked with a 5% skim milk solution in TBS-T for 1 h. Membranes were washed three times with TBS-T and probed with 5 μ g/ml of 4E12 diluted in blocking solution for 1 h at 37°C. Membranes were washed three times with TBS-T and incubated with a 1:2,000 dilution of an alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates Inc.) in blocking solution for 1 h at 37°C. After washing, membranes were developed with an ECL Western blotting substrate (Pierce Biotechnology) by following the manufacturer's directions. The immunofluorescence of live *H. capsulatum*, *C. neoformans* and *C. albicans* cells was performed as in [9] with a goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Southern Biotechnology Associates Inc.) used for immunofluorescence detection.

Radiolabeling of mAbs

The alpha particle emitter ^{213}Bi was eluted from an 225-Actinium generator, obtained from the Institute for Transuranium Elements, Karlsruhe, Germany [21]. ^{213}Bi was attached to the mAbs, which were conjugated in advance with the CHXA" chelator (Macrocyclics, San Antonio, TX) as previously described [2]. Excess CHXA" was removed by centrifugation through Centricon 50 filters (Millipore), and the conjugated mAbs were stored frozen until use. The beta particle emitter 188-Rhenium (^{188}Re) was eluted from 188-Tungsten generator procured from the Oak Ridge National Laboratory (Oak Ridge, TN). ^{188}Re was bound to the -SH groups generated on the mAbs by reduction with dithiothreitol as previously described [2].

RIT and mAbs Binding

For RIT using ^{213}Bi - (half-life 46 min) labeled mAbs, the fungal cells were prepared as described above, and then incubated with radiolabeled mAbs in PBS for 3 h at 37°C on a rotary shaker. The cells were then spun to remove the supernatant, the supernatant and pellet were counted in a gamma counter to measure the percent of mAbs which were bound by the cells, and the cells were diluted and plated on SAB agar to determine the number of CFUs. The percentage of surviving cells was calculated by dividing the CFUs from treated samples by the CFUs from untreated controls. For RIT with ^{188}Re - (half-life 17 h) labeled mAbs, the cells were suspended in PBS following an initial 3 h incubation at 37°C, and the incubation continued for 2–3 days at 4°C to allow for longer lived ^{188}Re to deliver the radiation dose to the cells and to prevent the division of the cells during the incubation. After that the cells were diluted and plated in duplicate to determine CFUs. The RIT results were analyzed by

one tail Student's *t* test. The differences between the treatment groups were considered significant when $P < 0.05$.

Results

Efficiency of Radiolabeling

The efficiency of radiolabeling of mAbs 2G8 and 4E12 (both IgGs) with ^{213}Bi was $>90\%$. In the case of mAb 6D2 (IgM) only 30% radiolabeling efficiency was achieved, which resulted in overall lower activity delivered to the fungal cells by the radiolabeled mAb. The radiolabeled 6D2 was used without purification from free ^{213}Bi . The attempts to radiolabel mAb B11 (IgM) with ^{213}Bi were not successful, which necessitated the switch to radiolabeling of this mAb with ^{188}Re .

RIT with mAb 2G8, Recognizing Glucan

The treatment of *C. albicans*, whose hyphal forms had been decreased in abundance by passage over a sucrose gradient led to an 80% decrease in the number of CFUs ($P = 0.01$) (Fig. 1a). In preparation of *C. albicans* that had about 50% pseudohyphae, RIT with ^{213}Bi -2G8 resulted in a more modest 30% reduction of CFUs ($P = 0.02$) (Fig. 1b). *C. neoformans* CFUs were reduced 100% by incubation with ^{213}Bi -2G8 (Fig. 1c). Under these conditions, unlabeled (“cold”) 2G8 mAb at the same concentrations had no effect on CFUs ($P = 0.07$) (Fig. 1a,b).

RIT with mAb 4E12, Recognizing Hsp60

As 4E12 mAb was raised against *H. capsulatum* Hsp60—before initiating RIT we performed western hybridization and immunofluorescence to ascertain that it will recognize Hsp60 in other fungi causing IFI. Figure 2a shows a Western blot detecting Hsp60 in the mitochondrial extracts and distinct cell wall fractions of *H. capsulatum*, *C. albicans* and *C. neoformans*. The bands in addition to the main Hsp60 band in Western blots of *H. capsulatum* extracts are probably due to the binding of the mAb to some Hsp60 fragments generated during the cell processing. Figure 2b displays the immunofluorescent images of 4E12 binding to live *H. capsulatum*, *C. albicans* and *C. neoformans* cells. Treatment of *C. albicans* samples containing 25% pseudohyphae with ^{213}Bi -4E12 caused a 90% decrease in CFUs ($P = 0.003$), with no reduction of CFUs following treatment with unlabeled 4E12 at the same concentration ($P = 0.06$) (Fig. 2c). Treatment with ^{213}Bi -4E12 mAb eradicated more than 90% of *C. neoformans* yeast cells ($P = 0.005$), while cold mAb treatment had no effect ($P = 0.1$) (Fig. 2d).

RIT with mAb B11, Recognizing Fungal Glucosylceramide

The IgM mAb B11 recognizing fungal glucosylceramide was labeled with the beta particle emitter ^{188}Re following unsuccessful attempts to label it with ^{213}Bi . The ^{188}Re -B11 decreased the CFUs of wild-type H99 cells by 40% ($P = 0.02$), while radiolabeled control isotype-matching IgM TEPC—did not ($P = 0.2$) (Fig. 3a). The specificity of killing as related to presence of glucosylceramide was further confirmed by inability of either ^{188}Re -B11 or ^{188}Re -TEPC to cause a decrease in the CFUs of the mutant strain lacking the ceramide ($P = 0.1$) (Fig. 3b).

RIT with mAb 6D2, Recognizing Melanin

Due to the poor radiolabeling of ^{213}Bi -6D2, the activities delivered by this mAb to the cells were 3–4 times lower than in the experiments with the other mAbs in this study.

Nevertheless, ^{213}Bi -6D2 had some cidal effect on melanized *C. albicans* cells, causing a 15% decrease in CFUs at the highest dose. However, the same decrease was seen using the cold 6D2 antibody, so the decrease was not significant ($P = 0.06$) (Fig. 4a). ^{213}Bi -6D2 caused a 22% ($P = 0.02$) decrease in CFUs of melanized *C. neoformans*, with no decrease occurring with the cold 6D2 ($P = 0.055$) (Fig. 4b).

Comparison of the Binding of the Various Antibodies

Both *C. albicans* and *C. neoformans* bound about 5% of the mAbs when incubated with radiolabeled 4E12 or 2G8, and about 12% of radiolabeled 6D2. The mAb 18B7 to *C. neoformans* capsular polysaccharide bound to *C. neoformans* cells at 70% under the same conditions (Fig. 5).

Discussion

We have identified several antigens shared by many human pathogenic fungi and shown here that the antibodies to these diverse cell surface antigens were able to deliver cidal amounts of radiation to both *C. albicans* and *C. neoformans* cells. While significant killing of *C. neoformans* was seen with all of the labeled mAbs, ^{213}Bi -labeled 2G8 and 4E12 mAbs to glucan and Hsp60, respectively, produced the best results in killing of both *C. neoformans* and *C. albicans* cells. Unlabeled antibody 2G8 has cidal activity to *C. albicans* [8], but the amount of antibody required to achieve this was more than 1,000 times higher than that utilized in our study, and no killing was detected with antibody to cells ratios that were 60 times higher than employed here. Our results consistently demonstrated the absence of killing by the matching concentrations of unlabeled antibodies, thus proving that the cytotoxic nature of the treatment was due to the presence of the radionuclides conjugated to the mAbs binding fungal antigens.

The cell binding experiments showed that 90–100% loss of CFUs can be achieved by using mAbs that have relatively few binding sites on the cells (4–5% binding, Fig. 5). With alpha particle radiation, a few “hits” per cell are sufficient to cause a cell to lose its ability to replicate. For comparison, 70% binding by antibody 18B7 to the capsular polysaccharide is required to achieve the same level of *C. neoformans* cell killing. The killing we observed with the antibodies to Hsp60 and to glucan therefore has higher efficiency (that is, less radioactivity per cell is needed to achieve killing) than the killing using antibody to the polysaccharide capsule. The antibody 18B7 binds to the cryptococcal capsule, in a spherical manner at a distance from the cell surface [22], and the intervening capsule itself probably provides some protection from the radioactivity. MAb 2G8 to glucan, on the other hand, penetrates the cryptococcal capsule and gets localized adjacent to the cell wall, inside the protective boundary of the polysaccharide, thus delivering cidal radioactivity closer to the cell nucleus and vital cellular machinery.

The radiolabeling results clearly showed the superiority of the IgG-isotype reagents for the universal RIT relative to IgM mAbs, as we consistently encountered difficulties in radiolabeling of IgM-isotype mAbs 6D2 and B11 with ^{213}Bi . In the past, we reported the same difficulty with the IgM mAb 9C7 to *H. capsulatum* [23]. One possible explanation for this could be sterical difficulties of incorporating CHXA" ligand into a complex IgM molecule. Thus, the potential of melanin and glucosylceramide as targets for universal RIT will need to be investigated further when better reagents in the form of IgG-isotype mAbs become available, which generally possess higher affinity.

C. neoformans seemed to be somewhat more sensitive to RIT than *C. albicans*. As 2G8 has been shown to bind well to hyphal forms [8]—the lack of binding is unlikely to be a reason for decreased killing. One possible explanation could be the presence of the pseudohyphae forms in *C. albicans* which could be either more resistant to targeted radiation, or might obstruct the mAbs penetration to all the cells. In the future, we will test the sensitivity of the log phase *C. albicans* to RIT which could be relevant for in vivo infection. We did not observe significant killing of *C. albicans* cells by 6D2, the mAb to melanin, which may be due to the melanin in *C. albicans* being insufficiently externalized [16] for effective mAb binding. Alternatively, the conditions for melanizing *C. albicans* in culture may produce cells that are no longer replicating and are therefore resistant to radiation, which most likely will not be the case in vivo.

Overall, these proof-of-principle in vitro experiments demonstrated the ability of radiolabeled mAbs to shared fungal antigens to deliver cytotoxic radiation to the cells and further in vivo experiments in animal models of IFI are warranted.

Current drugs often fail to eradicate IFI and hence the challenge is to create novel approaches to IFI treatment. By eradicating IFI with universal RIT—we will eliminate the need for specific mycological diagnosis, minimize toxicity of exposure to ineffective antifungals, potentially dramatically shorten treatment durations, and alleviate the need for alteration of immunosuppressive regimens taken by some immunocompromised patients. All existing treatments for IFI are chemotherapeutic drugs which interfere with certain biological processes in fungal cells and rely on the collaboration of patients' immune system. These are inherent drawbacks of antifungal drugs, especially as fungal cells have alternative pathways to the ones targeted by the drugs, which increases the potential for resistance. Plus, immune mechanisms are dysregulated in immunocompromised or patients on immunosuppressants or with sepsis, which reduces the effectiveness of the antifungals. The technology of attaching radionuclides to the mAbs is mature and can be easily translated from the cancer field into infectious diseases. Importantly, nuclear medicine departments in the US hospitals are now routinely treating cancer patients with RIT and are fully equipped for deployment of infectious diseases RIT. Portable imaging equipment is available for imaging patients receiving RIT in intensive care units (ICU) to ascertain the targeting of radiolabeled mAbs. Finally, RIT is known to have significantly lower general toxicity than any chemotherapeutic drugs, which is very important in managing immunosuppressed and cancer patients with IFI.

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Fig. 1. RIT of *C. albicans* and *C. neoformans* using ^{213}Bi -2G8 antibody recognizing glucan: **a** *C. albicans*, with the concentration of pseudohyphae reduced to 20%; **b** *C. albicans*, with 50% pseudohyphae; **c** *C. neoformans*. Each experiment was performed three times, the results shown are from one typical experiment, the CFUs for each antibody dose were plated in triplicate

Fig. 2. RIT with ^{213}Bi -4E12 antibody to Hsp60: **a** Western blot showing binding of 4E12 to mitochondrial and cell wall fractions of *C. albicans* and *C. neoformans*; **b** immunofluorescence of live *H. capsulatum*, *C. albicans* and *C. neoformans* cells with 4E12; **c** RIT of *C. albicans*; **d** RIT of *C. neoformans*. Each experiment was performed three times, the results shown are from one typical experiment, the CFUs for each antibody dose were plated in triplicate. Hc–*H. capsulatum*, Ca–*C. albicans* and Cn–*C. neoformans*

Fig. 3. RIT of *C. neoformans* with ^{188}Re -B11 antibody to glucosylceramide: **a** wild-type *C. neoformans* with ^{188}Re -B11; **b** glucosylceramide-depleted mutant *gcs* with ^{188}Re -B11. Each experiment was performed three times, the results shown are from one typical experiment, the CFU samples for each antibody dose were plated in triplicate

Fig. 4. RIT using ^{213}Bi -6D2 antibody to melanin: **a** *C. albicans*; **b** *C. neoformans*. Each experiment was performed three times, the results shown are from one typical experiment, the CFUs for each antibody dose were plated in triplicate

Fig. 5. Binding of the ^{213}Bi -2G8, ^{213}Bi -4E12 and control ^{213}Bi -18B7 antibodies to *C. neoformans* and *C. albicans* cells and respective killing of the cells. Each experiment was performed three times, the results shown are from one typical experiment, triplicates were used for binding measurements and for CFUs. CN *C. neoformans*, CA *C. albicans*

Table 1

MAbs used in the study and their sources

Antibody	Isotype	Antigen recognized	Reference or source
2G8	IgG2b	Beta (1,3) glucan	[8]
4E12	IgG2a	Hsp60	[9]
6D2	IgM	Melanin	[14]
B11	IgM	Fungal glucosyl ceramide	[15]
TEPC	IgM	Irrelevant control	MP Biomedicals