Published in final edited form as:
Future Microbiol. 2013 September ; 8(9): 1081–1089. doi:10.2217/fmb.13.79.

Radioimmunotherapy of *Cryptococcus neoformans* spares bystander mammalian cells

Ruth A Bryan[1], **Zewei Jiang**[1], **Alfred Morgenstern**[2], **Frank Bruchertseifer**[2], **Arturo Casadevall**[3], and **Ekaterina Dadachova**[*,1,3]

[1]Department of Radiology, 1695A Eastchester Road, Albert Einstein College of Medicine, Bronx, NY, USA

[2]Institute for Transuranium Elements, Karlsruhe, Germany

[3]Department of Microbiology & Immunology, 1300 Morris Park Avenue, Albert Einstein College of Medicine, Bronx, NY 10461, USA

Abstract

Aim—Previously, we showed that radioimmunotherapy (RIT) for cryptococcal infections using radioactively labeled antibodies recognizing the cryptococcal capsule reduced fungal burden and prolonged survival of mice infected with *Cryptococcus neoformans*. Here, we investigate the effects of RIT on bystander mammalian cells.

Materials & methods—Heat-killed *C. neoformans* bound to anticapsular antibodies, unlabeled or labeled with the β-emitter rhenium-188 (16.9-h half-life) or the α-emitter bismuth-213 (46-min half-life), was incubated with macrophage-like J774.16 cells or epithelial-like Chinese hamster ovary cells. Lactate dehydrogenase activity, crystal violet uptake, reduction of tetrazolium dye (2,3)-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-terazolium-5-carboxanilide and nitric oxide production were measured.

Results—The J774.16 and Chinese hamster ovary cells maintained membrane integrity, viability and metabolic activity following exposure to radiolabeled *C. neoformans*.

Conclusion—RIT of *C. neoformans* is a selective therapy with minimal effects on host cells and these results are consistent with observations that RIT-treated mice with cryptococcal infection lacked RIT-related pathological changes in lungs and brain tissues.

Keywords

bystander effects; *Cryptococcus neoformans*; fungal infection; NO production; particulate radiation; radioimmunotherapy

[*]Author for correspondence: Tel.: +1 718 405 8485, Fax: +1 718 405 8457, ekaterina.dadachova@einstein.yu.edu.

Financial & competing interests disclosure: This research was partially funded by the NIH grant AI060507 to E Dadachova. E Dadachova is a Sylvia and Robert Olnick Scholar in Cancer Research. A Morgenstern and F Bruchertseifer are funded by the European Commission. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research: The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Cryptococcus neoformans infections are among the most difficult to treat and lethal infections in HIV-infected individuals, with cryptococcal meningitis causing approximately 600,000 deaths/year in HIV patients in sub-Saharan Africa [1]. In addition, *C. neoformans* is a major pathogen for individuals with an impaired immune system, including organ transplant recipients and cancer patients [2]. *C. neoformans* is a ubiquitous organism that is acquired from the environment by inhalation of fungal spores into the lungs. It disseminates from the lungs by passing through the epithelial cells into the bloodstream and is able to infect the brain by penetrating the blood–brain barrier [3]. Existing treatments are not very effective, require a long course of treatment and often fail to eradicate the infection and thus require life-long therapy.

In the field of medical oncology, radioimmunotherapy (RIT) uses monoclonal antibodies (mAbs), specific for tumor-associated antigens, as vectors for radionuclides. Concentrated at the tumor site, the radionuclides release their tumoricidal dose of radiation to the tumor cells. The feasibility of RIT as a tumor therapy is already established, with US FDA-approved treatments currently clinically applied to primary, relapsed or refractory B-cell non-Hodgkin's lymphomas. We have pioneered RIT for the treatment of infectious diseases, including fungal infections. RIT for infectious diseases involves the delivery of particulate radiation to the microorganisms via microorganism-specific mAbs [4]. Previous studies have shown that RIT prolongs survival and lowers fungal burden in mice infected with *C. neoformans* [5]. RIT was effective in infected mice on two different genetic back-grounds: the AJC/r strain with reduced immune function and immunocompetent C57Bl6 mice [6]. The residual cryptococcal cells surviving post-RIT treatment in mice due to their intracellular location have been shown to be susceptible to the subsequent rounds of RIT, proving that RIT does not select for radiation-resistant mutants [7].

The mAb 18B7, used in the current study and previous studies, is a murine monoclonal IgG1 that binds to the polysaccharide glucuronoxylomannan, a major component of the *C. neoformans* capsule [8]. mAb 18B7 is opsonizing, allowing phagocytic cells to recognize and ingest microbes. The cryptococcal cells can be killed by the phagocytes, while the phagocytes themselves could be killed by the cryptococcal cells. In addition, cryptococcal cells can replicate within phagocytic cells and are then extruded, without damage to either themselves or the phagocytic cell [9]. Consequently, it is important to determine whether the phagocytic cells are damaged by ingested radioactivity bound to *C. neoformans*. Epithelial cells could also be affected by radiation as they can take up or be invaded by *C. neoformans* [3] and may come into close contact with *C. neoformans* carrying radioactive antibodies and be killed or damaged by ‘crossfire’ radiation. To study the effects of particulate radiation emanating from the antibodies bound to the cryptococcal capsule on epithelial and phagocytic cells, we utilized two mammalian cell lines: Chinese hamster ovary (CHO) cells, which have long been used for characterizing radiation damage, and J774.16 cells, a mouse macrophage-like line capable of nitric oxide (NO) production, which is a major component of the macrophage defensive arsenal. We employed four assays to assess the health of the mammalian cells: NO production assay; crystal violet assay as a measure of the cellular ability to proliferate; lactate dehydrogenase (LDH) assay for evaluating both cell proliferation and membrane integrity; and the tetrazolium dye (2,3)-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-terazolium-5-carboxanilide (XTT) assay, which is capable of assessing cellular metabolic status and is indicative of membrane integrity and mitochondrial activity. We found no evidence of damage to the epithelial or macrophage-like cells by the radiolabeled mAb bound to *C. neoformans*.

Materials & methods

Cells

C. neoformans strain 24067 was procured from ATCC (VA, USA). J774.16 cells are constantly maintained in our laboratories. They were propagated in Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS; Sigma, MO, USA) on Petri plates and passaged by scraping the cells up and diluting them into fresh media. CHO cells were obtained from the laboratory of J Pollard (Albert Einstein College of Medicine, NY, USA) and were propagated in DMEM with 10% FBS, and passaged by trypsinization. *Pseudomonas oleovorans* was obtained from ATCC.

Radiolabeling of 18B7 mAbs & radiolabeled mAb binding to *C. neoformans* cells

mAb 18B7, an IgG1 recognizing the polysaccharide capsule of *C. neoformans* [8], was labeled 'directly' with rhenium-188 (^{188}Re; 16.9-h physical half-life) eluted from a tungsten-188 generator (Oak Ridge National Laboratory, TN, USA) via reduction of some disulfide bonds on the antibody with dithiothreitol (Sigma), as described previously [5]. Labeling with bis-muth-213 (^{213}Bi; 46-min physical half-life) was accomplished by first attaching the ligand trans-cyclohexyldiethylenetriamine pentaacetic acid derivative CHXA-DTPA (Macrocyclics, TX, USA) to the antibody, then incubating with ^{213}Bi eluted from an actinium-225 generator (Institute for Transuranium Elements, Germany) [5]. For use as unlabeled controls in the cell treatment experiments, the 18B7 mAb was either treated with dithiothreitol without addition of ^{188}Re, or conjugated to CHXA"-DTPA without subsequent addition of ^{213}Bi. Following the radiolabeling, the antibodies were incubated with the heat-killed (70°C for 1 h) *C. neoformans* for 30 min, then the unbound antibodies were removed by centrifugation and the *C. neoformans* was added to the wells with the mammalian cells. We used heat-killed *C. neoformans* for radiation delivery in order to avoid the possible effects of viable *C. neoformans* on the mammalian cells, which could mask the radiation effects.

NO production

We performed several preliminary experiments to find the linear range of the assay where changes in NO concentration would be proportional to changes in cell number. Increasing the cell number from 25,000 to 75,000 cells/well produced a small increase in NO production, whereas there was a large increase in the wells with 75,000–100,000 cells (Figure 1A). Therefore, 100,000 cells/well were used in all experiments with the *C. neoformans* and mammalian cells. NO production was inhibited in the presence of aminoguanidine, an inhibitor of NO synthase, demonstrating that the nitrate measured was actually dependent on NO produced by the NO synthase (Figure 1A). NO production was dependent on the presence of lipopolysaccharide (Sigma) and FBS (not shown). We measured NO production at 20, 44 and 72 h in the presence of 1, 3 or 10% FBS, following addition of stimulus to the wells. With 10% FBS, NO production peaked at 24 h and declined after that. For 3% FBS, the highest levels of NO were detected at 48 h and stayed at that level up to 72 h, prompting us to use 3% FBS in the experiments with the *C. neoformans* and J774.16 cells.

To study the interaction of J774.16 cells with the radiation emanating from the antibodies on *C. neoformans*, J774.16 cells in DMEM/F12 were plated in 96-well plates at 10^5 cells/well and incubated overnight in the presence of 10% FBS and 500 U/ml IFN-γ (Cell Sciences, MA, USA) to induce adherence. On the following day, media was replaced with DMEM/F12 without phenol red, containing 3% FBS, 500 U/ml IFN-γ and 3 μg/ml lipopolysaccharide. Heat-killed *C. neoformans* bound to the radiolabeled antibodies was then added to the monolayers at a multiplicity of infection (MOI) of 2. For ^{213}Bi-labeled *C.*

neoformans, the supernatant was collected 48 h after addition of the *C. neoformans* to the wells, and for ^{188}Re-labeled *C. neoformans*, supernatant was collected at 72 h. NO has a half-life of only a few seconds, but can be converted to nitrate, which is stable in serum [10,11]. In turn, nitrate is converted to nitrite by 90-min treatment with nitrate reductase from cell extracts of *P. oleovorans*, as described by Granger *et al.* [11]. Nitrite was measured adding Griess reagent, 1% sulfanilamide, 0.1% *N*-1-naphthalenediamine and 2.5% phosphoric acid. Absorbance was measured at 535 nm and nitrite concentration in the cell supernatant was calculated from a standard curve of optical density (OD) as a function of nitrite.

Crystal violet assay

To determine the linear range for the crystal violet assay, we grew monolayers in 96-well plates with increasing numbers of cells. After 24-h growth, the assay was linear from 2250 to 40,000 cells/well. After 48-h growth, dye uptake was linear from 2250 to 17,000 cells/well; and after 72-h growth was recorded to be from 2250 to approximately 5000 cells/well (Figure 1B). The crystal violet uptake levels reached a plateau above the higher limits, probably because the cells had reached their growth limit.

Monolayers of CHO cells were grown up for 24 h in 96-well plates, then exposed for 12–72 h to heat-killed *C. neoformans* carrying radioactively labeled antibodies, at a MOI of 2. Monolayers were then washed and fixed with 100% ethanol, and crystal violet at 5% was added for 30 min, as described previously [12]. The crystal violet solution was removed and the cells were washed repeatedly in water. A total of 100 μl of ethanol was added to the wells to solubilize the crystal violet, 50 μl were removed and the OD at 595 nm was measured. For J774.16 cells, 50,000 cells/well were grown overnight, exposed to radiolabeled *C. neoformans* at a MOI of 2 and assayed for cell proliferation using crystal violet uptake as above.

LDH assay

Dose–response curves were generated to define the linear range of the assay as a function of starting cell number. LDH activity was very low in media from unlysed, untreated cells, and was linear as a function of cell number for wells seeded with 12,500–100,000 cells/well. To measure the total amount of LDH present within the cells, cells were lysed to release all LDH, using the lyzing reagent from the Roche Diagnostics kit (Germany). The amount of LDH in lysed cells was linear for wells seeded with 6250–50,000 cells/well for both CHO cells (Figure 1C) and for J774.16 cells (Figure 1D).

Fifty thousand J774.16 cells/well were grown overnight in 96-well plates with 500 U/ml IFN-γ to induce adherence. A total of 50,000 CHO cells/well were grown in media without IFN-γ. One hundred thousand heat-killed *C. neoformans* cells, with varying amounts of radioactively labeled or unlabeled 18B7 mAbs, were added to the J774.16 or CHO cells after 24 h. The cells were incubated for another 24 h, then assayed for LDH activity using the LDH cytotoxicity detection kit from Roche Diagnostics. Controls included untreated cells, cells treated with heat-killed *C. neoformans* and no antibodies and cells lysed to release all LDH.

XTT assay

The XTT assay was performed as described previously, with some modifications [13]. Preliminary experiments demonstrated that the XTT assay was linear in wells that had been seeded with 2000–30,000 cells/well and grown for 24 h. After 48-h growth, there were two linear portions of the response curve, one for wells seeded with up to 12,000 cells/well, and the second portion, with a different slope, for wells seeded with 12,000–22,000 cells/well.

After 72 h, the curve was linear from 2000 to 5000 cells/well (Figure 1E). The variations in the values at day 3 for the wells seeded with more than 10,000 cells/well were most probably caused by some senescence of the cells. CHO cells were seeded at 10,000 cells/well in 96-well plates in DMEM with 10% FBS and without phenol red. J774.16 cells at 10,000 cells/well were treated with 500 U/ml IFN-γ in order to make them adherent. The cells were grown up overnight, then heat-killed *C. neoformans* cells, at 10^5 cells/well with bound radiolabeled or unlabeled antibodies, were added and incubated for 24 h (^{213}Bi radiolabel) antibody) or 72 h (^{188}Re radiolabel). Wells were then washed and fresh media was added, along with 50 μl XTT (Sigma) at 1 mg/ml in phosphate buffered saline and 4 μl menadione (Sigma) at 1 mM in acetone. Cells were incubated for another 3 h, and the OD at 492 nm was read.

Statistical analyses

All assays were performed twice for both radionuclides, at a range of antibody concentrations, with three to six wells for each condition. The difference in the assay readouts between the various groups were analyzed by the two-tailed Student's t-test, with p-values of <0.05 considered statistically significant.

Results & discussion

NO production, a major defense of macrophage cells, is stimulated by the presence of the polysaccharide glucuronoxylomannan, a major component of the capsule of *C. neoformans*, and by the presence of heat-killed *C. neoformans* (Figure 1A). Our goal was to determine whether radioactivity emanating from the radiolabeled mAbs bound to the capsule of *C. neoformans* ingested by phagocytic cells would alter the ability of the cells to produce NO. We found that NO production was not decreased by either ^{213}Bi-labeled 18B7 or ^{188}Re-labeled 18B7 mAbs bound to heat-killed *C. neoformans* (Figure 2A & 2B).

As the level of the crystal violet dye uptake reflects the total number of cells, it can be used as a measure of cell proliferation. Any treatment that interferes with the ability of the cells to replicate is expected to cause a decrease in the crystal violet uptake. We found that crystal violet staining of CHO cells was not affected by the ^{213}Bi- or ^{188}Re-labeled 18B7 antibodies delivered by heat-killed *C. neoformans* (Figure 3A & 3B). The crystal violet uptake by J774.16 cells was not affected by ^{213}Bi-labeled 18B7 (Figure 3C). We were unable to evaluate crystal violet uptake by J774.16 cells following treatment with ^{188}Re-labeled 18B7, as the J774.16 cells lost adherence by the 72-h time point needed for treatment with ^{188}Re-labeled 18B7.

LDH is released from cells with leaky cell membranes and its detection in growth media is therefore indicative of cell damage. Levels of LDH released by CHO cells were not changed by the presence of heat-killed *C. neoformans* carrying either ^{213}Bi- or ^{188}Re-labeled 18B7, or unlabeled antibodies on its surface (Figure 4A & 4B). The same result was observed for J774.16 cells exposed to ^{213}Bi radiation (Figure 4C). We therefore concluded that the cells were not lysed by the radiation exposure. Similarly, the XTT assay detected no change in the reduction of XTT by CHO cells following incubation with heat-killed *C. neoformans* carrying either ^{213}Bi-or ^{188}Re-labeled 18B7 or unlabeled antibodies (Figure 5A & 5B). XTT levels remained stable following the exposure of J774.16 cells to ^{213}Bi delivered by heat-killed *C. neoformans* (Figure 5C).

In our previous studies on RIT treatment of mice that were infected either systemically and intratrachially with *C. neoformans*, we did not detect radiation damage via histological analyses of their lungs and brains – the organs where *C. neoformans* predominantly localizes during infection [6,14,15]. The current study was performed to take advantage of the

possibility of analyzing the early effects of bystander radiation on a large number of cells available in tissue culture, compared with the relatively few cells examined using histology following survival RIT studies *in vivo*. We assessed several different parameters of cell health, such as NO production, cellular ability to proliferate, membrane integrity, cellular metabolic status and mitochondrial activity. We used both the short-range α-emitter ^{213}Bi and the long-range β-emitter ^{188}Re, which have different emission ranges in tissues (μm vs mm, respectively) for labeling of the *C. neoformans*-specific mAbs. We expected that ^{188}Re might have a larger effect on mammalian cells than ^{213}Bi by virtue of its longer emission range. However, no assays used in this study showed any damage to the bystander cells by either radionuclide.

Strikingly, this absence of damage to the epithelial or macrophage-like cells was observed in the presence of doses of radiation that have been shown to be lethal in RIT of *C. neoformans* itself [16,17]. Possible explanations for these results are the following: targeted radiation (e.g., when the radioactivity is delivered directly to the target) is more likely to kill than bystander radiation. Fungal cells are smaller targets than mammalian cells and radiation delivered to their smaller volumes could conceivably do greater damage. In the field of oncology, the radiolabeled mAbs used for the treatment of certain types of cancer, such as non-Hodgkin's lymphoma, have demonstrated their efficacy and safety in patients, in spite of very pronounced uptake in such organs as the liver, spleen or kidneys.

Conclusion

Our findings show that RIT of *C. neoformans* is a selective and safe treatment that has potential for translation into the clinic.

References

Papers of special note have been highlighted as:

▪ of interest

▪ ▪ of considerable interest

1▪. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS. 2009; 23(4):525–530. Demonstrates the importance of *Cryptococcus neoformans* for public health. [PubMed: 19182676]

2. Sun HY, Alexander BD, Lortholary O, et al. Unrecognized pretransplant and donor-derived cryptococcal disease in organ transplant recipients. Clin Infect Dis. 2010; 51(9):1062–1069. [PubMed: 20879857]

3. Filler SG, Sheppard DC. Fungal invasion of normally non-phagocytic host cells. PLoS Pathog. 2006; 2(12):e129. [PubMed: 17196036]

4. Dadachova E, Casadevall A. Radioimmunotherapy of infectious diseases. Semin Nucl Med. 2009; 39(2):146–153. [PubMed: 19187806]

5▪ ▪. Dadachova E, Nakouzi A, Bryan RA, Casadevall A. Ionizing radiation delivered by specific antibody is therapeutic against a fungal infection. Proc Natl Acad Sci USA. 2003; 100(19): 10942–10947. Proof-of-principle study of the applicability of radioimmunotherapy (RIT) to the field of infectious diseases. [PubMed: 12930899]

6. Jiang Z, Bryan RA, Morgenstern A, Bruchertseifer F, Casadevall A, Dadachova E. Treatment of early and established *Cryptococcus neoformans* infection with radiolabeled antibodies in immunocompeten mice. Antimicrob Agents Chemother. 2012; 56(1):552–554. [PubMed: 22005995]

7▪. Bryan RA, Jiang Z, Huang X, et al. Radioimmunotherapy is effective against high-inoculum *Cryptococcus neoformans* infection in mice and does not select for radiation-resistant cryptococcal cells. Antimicrob Agents Chemother. 2009; 53(4):1679–1682. Proves that RIT of *C. neoformans* does not result in the selection of radiation-resistant cells. [PubMed: 19139285]

8. Casadevall A, Cleare W, Feldmesser M, et al. Characterization of a murine monoclonal antibody to *Cryptococcus neoformans* polysaccharide that is a candidate for human therapeutic studies. Antimicrob Agents Chemother. 1998; 42(6):1437–1446. [PubMed: 9624491]

9. Alvarez M, Casadevall A. Cell-to-cell spread and massive vacuole formation after *Cryptococcus neoformans* infection of murine macrophages. BMC Immunol. 2007; 8:16. [PubMed: 17705844]

10. Rivera J, Mukherjee J, Weiss LM, Casadevall A. Antibody efficacy in murine pulmonary *Cryptococcus neoformans* infection: a role for nitric oxide. J Immunol. 2002; 168(7):3419–3427. [PubMed: 11907100]

11. Granger DL, Hibbs JB Jr, Broadnax LM. Urinary nitrate excretion in relation to murine macrophage activation: influence of dietary l-arginine and oral NG-monomethyl-l-arginine. J Immunol. 1991; 146(4):1294–1302. [PubMed: 1991968]

12. Schweitzer AD, Howell RC, Jiang Z, et al. Physico-chemical evaluation of rationally designed melanins as novel nature-inspired radioprotectors. PLoS ONE. 2009; 4(9):e7229. [PubMed: 19789711]

13. Martinez LR, Bryan RA, Apostolidis C, Morgenstern A, Casadevall A, Dadachova E. Antibody-guided alpha radiation effectively damages fungal biofilms. Antimicrob Agents Chemother. 2006; 50(6):2132–2136. [PubMed: 16723575]

14▪▪. Bryan RA, Jiang Z, Howell RC, et al. Radioimmunotherapy is more effective than antifungal treatment in experimental cryptococcal infection. J Infect Dis. 2010; 202(4):633–637. Important back-to-back demonstration of the effectiveness and safety of RIT when compared with the current antifungal drugs. [PubMed: 20594103]

15▪▪. Dadachova E, Bryan RA, Frenkel A, et al. Evaluation of acute hematological and long-term pulmonary toxicity of radio-immunotherapy of *Cryptococcus neoformans* infection in murine models. Antimicrob Agents Chemother. 2004; 48(3):1004–1006. First *in vivo* study showing the safety of RIT with regards to the lungs and bone marrow. [PubMed: 14982795]

16. Dadachova E, Howell RW, Bryan RA, Frenkel A, Nosanchuk JD, Casadevall A. Susceptibility of the human pathogenic fungi *Cryptococcus neoformans* and *Histoplasma capsulatum* to gamma-radiation versus radioimmunotherapy with alpha- and beta-emitting radioisotopes. J Nucl Med. 2004; 45(2):313–320. [PubMed: 14960655]

17. Bryan RA, Huang X, Morgenstern A, Bruchertseifer F, Casadevall A, Dadachova E. Radio-fungicidal effects of external gamma radiation and antibody-targeted beta and alpha radiation on *Cryptococcus neoformans*. Agents Chemother. 2008; 52(6):2232–2235.

Executive summary

- Radioimmunotherapy (RIT) delivered to the fungal cells by antibodies specific to fungal capsule did not damage bystander Chinese hamster ovary or J774.16 mammalian cells:
  - Crystal violet uptake and (2,3)-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-terazolium-5-carboxanilide (XTT) assays demonstrated that there was no loss of the cells' ability to grow, measured by total crystal violet uptake and reduction of XTT.
  - Lactate dehydrogenase assay showed that there was no damage to cell membranes, as measured by release of lactate dehydrogenase.
  - XTT measurements ascertained that there was no loss of cellular energy, as measured by XTT activity.
  - Nitric oxide assay demonstrated that there was no loss of the J774.16 cells' ability to make nitric oxide in response to the presence of Cryptococcus neoformans.
- RIT of *C. neoformans* is a selective therapy with minimal effects on host cells and these results are consistent with observations that RIT-treated mice with cryptococcal infection lacked RIT-related pathological changes in lung and brain tissues.

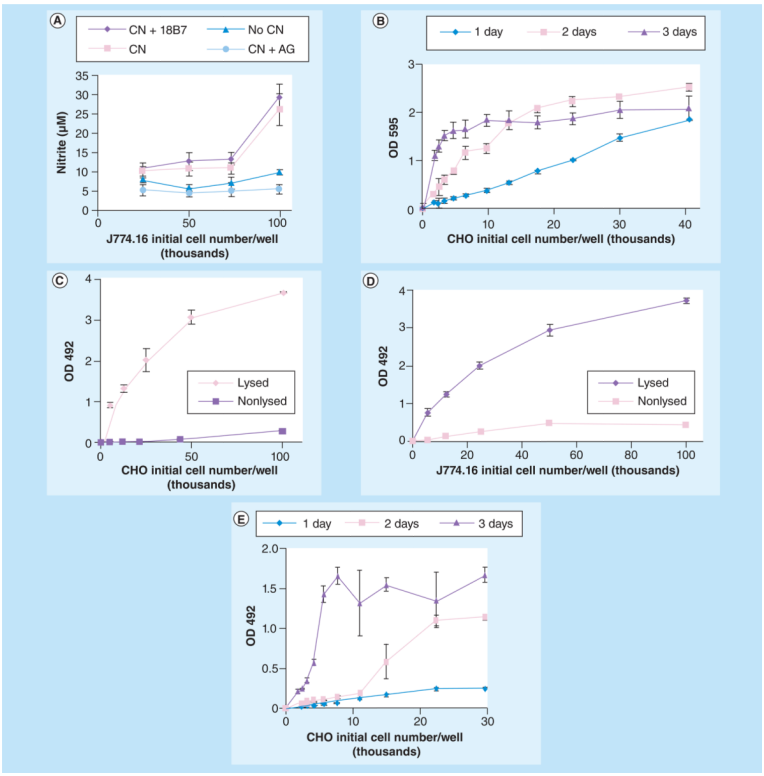

Figure 1. Linearity of assays as a function of starting cell number

(A) Nitric oxide production by J774.16 cells grown in 96-well plates in the presence and absence of heat-killed CN, showing inhibition by AG. J774.16 cells were grown for 24 h; CN (at a multiplicity of infection of approximately 2, assuming one doubling of J774.16 cells in the 24-h incubation) and AG (at a final concentration of 2 mM) were added and incubation continued for 24 h before determination of the nitrite concentration. Control wells had cells only and no CN, AG or 18B7 ('No CN'). **(B)** Crystal violet staining of CHO cells in 96-well plates as a function of starting cell number and time of incubation. **(C)** Lactate dehydrogenase activity released by lysed and nonlysed CHO cells. **(D)** Lactate dehydrogenase activity released by lysed and nonlysed J774.16 cells. **(E)** (2,3)-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-terazolium-5-carboxanilide reduction as a function of starting CHO cell number and time of incubation. Error bars in each graph indicate standard deviations.

AG: Aminoguanidine; CHO: Chinese hamster ovary; CN*: Cryptococcus neoformans*; OD 492: Optical density at 492 nm; OD 595: Optical density at 595 nm.

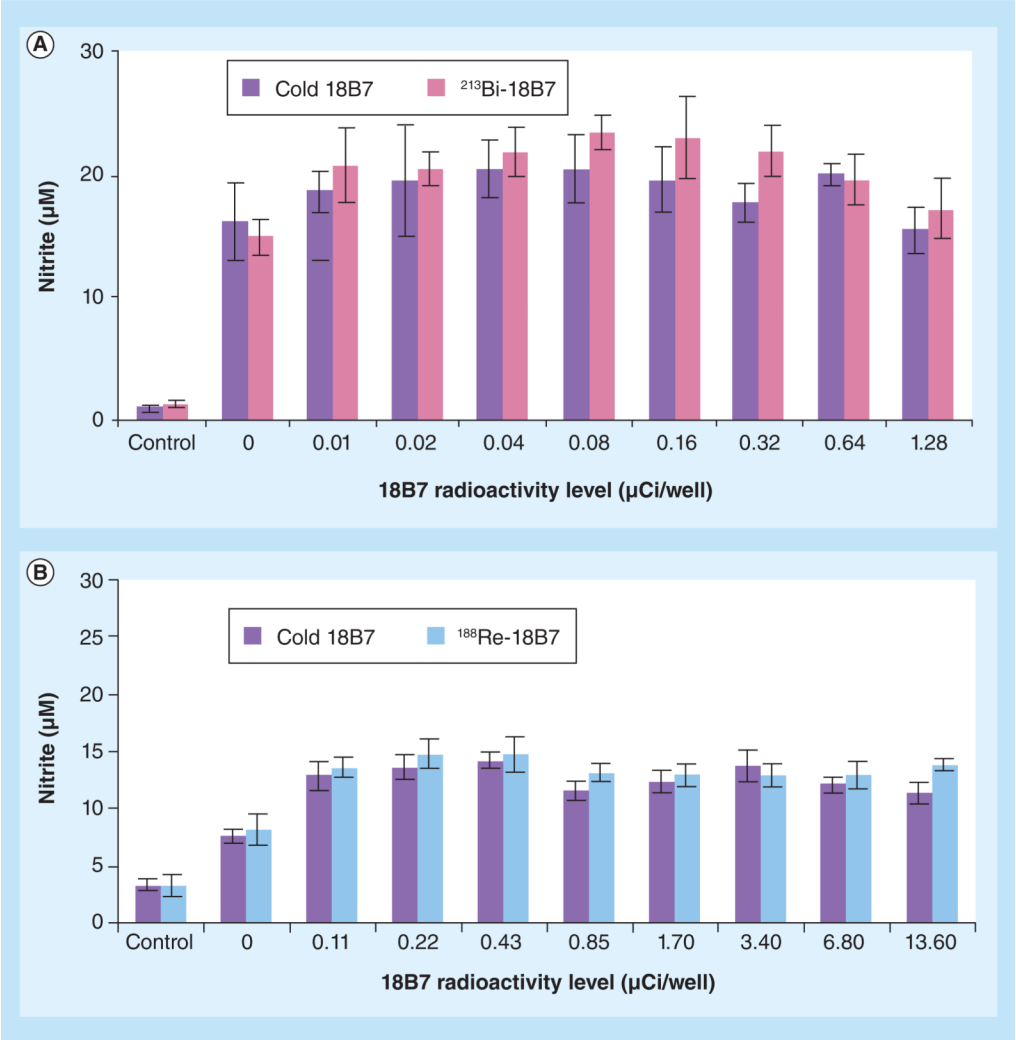

Figure 2. Nitric oxide production by J774.16 cells at 100,000 cells/well in 96-well plates

J774.16 cells were incubated with **(A)** 400,000 cells/well (multiplicity of infection of ~2) of heat-killed *Cryptococcus neoformans* carrying ^{213}Bi-labeled 18B7, or matching amounts of unlabeled 18B7, for 24 h, or **(B)** 400,000 *C. neoformans* cells/well carrying ^{188}Re-labeled 18B7 monoclonal antibodies, or matching amounts of unlabeled 18B7, for 72 h. Control wells had J774.16 cells but no *C. neoformans*. Error bars in each graph indicate standard deviations. ^{188}Re: Rhenium-118; ^{213}Bi: Bismuth-213.

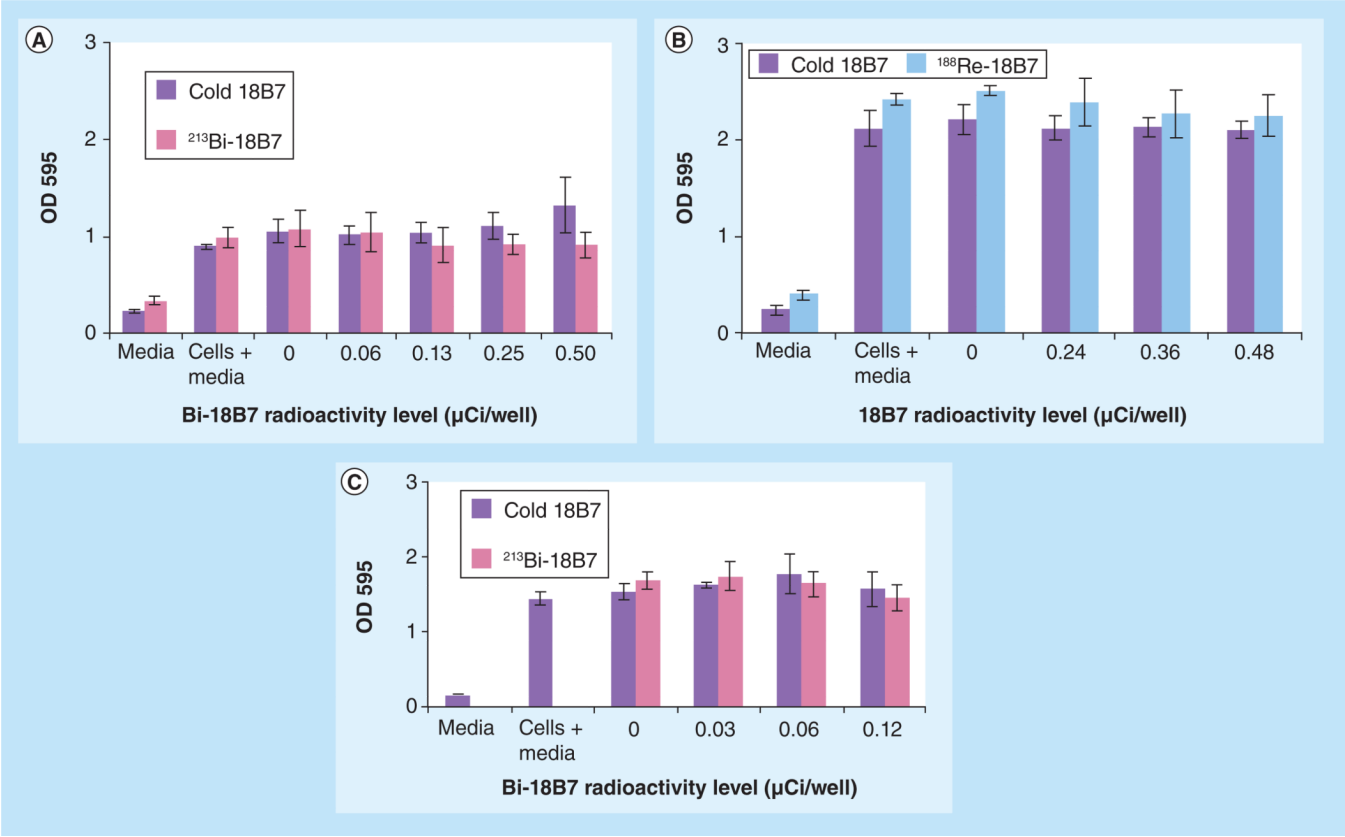

Figure 3. Crystal violet staining of 50,000 Chinese hamster ovary or J774.16 cells/well, incubated for 24 h before addition of 100,000 heat-killed *Cryptococcus neoformans* cells per well, carrying radiolabeled or unlabeled 18B7

(A & B) Chinese hamster ovary (CHO) cells and **(C)** J774.16 cells. **(A)** CHO cells (50,000 cells/well) were grown for 24 h, then incubated for another 24 h with heat-killed *Cryptococcus neoformans* (100,000 cells/well) carrying ^{213}Bi-labeled 18B7 monoclonal antibodies (mAbs), then assayed for crystal violet uptake. **(B)** CHO cells (50,000 cells/well) were incubated for 24 h, then incubated for another 48 h with heat-killed *C. neoformans* (100,000 cells/well) carrying ^{188}Re-labeled 18B7 mAbs. **(C)** J774.16 cells (50,000 cells/well) were incubated for 24 h, then incubated for another 24 h with heat-killed *C. neoformans* (100,000 cells/well) carrying ^{213}Bi-labeled 18B7 mAbs. Control wells had media alone ('Media'), cells and media ('Cells + media') or cells with heat-killed *C. neoformans* carrying unlabeled 18B7 antibody ('Cold 18B7'). Error bars in each graph indicate standard deviations.

^{188}Re: Rhenium-188; ^{213}Bi: Bismuth-213; OD 595: Optical density at 595 nm.

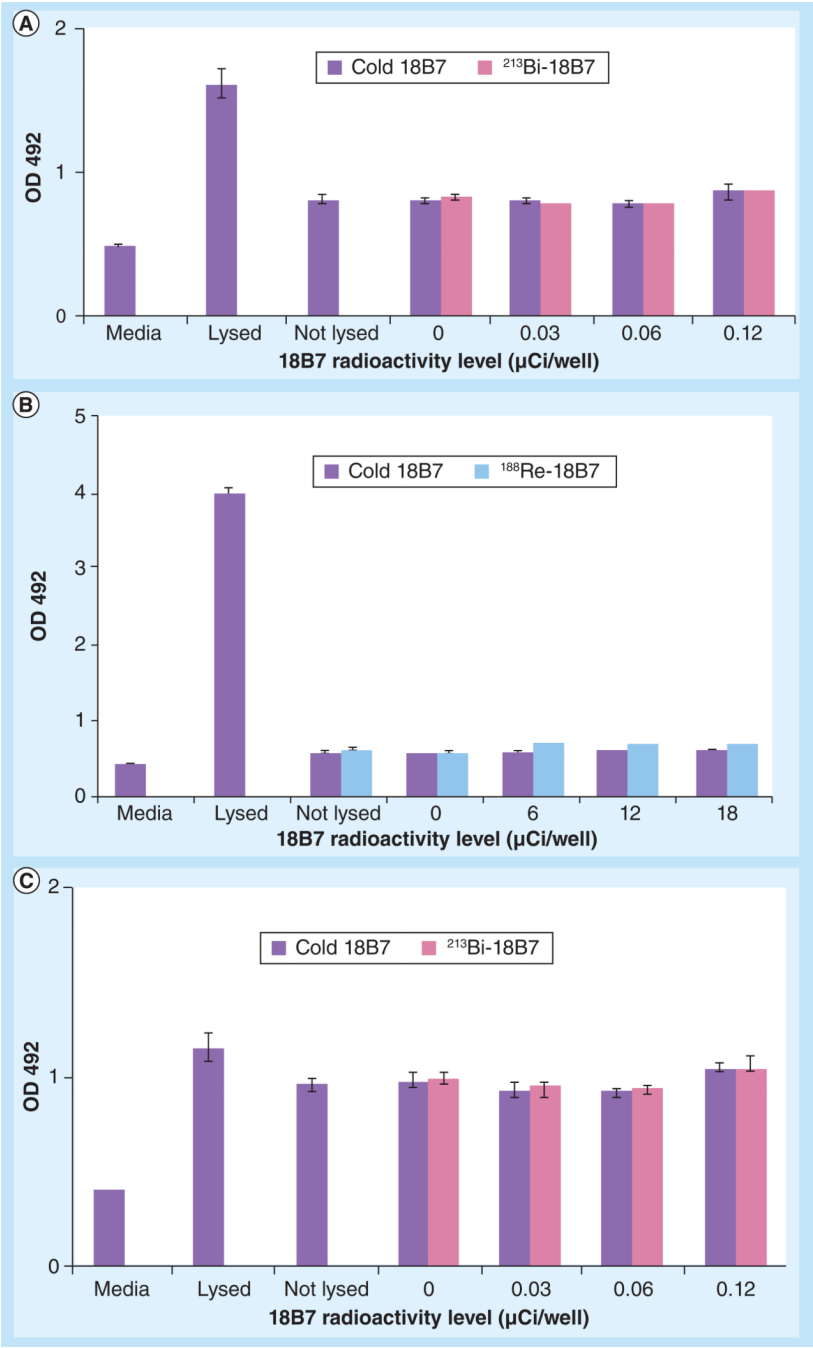

Figure 4. Lactate dehydrogenase released by 50,000 cells/well incubated for 24 h before the addition of 100,000 heat-killed *Cryptococcus neoformans* cells carrying radiolabeled or unlabeled 18B7

(A) Chinese hamster ovary (CHO) cells were incubated with antibodies labeled with ^{213}Bi and lactate dehydrogenase release was measured 24 h after the addition of *Cryptococcus neoformans*. **(B)** CHO cells were incubated for 48 h after the addition of the *C. neoformans* carrying ^{188}Re-labeled antibodies before measurement of lactate dehydrogenase release. **(C)** J774.16 cells were incubated for 24 h after the addition of *C. neoformans* carrying ^{213}Bi-labeled antibodies before measurement of lactate dehydrogenase release. Control wells had media alone ('Media'), media with lysed cells without *C. neoformans* ('Lysed'), media with

cells without *C. neoformans* (‘Not lysed’) or cells with heat-killed *C. neoformans* carrying unlabeled 18B7 antibody (‘Cold 18B7’). Error bars in each graph indicate standard deviations.
^{188}Re: Rhenium-188; ^{213}Bi: Bismuth-213; OD 492: Optical density at 492 nm.

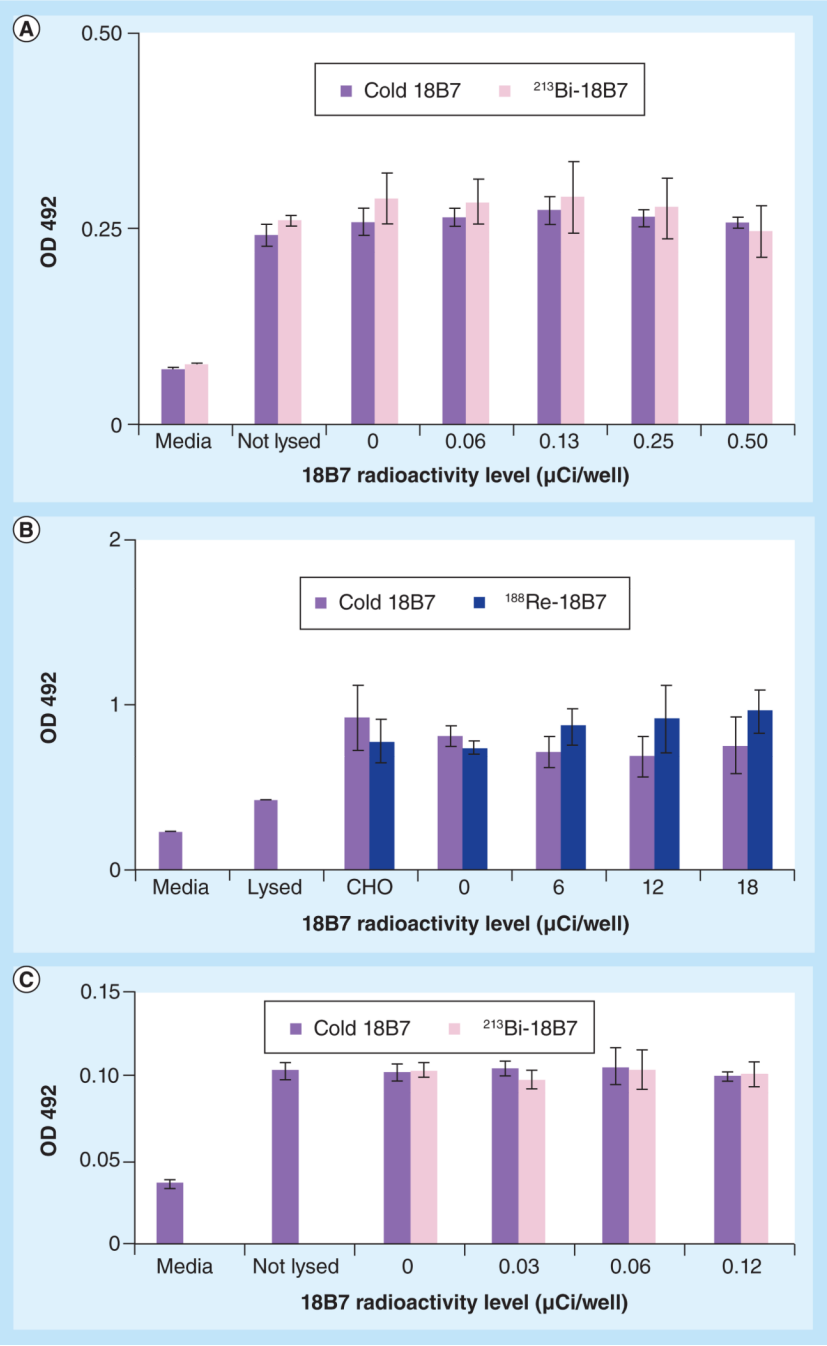

Figure 5. (2,3)-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-terazolium-5-carboxanilide reduction by 50,000 cells/well incubated for 24 h before the addition of 100,000 heat-killed *Cryptococcus neoformans* cells/well

(A) CHO cells were inclubated with antibodies labeled with ^{213}Bi, or unlabeled, and XTT reduction was measured 24 h after addition of *Cryptococcus neoformans*. **(B)** CHO cells were incubated for 48 h after the addition of the *C. neoformans* carrying ^{188}Re-labeled antibodies, or unlabeled, before XTT activity was measured. **(C)** J774.16 cells were incubated for 24 h after the addition of *C. neoformans* carrying ^{213}Bi-labeled antibodies, or unlabeled, before measurement of XTT reduction. Control wells had media alone ('Media'),

media with lysed cells without *C. neoformans* (‘Lysed’) or media with cells without *C. neoformans* (‘Not lysed’). Error bars in each graph indicate standard deviations. ^{188}Re: Rhenium-188; ^{213}Bi: Bismuth-213; CHO: Chinese hamster ovary; OD 492: Optical density at 492 nm; XTT: (2,3)-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-terazolium-5-carboxanilide.