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Detection of *Aspergillus fumigatus* pulmonary fungal infections in mice with ^{99m}Tc-labeledMORF oligomers targeting ribosomal RNA

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Abstract

Purpose—Invasive aspergillosis is a major cause of infectious morbidity and mortality in immunocompromised hosts. The fungus *Aspergillus fumigatus* (*A. fumigatus*) is the primary causative agent of invasive aspergillosis. However, *A. fumigatus* infections remain difficult to diagnose particularly in the early stages due to the lack of a rapid, sensitive and specific diagnostic approach. In this study, we investigated ^{99m}Tc labeled MORF oligomers targeting fungal ribosomal RNA (rRNA) for the imaging detection of fungal infections.

Procedures—Three phosphorodiamidate morpholino (MORF) oligomer (a DNA analogue) probes were designed: AGEN, complementary to a sequence of the fungal 28S ribosomal RNA (rRNA) of *Aspergillus*, as a genus-specific probe; AFUM, complementary to the 28S rRNA sequence of *A. fumigatus*, as a fungus species-specific probe; and cMORF, irrelevant to all fungi species, as a control probe. The probes were conjugated with Alexa Fluor 633 carboxylic acid succinimidyl ester (AF633) for fluorescence imaging or with NHS-mercaptoacetyl triglycine (NHS-MAG3) for nuclear imaging with ^{99m}Tc and then evaluated in vitro and in vivo.

Results—The specific binding of AGEN and AFUM to fungal total RNA was confirmed by dot blot hybridization while specific binding of AGEN and AFUM in fixed and live *A. fumigatus* was demonstrated by both fluorescent in situ hybridization (FISH) analysis and accumulation in live cells. SPECT imaging of BALB/c mice with pulmonary *A. fumigatus* infections and administered ^{99m}Tc labeled AGEN and AFUM showed immediate and obvious accumulation in the infected lungs, while no significant accumulation of the control ^{99m}Tc-cMORF in the infected lung was observed. Compared to non-infected mice, with sacrifice at 1 hour, the accumulation of ^{99m}Tc-AGEN and ^{99m}Tc-AFUM in the lungs of mice infected with *A. fumigatus* were 2 and 2.7 fold higher respectively.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Conclusions—In vivo targeting fungal ribosomal RNA with ^{99m}Tc labeled MORF probes AGEN and AFUM may be useful for *A. fumigatus* infection imaging and may provide a new strategy for the noninvasive diagnosis of invasive aspergillosis and other fungal infections.

Keywords

invasive aspergillosis; *A. fumigatus*; rRNA-targeted probe; phosphorodiamidate morpholino (MORF); SPECT

Introduction

A. fumigatus is one of the most prevalent pathogens causing invasive aspergillosis, a common fungal infection in immune compromised patients and a major cause of mortality [1–3]. Early diagnosis is critical for effective treatment [4, 5]. However, the infection is generally difficult to diagnose due to the lack of a rapid diagnostic test that is both sensitive and specific especially in the early stages of the disease.

Since first introduced by Stahl et al. in 1988 [6], targeting ribosomal RNAs (rRNAs) with oligonucleotide probes have become widely used for the specific detection and identification of bacterial microorganisms in vitro. This approach has more recently been applied to the in vitro detection of fungal infections [7, 8]. Targeting rRNAs has several advantages over, for example, targeting receptors. First, the base sequences of the rRNAs are known for many fungi and consist of variable regions, potentially useful to target specific fungi, and conserved regions that can be used to design universal probes. Second, rRNAs are present in high copy numbers in each replicating and metabolically active cell, providing the potential for a strong signal. Finally, unlike receptors whose expression level may depend upon many confounding factors, rRNAs are present at a constant level. For these reasons, rRNA-targeted probes carrying a radiolabel are being considered here, to our knowledge for the first time, for the detection of specific fungal infection in vivo through SPECT imaging. Because probe stability is critical for in vivo detection and the conventional phosphodiester DNA with a native diester backbone is unstable to nucleases, a DNA analogue was used in this research. Morpholino oligomers (MORFs), are DNA analogs in which the sugar is replaced by a morpholino moiety connected by a phosphorodiamidate linkage [9, 10]. MORFs bind to their complementary DNA or RNA by Watson-Crick base pairing with high affinity, and because of the altered backbone, MORFs are resistant to nucleases [9–12]. Additionally, because of the nonionic structure, MORFs show low binding to serum proteins, and can both enter cells and be cleared rapidly from circulation. For these reasons MORF oligomers have been used successfully by this laboratory for detection of tumors [13–15] and bacterial infection detection through imaging [16].

In this study, we investigated two ^{99m}Tc labeled 28S fungal rRNA-targeted MORF probes, a genus specific probe and a species specific probe, with both sequences selected from the literature on fungal detection in vitro. The probe AGEN was designed as a genus specific probe against *Aspergillus*, and AFUM was designed as a species specific probe against *A. fumigatus*, while the probe cMORF was used as a control probe. The specific binding of the probes in fungi was confirmed by RNA dot blot hybridization, fluorescent in situ hybridization (FISH) and accumulation into live cells. Their biodistributions by sacrifice and by SPECT/CT imaging were performed in BALB/c mice with an *A. fumigatus* infection in the lungs.

Materials and methods

Reagents and fungal strains

All fungi used in this study: *A. fumigatus* (strain ATCC 9197); *Aspergillus flavus* (*A. flavus*, strain ATCC 9643) and *Candida albicans* (*C. albicans*, strain ATCC10231) were from the American Type Culture Collection (ATCC). Sabouraud dextrose broth and Sabouraud dextrose agar were from BD (Franklin Lakes, NJ). Alexa Fluor 633 carboxylic acid succinimidyl ester, salmon sperm DNA, and 50× Denhardt's solution were from Invitrogen (Eugene, OR). RNeasy Plant Mini Kit was from Qiagen (Valencia, CA). Cyclophosphamide and cortisone acetate were from Sigma-Aldrich (St Louis, MO). The NHS-MAG3 was synthesized in house [17]. The technetium-99m (^{99m}Tc) pertechnetate was eluted from a ⁹⁹Mo-^{99m}Tc generator (Perkin Elmer Life Science Inc, Boston, MA). All other chemicals were analytical grade from standard suppliers.

Probes and labeling

Two oligonucleotide probes (Table 1), named here AGEN and AFUM, were selected from sequences identified and confirmed in the literature and further verified in GeneBank. NCBI BLAST software was used to search the target sequences in all other fungi strains to check for genus or species specificity. Oligomers were purchased with the phosphoramidate morpholino backbone with an amine modification on the 3' equivalent end via a 6-aminohexanoic acid linker from GeneTools, LLC (Philomath, OR). Probe AGEN (5'-AGTGCTTTTCATCTTTCGATCAC-linker-amine) is complementary to a sequence in a highly conserved region of the 28S rRNA gene (GeneBank accession no. Z48339) of clinically relevant *Aspergillus* [18], and was used here as a genus-specific probe against *Aspergillus*. Probe AFUM (5'-TGATACATTCCGAG-linker-amine) is complementary to the 28S rRNA of species *A. fumigatus* (GeneBank accession no. U26871), and served as a species-specific probe [19]. In both cases, evidence by hybridization and PCR showed specific binding to their respective DNA transcription templates of their rRNAs. The probe named cMORF (5'-TAGTTGTGAAGTAGAAGA-linker-amine) is an arbitrary sequence used by this laboratory in other studies [15], and has a sequence that is not found within the fungal genome (verified by NCBI BLAST software), and was used here as a control.

The probes were conjugated with Alexa Fluor 633 carboxylic acid succinimidyl ester (AF633) (Invitrogen, Eugene, OR) on the terminal amine for fluorescent in situ hybridization. Briefly, each probe was dissolved in 0.1 M sodium bicarbonate buffer, pH 8.5, at 5 µg/ul. Immediately before use, the AF633 ester was dissolved in N-methylpyrrolidone (NMP, Sigma Aldrich, MO) at a concentration of 10 µg/µl, and 100 µg of the MORFprobe was added to 16 µl of the AF633 solution. The solution was mixed immediately on a vortex at low speed, and then incubated for at least 6 h at room temperature with mixing every half-hour for the first 2 h. The AF633 conjugated probes were then purified on a 0.7 × 20 cm P-2 size exclusion column (Bio-Rad) using 0.25 M ammonium acetate buffer pH 7.0 as eluant.

For radiolabeling with ^{99m}Tc, the amine-modified probes were conjugated with NHS-MAG3 and radiolabeled following the methods used in this laboratory [20, 21]. Radiochemical purity was confirmed by size exclusion HPLC on a Superose-12 column (Amersham Pharmacia Biotech, Piscataway, NJ), using 20% acetonitrile in 0.1 M Tris-HCl pH 8.0 as eluant at a flow rate of 0.6 ml/min. Radioactivity recovery was routinely measured.

RNA extraction and dot blot hybridization

Total RNA was extracted from *A. fumigatus*, *A. flavus* and *C. albicans*, using an RNeasy Plant Mini Kit (Qiagen Sciences, Maryland) using fungi grown in Sabouraud dextrose broth at 30 °C for 24 h. Dot blot hybridization of the ^{99m}Tc -labeled MORF probes to the extracted total RNA was according to that described in Current Protocols in Molecular Biology [22]. In brief, the extracted RNA was denatured by adding three volumes of denaturing solution (500 μl formamide, with 162 μl 12.3 M (37%) formaldehyde, and 100 μl MOPS buffer plus 0.025% (v/v) of bromophenol blue dye to serve as a marker). After heating for 15 min at 65 °C, the denatured RNA samples were placed on ice immediately and two volumes of ice cold 20 $\times$ SSC (3 M sodium chloride, 300 mM sodium citrate, pH 7.0) was added. The denatured RNA samples were transferred onto a nylon membrane (Invitrogen, Eugene, OR) with 3 μg per dot using a Bio-Dot Apparatus (Bio-Rad), and immobilized by UV (254 nm wavelength) treatment for 1 min. The samples were then pre-hybridized by treatment of the membrane with hybridization buffer (0.75 M NaCl, 25 mM sodium dihydrogen phosphate, 2.5 mM EDTA, 5 $\times$ Denhardt solution, 20% (v/v) formamide, 0.5% (w/v) sodium dodecyl sulfate (SDS), 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA) at 42 °C for 2 h. The ^{99m}Tc labeled probes were then added to the hybridization buffer to a concentration providing about $1\text{--}2 \times 10^7\text{cpm}/\text{ml}$. After incubation at 42 °C for 4 h, the membrane was washed twice with 2 $\times$ SSC containing 0.1% SDS for 5 min with rotation at room temperature. Subsequently, the membranes were washed twice with 0.5 $\times$ SSC containing 0.1% SDS as above. Finally the membranes were washed with 0.1 $\times$ SSC containing 0.1% SDS for 10 min at 42 °C on a rotation bed. The membranes were then air dried, sealed in plastic wrap and set on a phosphor screen (Molecular Dynamics) for exposure overnight. The screen was scanned on a Molecular Dynamics Storm 840 Image Analyzer/Scanner (Sunnyvale, CA).

Fluorescent in situ hybridization

A. fumigatus was grown in Sabouraud Dextrose medium at 30 °C, 200 rpm overnight, harvested and washed with PBS, pH 7.2, and then fixed with fresh, cold 3% paraformaldehyde in PBS. Several fixed colonies were placed on a poly L lysine coated slide, air dried, and dehydrated by immersion in a series of ethanol solutions of 50, 80, and 100% (v/v) for 3 min each. Subsequently, a Secure Seal hybridization chamber (Grace Bio-Labs, Inc., Bend, OR) was placed over the fixed colonies. Then 200 μl of PBS containing 0.1% Triton X-100 was added to the hybridization chamber. After incubation for 30 min at room temperature, the fixed colonies were pre-hybridized in hybridization buffer (900 mM NaCl, 20 mM Tris-HCl pH 7.2, 0.01% SDS, 50% (v/v) formamide (Sigma), and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA) at 45 °C for 1 h. Following the pre-hybridization, AF633 conjugated probes were added to the hybridization buffer at a concentration of 2 ng/ μl , and left at 45 °C for 4 h. The excess probes were removed by washing twice with washing buffer (900 mM NaCl, 20 mM Tris-HCl pH 7.2, 0.01% SDS) at 45 °C for 10 min. The slide was air dried and samples mounted with ProLong® Gold anti-fade reagent (Invitrogen). Fluorescence was detected on an Olympus IX 70 Inverted Light Microscope (Olympus America, Inc., NY) using a Cy5 filter.

Accumulation of MORF probes in live *A. fumigatus*

Fungus *A. fumigatus* was grown in Sabouraud dextrose medium at 30 °C, with shaking at 200 rpm until the colonies were about 1 mm in diameter. The colonies were then transferred to a 96-well filtration system plate (MSHVN4B50, Millipore, MA), with about 100 colonies per well. After washing twice with PBS using a Multiscreen vacuum manifold (Millipore), the colonies were incubated with the ^{99m}Tc -labeled probes in PBS (100 μl per well) using several different concentrations (from 0.1 nM to 90 nM, with an $N=5$). After incubation at 37 °C for 2 h, the colonies were washed with PBS containing 0.1% (v/v) Tween-80 three times and then with PBS alone for an additional three times. Finally, the washed colonies on

the filter at the bottom of each well were punched directly into tubes for counting in a gamma well counter Na (TI) (Cobra II Auto-Gamma, Packard Instrument Co, Downer Grove, IL).

A. fumigatus conidia preparation

A. fumigatus was grown on a Sabouraud dextrose agar plate at 30 °C for 1 week, after which time the conidia were collected by flooding the plate with 10 ml Tween-saline (0.9%, w/v, NaCl containing 0.1% (v/v) Tween-80) and drawing a sterile inoculating loop across the agar surface. The suspension was transferred to a sterile tube, mixed vigorously on a vortex to release the conidia, and centrifuged at 5,000 g for 5 min to pellet the single-cell conidia. The conidia were washed with PBS once and resuspended in Tween-saline for a concentration of 10^9 conidia per ml in preparation for administration into mice. Conidia number was counted with a hemocytometer.

Animal experiments

Mice—Male BALB/c mice from Charles River Laboratories International, Inc. (Wilmington, MA), weighing 18 to 22 g, about 8 week-old, were fed standard mouse chow and water, and housed under standard conditions. All animal experiments were approved by the Institutional Animal Care and Use Committee of the university.

Mice infection with *A. fumigatus* conidia—Prior to infection, mice were immunosuppressed according to Svirshchevskaya, et al [23] with some modification. Briefly, cyclophosphamide dissolved in sterile saline at 20 mg/ml was administered intraperitoneally at 200 mg/kg on days -4 and -1, and 2 days after infection. One day before infection, cortisone acetate at 25 mg/ml suspended in sterile PBS containing 0.1% Tween-80 was administered subcutaneously at 125 mg/kg.

The immunosuppressed mice were infected with *A. fumigatus* following the protocol described by Svirshchevskaya, et al. and Dixon, et al. [23, 24]. In brief, mice were anesthetized with isoflurane, and 20 μ l of a suspension containing 2×10^7 conidia in Tween-saline was slowly applied to the nares using a micropipette. During inoculation the mice were held in an upright position until the suspension was completely delivered. Mice were observed and weighed once daily during the study from time of pretreatment.

Biodistribution and SPECT/CT imaging—The biodistribution of ^{99m}Tc labeled probes was evaluated in non-infected mice and in infected mice on day 3 or 4 after infection with *A. fumigatus* conidia. Each mouse received 1 μ g of ^{99m}Tc -labeled MORF probe with 1.1 MBq (specific activity 1.1 MBq/ μ g) in 100 μ l saline via tail vein injection. The mice were sacrificed at 30 min or 1 h after injection, with four mice per time point. After sacrifice, blood and organs of interest were removed, weighed and counted in the NaI(Tl) well counter against a standard of the injectate. All counts were corrected for physical decay.

To confirm that the infection was established in the lungs, the lungs from infected mice were fixed in 4% formalin and embedded in paraffin for histological evaluation. The paraffin sections were stained with Grocott methenamine silver (GMS), a specific fungal stain, and hematoxylin-eosin (HE), a common cell nucleus stain.

Mice infected with *A. fumigatus* were imaged on a NanoSPECT/CTTM small animal imaging camera (Bioscan, Inc., Washington, D.C.) at 1, 2 and 3 h after injection of about 10 μ g with 37 MBq of ^{99m}Tc labeled MORF probes AGEN, AFUM, or cMORF as control in 100 μ l of saline. The specific activity of the probes was about 3.7 MBq/ μ g. The CT scanning was performed first at standard resolution, using a 45 kVp voltage and 500 milliseconds

exposure time. The SPECT image parameters were 1.0 mm/pixel, 256×256 frame size and 60 sec per projection with 24 projections. Acquisition time was approximately 30 min. During imaging, the animals were anesthetized with 1–2 % isoflurane in 1.5 l/min oxygen. The CT and SPECT reconstructions and volume-of-interest (VOI) analysis of the SPECT acquisitions was performed by InVivoScope 1.43 software (Bioscan, Washington, DC).

Results

Confirmation of MORF probe specificity

The radiolabeling efficiency of the MORF oligomers was always greater than 90% as shown by size exclusion HPLC (Fig. 1), with radioactivity recovery always greater than 90%. The specificity of the ^{99m}Tc -labeled MORF probes was confirmed by dot blot hybridization of ^{99m}Tc labeled AGEN, AFUM and cMORF to the total RNA from fungi *A. fumigatus*, *A. flavus* and *C. albicans* (Fig. 2). Phosphor screen exposure of the dot blot membrane showed strong specificity of ^{99m}Tc -AGEN, as a genus-specific probe to the total RNA from both *A. fumigatus* and *A. flavus*, and a weak signal to the total RNA from *C. albicans*. The ^{99m}Tc -AFUM as a species-specific probe showed specificity only for the total RNA of *A. fumigatus*. Thus this probe showed no specificity for the total RNA from both *A. flavus* and *C. albicans* as expected. Also as expected, ^{99m}Tc labeled cMORF as a control showed no specificity for the total RNA from any of the three fungal strains. The figure also shows higher specificity of the genus specific probe AGEN to *A. fumigatus* than the species specific probe AFUM. These results confirm that the sequences taken from the literature and used here with the MORF backbone and radiolabeled with ^{99m}Tc have the ability to bind to their isolated fungal RNA.

Fluorescent in situ hybridization

To evaluate hybridization of the MORF probes to RNA within the fixed fungi, fluorescent in situ hybridization was performed in *A. fumigatus* with the AF633 conjugated MORFs. When compared to the phase-contrast images, the fluorescence shown in red (Fig. 3) indicates binding of both the AGEN and AFUM to the fixed preparations of *A. fumigatus* almost certainly retained there by the hybridization of the MORFs to the fungal RNA. The fluorescence signal is stronger for AF633-AGEN than for AF633-AFUM, which is consistent with the results obtained in the dot blot hybridization study (Fig. 2). Furthermore, no fluorescence was detected with the control AF633 conjugated cMORF. These results further confirm the specific binding of these MORF probes to their respective fungal RNA, now in fixed cells.

Accumulation of MORF probes into live *A. fumigatus*

To demonstrate accumulation of the MORF probes in live fungi, an assay was performed with live *A. fumigatus* and ^{99m}Tc labeled AGEN, AFUM, and cMORF. Live *A. fumigatus* was incubated with increasing concentrations of the ^{99m}Tc -labeled probes. After incubation and removal of the non-specifically bound probes by washing with PBS containing 0.1% Tween-80 and PBS, the accumulation of ^{99m}Tc -labeled probes was evaluated by counting the samples in the NaI(Tl) well counter. As shown in Fig. 4, the percent accumulation of both ^{99m}Tc -labeled AGEN and ^{99m}Tc -labeled AFUM in live *A. fumigatus* was higher than that of the ^{99m}Tc -labeled control cMORF. The difference between the specific and control probe is more pronounced at the lowest probe concentrations, and with the increasing probe concentration the difference between the specific and control probes declines as the binding becomes saturated. Student *t* test ($P<0.05$) showed that the difference between the specific probe AGEN and the control probe was significant ($P<0.05$) at concentrations below 20 nM, and for the specific probe AFUM the difference was significant ($P<0.05$) at concentrations below 10 nM.

Biodistribution of ^{99m}Tc labeled MORF probes

Biodistributions were obtained by sacrifice and dissection at 30 min or 1 h after injection via a tail vein with ^{99m}Tc -labeled AGEN or AFUM in non-infected mice and in mice infected 3 days earlier with *A. fumigatus*. The results are presented in Tables 2 and 3 as the percent of the injected dose per gram of tissue. A lung infection was verified by GMS (Fig. 5) and HE staining of formalin fixed tissues. Both ^{99m}Tc -labeled AGEN and AFUM were cleared rapidly from circulation with no significant accumulations in liver, heart, spleen, stomach, intestine and muscle in either infected or non-infected mice. In particular, accumulation in the non-infected lung was low. In most organs, the accumulation of ^{99m}Tc -AFUM was slightly lower than ^{99m}Tc -AGEN. For both MORFs the highest accumulation was in kidneys in both infected and non-infected mice, with ^{99m}Tc -labeled AGEN accumulating at about twice that observed for ^{99m}Tc -labeled AFUM. As expected, and consistent with our prior experience with MORF oligomers of this size, these MORFs are primarily excreted through the kidneys [13].

The ratio of lung accumulation between infected and non-infected mice increased steadily from 30 min to 1 h for both ^{99m}Tc labeled AGEN and AFUM. The infected to non-infected lung ratio for ^{99m}Tc -AGEN was 1.6 at 30 min and increased to 2.0 at 1 h; while for ^{99m}Tc -AFUM, this ratio was 1.4 at 30 min and increased to 2.7 at 1 h. The student *t* test showed that the differences were significant ($P < 0.05$) for uptake in the lungs between the infected mice and uninfected mice at all time points. Although the ratios for ^{99m}Tc -AGEN were lower than that for ^{99m}Tc -AFUM at 1 h, the percent accumulation in infected lungs was slightly greater with ^{99m}Tc -AGEN than with ^{99m}Tc -AFUM.

SPECT/CT imaging

SPECT/CT imaging was performed on mice infected with *A. fumigatus* 3–4 days earlier. Fig. 6 presents upper body images at 1, 2 and 3 h after injection of ^{99m}Tc -labeled AGEN, AFUM or cMORF. The infected lungs are clearly defined at 1 h with both ^{99m}Tc -AGEN and ^{99m}Tc -AFUM and the signal persists through 3 h in both cases. In addition, the contrast between infected lungs and surrounding tissue increased from 1 h to 3 h, whereas with the control cMORF lung accumulation is less intense at each imaging time. Volume of interest (VOI) quantification for these single images showed that the accumulation of ^{99m}Tc labeled AGEN in infected lungs was 3.23 % ID/g at 1 h, 1.32 % ID/g at 2 h and 0.8 % ID/g at 3 h, while for ^{99m}Tc labeled AFUM the values were 1.5 % ID/g, 0.74 % ID/g and 0.38 % ID/g respectively. For the control ^{99m}Tc -cMORF the respective values were 0.57 % ID/g, 0.23 % ID/g and 0.08 % ID/g. The result is consistent with the in vitro evaluation.

Discussion

A. fumigatus is the primary causative agent of invasive aspergillosis responsible for severe and often fatal infection in immunosuppressed patients, such as those undergoing treatment for cancer, organ transplant or HIV. The incidence of invasive aspergillosis has increased over the years as increasing number of patients undergo treatments that lower their immune response. While early treatment of invasive aspergillosis can be critical to patient survival, treatment at present is often delayed by the time required to confirm the diagnosis. Furthermore, a fungal specific imaging approach could define the location, extent of infection, and potentially the causative agent. Imaging approaches that have been investigated for the detection of fungal infections include CT, MRI, and PET studies with ^{18}F -FDG, and ^{68}Ga -sidephores, and SPECT with agents such as ^{99m}Tc -antimicrobial peptides, ^{99m}Tc -fluconazole, and ^{99m}Tc -chitin [25–30]. In some cases, these agents are highly sensitive but lack either suitable pharmacokinetics or specificity and accuracy, especially in the early stages of infection. In this study, we investigated ^{99m}Tc labeled

MORF probes targeting fungal rRNA for the detection of pulmonary aspergillus infection in a mouse model by SPECT imaging.

The base sequences for MORF probes AGEN and AFUM were taken from the literature and confirmed to be specific by NCBI BLAST software. The probes lengths were not varied in this study from that taken from the literature, except for probe AGEN in which 3 bases were removed from that reported to a sequence length under 25 mer. From our prior experience, the MORF oligomers of 12–25 mer have been most successful in vivo study. The control sequence with a base length of 18 mer between that of AGEN (23 mer) and AFUM (14 mer), and used by this laboratory in previous studies [15] is arbitrary and not found within the fungal genome, thus was used as a control for both probes AGEN and AFUM. The three probes were synthesized using the morpholino backbone chemistry for in vivo stability and low non specific binding. To confirm that the selected sequences with the MORF backbone and carrying ^{99m}Tc via a MAG3 chelator could hybridize to fungal RNA, a dot blot hybridization study on total RNA was performed (Fig. 2). The results were consistent with the observations of others that these sequences target their respective rRNA in vitro [18, 19]. Specifically, the genus-specific probe AGEN bound to the total RNA from *A. fumigatus* and *A. flavus* as expected since both fungi share the same genus. However weak binding was observed to the total RNA from *C. albicans*, an unexpected observation since this fungi belongs to a different genus, *Candida*. However, a further and more extensive NCBI BLAST search of the *C. albicans* complete genome identified a match for the probe AGEN but with a two base mismatch. This overlap most likely explains the low level binding. The species-specific rather than the genus-specific probe AFUM was found to bind only to the total RNA of *A. fumigatus* as expected since AFUM was designed to specifically bind this fungus. However, a stronger signal was observed for probe AGEN than AFUM, possibly because the target region for the probe AGEN may be more readily accessible. No signal and thus no binding was observed with the control, as expected. The specific binding of the MORF probes in *A. fumigatus* was confirmed by fluorescent in situ hybridization (Fig. 3) and accumulation in live cells. Both AF633 labeled AGEN and AFUM recognized specifically *A. fumigatus*, and the results are consistent with the dot blot hybridization results. The specific binding study in live *A. fumigatus* indicated that both ^{99m}Tc labeled AGEN and AFUM showed higher uptake in live fungi than the control cMORF at low concentrations. The differences were less obvious at the higher probe concentrations probably due partly to the binding becomes saturated. Biodistributions of ^{99m}Tc labeled AGEN and AFUM were performed in non-infected mice and in infected mice with *A. fumigatus* in the lungs (Table 2, 3). Both study MORFs showed rapid clearance through the kidneys with minimal accumulation in non-targeted organs, as has been repeatedly observed in this laboratory for MORFs of these lengths [13]. Furthermore HPLC analysis of urine collected from mice following injection of the ^{99m}Tc labeled MORFs indicated that the ^{99m}Tc labeled probes were excreted intact into urine and thus stable in vivo (data not shown), which is consistent with previous studies from this laboratory [13]. Accumulations of both ^{99m}Tc -AGEN and AFUM were higher in the infected lungs than non-infected lungs (Table 2, 3) by about 2 fold and, in both cases, the ratio of accumulation between infected and non-infected lungs increased from 30 min to 1 h. As already noted, the highest radioactivity accumulations were in kidneys. The standard deviations in the infected group with ^{99m}Tc -AGEN at 30 min post injection were high due to the different severity of infections between mice.

SPECT/CT imaging of normal mice with the three ^{99m}Tc -MORFs showed at 1 h after injection no accumulation in the lungs and other tissues except kidneys and bladder (data not shown) as expected. SPECT/CT imaging of *A. fumigatus* infected mice performed with the ^{99m}Tc labeled MORFs clearly showed the infected lungs even at 1 h with both AGEN and AFUM (Fig. 6). The contrast between the lungs and the surrounding tissues was strongest at 2 h for both ^{99m}Tc -AGEN and ^{99m}Tc -AFUM. Furthermore, the VOI

quantification for the images and the biodistribution data showed higher accumulation for ^{99m}Tc -AGEN vs ^{99m}Tc -AFUM in infected lungs, which is consistent with the results from the in vitro evaluation by dot blot hybridization and FISH. More importantly, no important accumulation in the lungs of infected mice of the ^{99m}Tc -control cMORF was observed at any time point.

Conclusion

In summary, in this study we investigated ^{99m}Tc labeled rRNA-targeted MORF probes for detection of *A. fumigatus* infection through diagnostic imaging: a novel strategy utilizing novel probes for the detection and diagnosis of fungal infection in vivo. Our results with the two radiolabeled MORF probes AGEN and AFUM support our strategy of using oligomer probes for the detection and diagnosis of fungal infection in vivo by SPECT imaging. We conclude that in vivo targeting of fungal ribosomal RNA with ^{99m}Tc labeled MORF probes AGEN and AFUM may be useful for *A. fumigatus* infection imaging and may provide a new strategy for the noninvasive diagnosis of invasive aspergillosis and other fungal infections.

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FIGURE 1. Size exclusion HPLC radiochromatograms of ^{99m}Tc labeled AGEN, AFUM and cMORF. Radioactivity recovery was greater than 90% in all cases.

FIGURE 2.

RNA dot blot hybridization using total RNA from *A. fumigatus*, *C. albicans*, and *A. flavus* incubated with ^{99m}Tc labeled: AGEN, AFUM and cMORF in 20 % formamide hybridization buffer. ^{99m}Tc -AGEN, used as a genus-specific probe for the genus *Aspergillus*; ^{99m}Tc labeled AFUM, as a species-specific probe; and ^{99m}Tc labeled cMORF, as a control probe.

FIGURE 3. Fluorescent in situ hybridization in fixed *A. fumigatus* with AF633 labeled AGEN, AFUM and cMORF. The phase-contrast images are shown on the right, and the fluorescence images are on the left. Magnification: 200 $\times$.

FIGURE 4. Percent accumulation with increasing concentrations of ^{99m}Tc -labeled AGEN (panel A), AFUM (panel B), and cMORF in live *A. fumigatus*, N=5.

FIGURE 5.

GMS staining of lung slices from an *A. fumigatus* infected mouse (A) on day 3 after infection with *A. fumigatus* and an uninfected mouse (B). The arrows indicate the mycelium growing mainly in the bronchiolar spaces. Magnification: 100 $\times$.

FIGURE 6. SPECT/CT images (sagittal section) at 1, 2 and 3 h post injection of 37 MBq ^{99m}Tc -AGEN, ^{99m}Tc -AFUM, or ^{99m}Tc -cMORF in mice infected with *A. fumigatus* 3–4 days earlier. The CT scanning was performed at standard resolution, using a 45 kVp voltage and 500 milliseconds exposure time. The SPECT image parameters were 1.0 mm/pixel, 256×256 frame size and 60 sec per projection with 24 projections. Acquisition time was approximately 30 min. During imaging, the animal was anesthetized with 1–2 % isoflurane in 1.5 l/min oxygen.

TABLE 1

MORF oligomer probes used in this study.

Probe	Specificity	Sequence (5'-3')	Reference
AGEN	Genus <i>Aspergillus</i>	AGTGCTTTTCATCTTTCGATCAC	18
AFUM	Species <i>A. fumigatus</i>	TGATACATTCCGAG	19
cMORF	Negative control	TAGTTGTGAAGTAGAAGA	15

TABLE 2

Biodistribution of ^{99m}Tc -AGEN in *A. fumigatus* infected mice and uninfected mice at 30 min and 1 h in percent ID per gram. Presented as the mean of N=3–4 mice with one standard deviation (SD).

ID%/g	Infected mice				Uninfected mice			
	30 min		1 h		30 min		1 h	
	mean	SD	mean	SD	mean	SD	mean	SD
Liver	1.18	0.55	0.55	0.05	0.71	0.05	0.51	0.04
Heart	1.29	0.80	0.28	0.09	0.59	0.05	0.24	0.04
Kidney	19.80	9.24	34.43	2.01	42.44	1.62	48.91	1.25
Lungs	2.39	0.39	0.96	0.13	1.48	0.36	0.49	0.10
Spleen	1.33	0.92	0.80	0.45	0.51	0.08	0.27	0.03
Stomach	1.09	0.77	0.24	0.05	0.80	0.18	0.90	0.55
Small intestine	0.95	0.48	0.51	0.06	1.04	0.18	1.13	0.10
Large intestine	0.87	0.48	0.16	0.06	0.38	0.11	0.23	0.18
Muscle	0.53	0.17	0.12	0.02	0.54	0.28	0.33	0.20
Blood	4.06	3.06	0.55	0.18	1.50	0.22	0.40	0.11

TABLE 3

Biodistribution in percent ID per gram of ^{99m}Tc -AFUM in *A. fumigatus* infected mice and uninfected mice at 30 min and 1 h. Presented as the mean of n=3–4 mice with one standard deviation (SD).

ID%/g	Infected mice				Uninfected mice			
	30 min		1 h		30 min		1 h	
	mean	SD	mean	SD	mean	SD	mean	SD
Liver	0.52	0.08	0.27	0.02	0.51	0.09	0.26	0.03
Heart	0.64	0.07	0.18	0.02	0.57	0.15	0.13	0.03
Kidney	14.94	2.08	12.82	0.77	12.55	0.55	12.23	0.94
Lungs	1.67	0.19	1.04	0.24	1.17	0.25	0.39	0.09
Spleen	0.74	0.15	0.33	0.07	0.39	0.04	0.17	0.02
Stomach	0.49	0.08	0.21	0.02	0.88	0.23	0.31	0.14
Small intestine	0.70	0.02	0.51	0.14	1.37	0.30	1.14	0.15
Large intestine	0.32	0.04	0.12	0.01	0.31	0.08	0.13	0.05
Muscle	0.46	0.07	0.11	0.03	0.42	0.17	0.08	0.01
Blood	1.90	0.14	0.51	0.06	1.68	0.42	0.31	0.07