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Research Article

Peptidoglycan aptamers biodistribution in infection-bearing mice

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Abstract

Introduction: Acid Nucleic aptamers are short single-stranded oligonucleotides that display high affinity and selectivity for a given target. Aptamers contain many features that are advantageous for radiopharmaceuticals development. Peptidoglycan is a cell wall polymer common to both Gram-positive and Gram-negative bacteria. In the present study, the potential of two peptidoglycan aptamers for bacterial infection foci identification was evaluated.

Material and methods: The peptidoglycan aptamers were labeled with ^{99m}Tc by the direct method and the stability of each ^{99m}Tc-aptamer complex was evaluated in saline, plasma and in presence of cysteine. The aptamers degradation by plasma nucleases was also assessed. Bacterial-infected (*Staphylococcus aureus)* mice and fungal-infected mice *(Candida albicans)* were used for the ex vivo biodistribution studies with the ^{99m}Tc-aptamers.

Results and discussion: The aptamers were not degraded by plasma nucleases. High radiolabel yields were obtained by the direct method and the complexes were stable in presence of saline and plasma. Some trans chelation was observed in the presence of cysteine. The ^{99m}Tc-pepdigoglycan aptamers uptake in the bacterial infection foci were significantly higher than the control (a radiolabeled oligonucleotide library) and their uptake in the fungal infection model.

Conclusion: Both radiolabeled peptidoglycan aptamers present specific uptake in the bacterial infection foci highlighting the potential of these molecules as radiotracer for bacterial infection.

Introduction

Nuclear imaging of bacterial infections has been a developing field for more than 50 years. In the last decade, significant efforts have been made to develop bacteria-specific radiolabeled tracers that bind or accumulate only in the bacterial cells differentiating bacterial infection from inflammation and from other types of infection. The most used imaging agents as ^{18}F-fluorodeoxyglucose (^{18}F-FDG), ^{67}Ga-citrate, and in vitro-radiolabeled leukocytes are not specific for infection, targeting the infection associated inflammation, and so not solving a frequent clinical problem that is differentiating active infection from other causes of inflammation [1].

Acid Nucleic aptamers are short single-stranded oligonucleotides that display high affinity and selectivity for a given target. Aptamers contain many features that are advantageous for radiopharmaceuticals development. They seem to be non-toxic and non-immunogenic, have small size (10 to 20 kDa) and fast clearance, allowing superior target-to-noise ratios at early time points. Since their discovery, several aptamers have been used as targeting molecule of radiopharmaceuticals in preclinical studies. The majority has been radiolabeled with ^{99m}Tc for SPECT (single-photon emission computed tomography) imaging of cancer-related targets [2]. Our research group has explored radiolabeled aptamers for infection diagnosis [3-6]. Radiolabeled aptamers specific for infectious agents could give an important contribution to infections diagnosis through scintigraphy, allowing distinguishing between infection and inflammation and identifying the microorganism causing the infection. Pathogen-specific imaging techniques could avoid the inappropriate use of antibiotics for noninfectious entities or for non-bacterial infections and provide a way to monitor antibiotic treatments.

Peptidoglycan is a cell wall polymer common to both Gram-positive and Gram-negative bacteria that coats the entire cell. In the peptidoglycan structure, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) alternate to form the carbohydrate polymer that is connected by multiple peptide cross-links [7]. The particular composition of peptidoglycan makes it a possible target for specific bacterial recognition. In a previous work, Ferreira et al. (2014) [8] selected two aptamers for peptidoglycan. The radiolabeled aptamers showed high binding capacity for *S. aureus* and *E. coli* cells *in vitro,* but the binding to *C. albicans* and human fibroblasts was negligible. Graziani *et al.* [9] demonstrated that the both peptidoglycan aptamers bind with high efficiency to all Gram-positive and Gram-negative

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species tested. In the present study, the peptidoglycan aptamers potential for bacterial infection foci identification was evaluated by ex vivo biodistribution studies. Two different experimental infection models were used: Bacterial-infected mice (*S. aureus*) and fungal-infected mice (*C. albicans).*

Material and Methods

Microorganisms and culture

Staphylococcus aureus (ATCC 25923) and *Candida albicans* (ATCC 18804) were cultured on BHI solid (HI media Laboratories Pvt Ltd.) in Petri dishes at 37 °C and sub-cultured every seven days.

Animals

The mice were kept in cages with wood shavings, water and common food (ad libitum) in ordinary shelves. The Swiss mice infected with *C. albicans* were firstly immunosuppressed by gamma radiation in a uniform ^{60}Co source at the Gamma Irradiation Laboratory of the Center of Nuclear Technology Development (CDTN, Brazil). A dose of 2.5 Gray and a dose rate of 75 Gray/h were used. After irradiation, the animals were maintained in autoclaved cages with wood shavings, water and food (ad libitum). All protocols were approved by the local Ethics Committee for Animal Experimentation of the Federal University of Minas Gerais (CETEA / UFMG), Protocol n° 108/2014.

Chemicals

The ^{99m}Tc was obtained from a molybdenum generator (IPEN/Brazil). Reagents, including tricine, ethylenediamine-N´, N´-diacetic acid (EDDA), $SnCl_2.2H_2O$ were purchased from Sigma-Aldrich (São Paulo, Brazil). The aptamers Antibac1 (5'TCGCGCGAGTCGTCTGGGGA-CAGGGAGTGCGCTGCTCCCCCCGCACGTCCTCCC 3') and Antibac2 (5'TCGCGCGAGTCGTCTGGGGGACTAGAGGACTT-GTGCGGCCCCGCATCGTCCTCCC3'), previously selected by Ferreira *et al.* (2014), were synthesized by Integrated DNA Technologies (IDT) with the introduction at the 3´end of an amino group with a 6 carbons spacer and at the 5' end of an inverted thymidine.

Evaluation of aptamer degradation by plasma nucleases

Blood (3 mL) was collected from Swiss mice and the plasma fraction was separated by centrifugation (700g). EDTA (0.1M) was used as anticoagulant. The aptamers Antibac1 and Antibac2 modified at the 3' and 5' ends were incubated separately with the plasma at 37 °C in the following ratio: 1 µL of aptamer solution (200 pmol / µL) for 9 µl of plasma. Aliquots of 10 µl were removed 5 m, 1 h, 3 h, 6 h and 24 h after and submitted to electrophoresis on 2% agarose gel stained with ethidium bromide.

Aptamer radiolabeling with ^{99m}Tc and radiolabel yield determination

Labeling with ^{99m}Tc was performed by the direct method according to Correa *et al* (2014) [10]. For the labeling reaction 111.6 mol of tricine and 28.3 mol of EDDA were added to 300 µL of 0.9% saline. Then, 10 µL of aptamer (200 pmol/µL) followed by 100 µL of $SnCl_2{\cdot}2H_2O$ (8.9 mM in HCl 0.25 N) were added to the solution. The pH was adjusted to 7.0 with 1.0 N NaOH. The bottle was sealed, and vacuum was applied with a syringe. The activity of 296 MBq of a ^{99m}Tc-pertechnetate solution ($Na^{99m}TcO_4^-$) was added. Then, the solution was boiled in water bath for 15 min and next cooled in running water. The injected activity for each animal was 14.8 MBq. An oligonucleotide DNA library (random sequences) was labeled in the same way and used as control.

The radiolabel yield of ^{99m}Tc-aptamer complex was assessed by ascending instant thin-layer chromatography (TLC) using silica gel-coated fiberglass sheets and two solvent systems: (1) 100% acetone to determine the percentage of TcO_4^- and (2) 0.9% NaCl solution with 5% NH_4OH to determine the percentage of TcO_2. The labeled product (^{99m}Tc-aptamer) remained at the point of application when 100% acetone was used as the mobile phase (Rf=0) and the labeled product moved with the solvent front when 0.9% NaCl solution with 5% NH_4OH was used as the mobile phase (Rf=1). The radiolabel yield was determined according to the following equation: Labeling percentage = 100 – (% TcO_4^- + % TcO_2).

Stability of ^{99m}Tc labeled aptamers

The stability of each ^{99m}Tc-aptamer complex was evaluated in saline, plasma and cysteine excess (50 mol of cysteine per mol of aptamer) by TLC. Analysis of stability was performed by adding 100 µL of the radiolabeled aptamers solution in tubes containing 1.1 mL of 0.9% NaCl, mice plasma or cysteine solution. The solutions of saline and cysteine were stored at room temperature and the plasma was incubated at 37 °C. All solutions were analyzed at 5 min, 1 h, 3 h, and 6 h later by TLC.

Biodistribution

The animals were anesthetized with a mixture of xylazine (15 mg / kg) and ketamine (80 mg / kg). The mice were infected intramuscularly in the right thigh with 1 x 10^6 cells of *S. aureus* (ATCC 25923) suspended in 100 µL of saline or infected in the same way with 1 x 10^5 cells of *C. albicans* (ATCC 18804). Groups of Swiss mice (20-25 g weight) containing 6 animals each (n = 6) were used. The animals infected with *C. albicans* were immunosuppressed before infection as described earlier. A visible swelling was observed on the infected thigh of all animals at 24 h after the intervention. So, 100 µL (14.8 MBq) of the radiolabeled aptamer solution or the radiolabeled oligonucleotide library (control) were injected by the tail vein in each animal. The mice were euthanized at 3 h post-injection and tissue samples (blood, liver, spleen, stomach, heart, lung, kidney, infected thigh muscle, and non-infected thigh muscle) were dissected, weighed, and their activities measured in a gamma counter. At the time of euthanasia, samples from infection foci were obtained for microbial culture and only the animals that tested positive were considered in this study. The results were expressed as the percentage of injected dose per gram of tissue (%ID/g). Target/non-target ratios were obtained from the analysis of radiation measured in the right thigh infected muscle in relation to radiation measured in the left thigh muscle.

Statistical analysis

All data were expressed as mean ± SD and analyzed by GraphPad PRISM version 5.01 software. The analysis of variance (ANOVA) with a confidence interval of 95% and Tukey multiple comparison test were used. A P value <0.05 was considered to indicate a statistically significant difference.

Results and Discussion

The Figure 1 shows the results of the assay of aptamers degradation by plasma nucleases. The aptamers Antibac1 and Antibac2, modified with the introduction at the 3´end of an amino group with a 6 carbons spacer and at the 5' end of an inverted thymidine, were not degraded by plasma nucleases and remained stable up to 24 h. This stability in plasma is an important property for a radiopharmaceutical candidate.

The aptamers were labeled with ^{99m}Tc by the direct method developed by Correa *et al* (2014), which allows high radiolabel yields. In

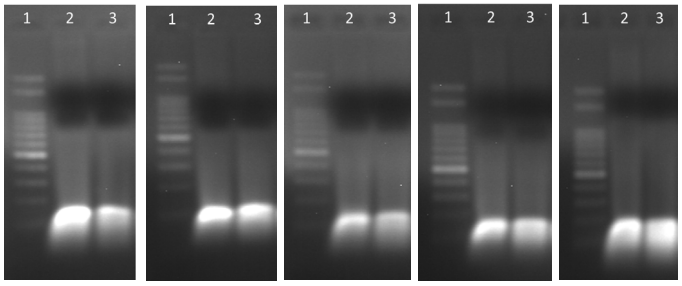

Figure 1. Evaluation of Antibac1 and Antibac2 degradation by plasma nucleases.

The aptamers Antibac1 and Antibac2 modified at the 3' and 5' ends were incubated separately with the plasma at 37 °C. Aliquots of 10 µl were removed 5 m (A), 1 h (B), 3 h (C), 6 h (D) and 24 h (E) after and submitted to electrophoresis on 2% agarose gel stained with ethidium bromide. (1) DNA Ladder of 50 pb, (2) Antibac1 and (3) Antibac2

the present study, only radiotracer preparations presenting radiolabel yields higher than 90% were used in the experiments. The stability of each ^{99m}Tc-aptamer complex was evaluated in saline, plasma and in presence of cysteine. A high complex stability was verified in presence of saline and plasma for both aptamers complexes, since the percentage of radiolabeling was kept above 90% up to 6 h. Some trans chelation was observed in the presence of cysteine (50 mol of cysteine per mol of aptamer). The radiolabeled yields percentages in cysteine excess for Antibac1 and Antibac2 were 73.1 ± 0.1 and 81.9 ± 1.9 at 3 h, respectively, and after 6 h, these values were 71.9 ± 0.6 and 73.8 ± 1.6 (Tables 1 and 2). Based on these findings it was concluded that the ^{99m}Tc-aptamers complexes were suitable for further in vivo assays.

The biodistribution studies were carried out in two different experimental infection models: Bacterial-infected mice (*S. aureus)* and fungal-infected mice (*C. albicans*). The fungal-infected mice group was used to confirm the aptamers specificity for the bacterial infection foci. This control group was also useful to measure the radiotracer uptake due to the local inflammatory process that accompanies an infection, producing vasodilatation and increased capillary permeability. Many radiopharmaceuticals used for infection diagnosis actually accumulate in the infection site mainly due to these vascular effects and they are not specific to infection [Ferro-Flores *et al.*, 2012]. To help the evaluation of this nonspecific uptake, a ^{99m}Tc-radiolabeled library consisting of oligonucleotides with random sequences was also used as a control in both infections models. These oligonucleotides without specificity for the microbial targets act as blood flow markers and non-specific uptake indicators.

Figure 2 presents the biodistribution for ^{99m}Tc-Antibac1, ^{99m}Tc-Antibac2 and the ^{99m}Tc-library in the *S. aureus*-infected mice. The results showed a higher uptake of the ^{99m}Tc-Antibac1 and ^{99m}Tc-Antibac2 in the infected thigh compared to the radiation measured in the left thigh muscle. The target to non-target (T/NT) ratio for ^{99m}Tc-Antibac1and ^{99m}Tc-Antibac2 were of 3.2 ± 0.2 and 2.6 ± 0.7, respectively. These ratios were statistically higher ($p < 0.05$) than that found for the ^{99m}Tc-library (1.5 ± 0.1).

The biodistribution in the fungal-infected model is shown in Figure 3. No statistical difference ($p > 0.05$) was observed between ^{99m}Tc-Antibac1, ^{99m}Tc-Antibac2 and ^{99m}Tc-library uptake. The T/NT ratios were of 1.5 ± 0.1, 1.7 ± 0.2 and 1.5 ± 0.2, respectively, indicating non-specificity of peptidoglycan aptamers to *C. albicans* infection foci.

All biodistribution studies showed a high percentage of radioactivity in the kidneys. This finding indicates a main renal excretion rout, which is consistent with the hydrophilic nature and small size of aptamers. Because chelating agents were not used in the labeling process, the biodistribution profile seemed to reflect mainly the aptamer properties.

The uptake of the ^{99m}Tc-library in the infection foci can be correlated to the increased capillary permeability and vasodilatation triggered by the inflammation associated to the infection, since T/NT ratios due the ^{99m}Tc-library were similar in both infection models (1.5). These ratios were also comparable to the T/NT ratios of ^{99m}Tc-Antibac1 and ^{99m}Tc-Antibac2 in the fungal infection model, in which the aptamers have no affinity for the microorganism causing the infection. By the other side ^{99m}Tc-Antibac1 and ^{99m}Tc-Antibac2 allowed high T/NT in the bacterial infection model, statistically higher than found for the ^{99m}Tc-library and the T/NT ratios verified for these aptamers in the fungal infection model.

Aptamers are molecules that provide high specificity for pre-selected targets. In this work, we evaluate aptamers for the peptidoglycan, present in the cell walls of all bacterial species, and therefore they work as generic probes for bacteria identification. However, aptamers can also be used for scintigraphy identification of a particular bacterial specie [5], highlighting the potential of these molecules for the development of radiopharmaceuticals for infection diagnosis.

Table 1. In vitro stability of the ^{99m}Tc-Antibac1 complex in the presence of 0.9% saline, plasma, and molar excess of cysteine (% radiolabeled yield).

Time	Saline	Plasma	Cys 50:1
5 min	95.50 ± 1.07	93.87 ± 0.21	96.09 ± 0.14
1 h	92.49 ± 2.09	97.49 ± 0.09	72.48 ± 1.00
3 h	92.93 ± 0.19	97.81 ± 0.04	73.09 ± 1.00
6 h	87.00 ± 0.43	97.34 ± 0.26	71.89 ± 0.61

Cys- Cysteine

Table 2. In vitro stability of the ^{99m}Tc-Antibac2 complex in the presence of 0.9% saline, plasma, and a molar excess of cysteine (% radiolabeled yield).

Time	Saline	Plasma	Cys 50:1
5 min	95.46 ± 1.08	95.70 ± 0.74	89.39 ± 1.24
1 h	91.96 ± 0.28	97.62 ± 0.09	89.71 ± 1.26
3 h	90.01 ± 0.51	98.06 ± 0.74	81.94 ± 1.86
6 h	91.25 ± 0.96	97.01 ± 1.46	73.81 ± 1.62

Cys- Cysteine

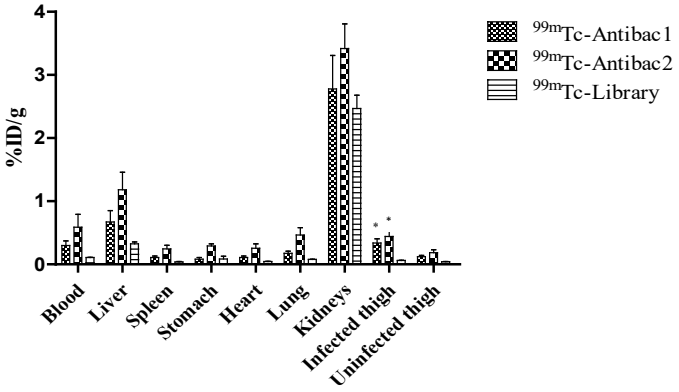

Figure 2. Biodistribution in the bacterial-infected model.

The Antibac1, Antibac2 and the library were labeled with ^{99m}Tc and injected into the tail vein of *S. aureus* infected mice. The mice were euthanized at 3 h after injection, tissue samples were dissected, and their activities were measured in a gamma counter. The symbol (*) indicates a statistical difference in the radiation uptake between the infected right thigh and the uninfected left thigh ($p < 0.05$).

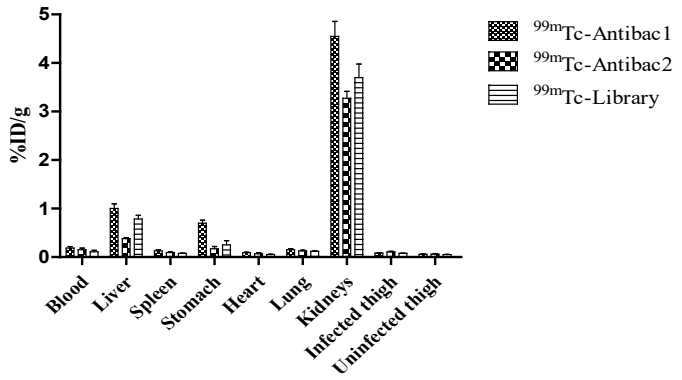

Figure 3. Biodistribution in the fungal-infected model

The Antibac1, Antibac2 and the library were labeled with ^{99m}Tc and injected into the tail vein of *Candida albicans* infected mice. The mice were euthanized at 3 h after injection, tissue samples were dissected, and their activities were measured in a gamma counter.

Conclusions

Both peptidoglycan aptamers were successfully labeled with ^{99m}Tc, showing stability for in vivo studies. By using two different infection models and a control based on a radiolabeled oligonucleotide library was possible to demonstrate that both peptidoglycan aptamers present specific uptake in the bacterial infection foci.

Conflict of interest

None

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