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Nuclear Medicine and Biology 39 (2012) 361–369

NUCLEAR MEDICINE — AND — BIOLOGY

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In vitro and in vivo evaluation of selected ^{68}Ga-siderophores for infection imaging☆

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Received 28 July 2011; received in revised form 20 September 2011; accepted 29 September 2011

Abstract

Introduction: Siderophores are low-molecular-mass iron chelators serving as iron transporters for almost all bacteria, fungi and some plants. Iron is an essential element for majority of organisms and plays an important role in virulence of pathogenic organisms. ^{68}Ga is a positron emitter with complexing properties comparable to those of Fe(III) and readily available from a generator. Initial studies with ^{68}Ga-triacetylfusarinine C (TAFC) showed excellent targeting properties in a rat infection model. We report here on the in vitro and in vivo evaluation of other siderophores radiolabelled with ^{68}Ga as potential radiopharmaceuticals for infection imaging.

Methods: ^{68}Ga labelling was performed using acetate buffer. Stability, log *P* and protein binding values were determined. In vitro uptake was tested using iron-deficient and iron-sufficient *Aspergillus fumigatus* (*A.f.*) cultures. Biodistribution of ^{68}Ga-siderophores was studied in Balb/c mice.

Results: Significant differences among studied siderophores were observed in labelling efficiency, stability and protein binding. Uptake in *A.f.* cultures was highly dependent on iron load and type of the siderophore. In mice, ^{68}Ga-TAFC and ^{68}Ga-ferrioxamine E (FOXE) showed rapid renal excretion and low blood values even at a short period after injection; in contrast, ^{68}Ga-ferricrocin and ^{68}Ga-ferrichrome revealed high retention in blood and ^{68}Ga-fusarinine C showed very high kidney retention.

Conclusions: Some of the studied siderophores bind ^{68}Ga with high affinity and stability, especially ^{68}Ga-TAFC and ^{68}Ga-FOXE. Low values of protein binding, high and specific uptake in *A.f.*, and excellent in vivo biodistribution make them favourable agents for *Aspergillus* infection imaging.

Keywords: Siderophores; *Aspergillus fumigatus*; ^{68}Ga; PET imaging; Invasive aspergillosis

1. Introduction

Siderophores are low-molecular-weight (500–1500 Da), iron-chelating molecules produced by nearly all bacteria, fungi and some plants [1]. Since 1970, a large number of siderophores have been characterized. The majority possess hydroxamate, catecholate or α-hydroxycarboxylate functional groups and form six coordination complexes with extremely high affinity (binding constant of $>10^{30}$) and selectivity for ferric ions [1]. Their biosynthesis is regulated by the iron levels of the environment where the organism is located, and they serve to deliver iron into the microbial cells [2].

Iron is an essential nutrient for almost all organisms. For prime producers, such as bacteria, fungi and plants, iron bioavailability is limited by the inherently low solubility of ferric ions. Under aerobic conditions, iron exists mainly in the form of Fe(III), as hydroxide and oxyhydroxide colloid particles that have a solubility below 10^{-9} M at neutral pH [3]. This is far below the level of demand for the iron supply of living cells. Therefore, iron-dependent microorganisms have evolved different strategies to solve the bioavailability

☆ This project was financially supported by the Austrian Science Fund (FWF) L676-B18 and partly by P21643-B11.

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doi:10.1016/j.nucmedbio.2011.09.012

problem. These strategies usually involve biosynthesis of siderophores. Extracellular siderophores serve microorganisms to acquire iron from the environment, while intracellular siderophores have been proposed to play a role in iron storage and have been recognized as asexual spore germination factors of several microorganisms (*Neurospora crassa*, *Penicillium chrysogenum*, *Aspergillus nidulans*, etc.) [4].

After synthesis and excretion of an iron-free siderophore (desferri-siderophore) followed by chelation of iron, the siderophore–iron complex (ferri-siderophore) is taken up into the cell. Highly specific iron uptake systems [2] recognize the specific siderophore as well as its chirality. They transport the ferric complexes into the cell in an active and energy-dependent way. The ferric ions once collected here are then handed over to the intracellular transport and storage components [5].

In recent years, it has become clear that iron acquisition is also one of the important factors of virulence of pathogenic microorganisms [6]. Schrettl et al. [7] demonstrated that siderophores play a fundamental role as a virulence determinant of *Aspergillus fumigatus (A.f.)*. *A.f.* is one of the most common airborne fungi, and humans constantly inhale numerous conidia of this fungus. Usually, these are eliminated in the immunocompetent host by innate immune mechanisms. However, for the immunosuppressed patients, invasive aspergillosis (IA) mainly caused by *A.f.* represents life-threatening and often fatal infection. The prevalence of IA has increased significantly during the past decades, currently being the most common mold infection worldwide [8,9]. Early diagnosis is critical to a favourable outcome of IA, but is difficult to achieve with currently available diagnostic methods, which lack specificity and/or sensitivity.

A.f. produces four structurally different hydroxamate peptide siderophores [7,10]: it excretes fusarinine C (FUS) and triacetylfusarinine C (TAFC) to acquire extracellular iron and employs ferricrocin (FC) and hydroxyferricrocin for hyphal and conidial iron storage, respectively [7,11]. The *A.f.* genome encodes seven putative siderophore transporters [12], five of which are up-regulated during iron starvation conditions [13]. As *A.f.* excretes only two siderophore types, FUS and TAFC, these data indicate either high redundancy of siderophore uptake or additional uptake of structurally different siderophores. In this regard, it is interesting to note that several fungal species are able to utilize siderophores produced by other fungi, termed xenosiderophore, e.g., *Saccharomyces cerevisiae, Candida albicans* and *Aspergillus nidulans* [10,14–16].

^{68}Ga is a positron emitter that has recently gained great interest for molecular imaging applications using positron emission tomography (PET) [17]. It is readily available from a radionuclide generator, has a suitable short half-life of 68 min and comparable chemistry to Fe(III). In a proof-of-principle study, we recently showed that a ^{68}Ga-labelled siderophore (TAFC) can detect *A.f.* infections in a rat animal model using PET imaging [18]. Consequently, we characterized in this study the in vitro and in vivo uptake of endogenous and selected xenosiderophores and evaluate the potential of these compounds as radiopharmaceuticals for PET imaging of IA.

2. Materials and methods

2.1. Chemicals

All commercially available reagents were of analytical grade and used without further purification. Desferri-siderophores were obtained from Genaxxon Bioscience (Ulm, Germany). ^{68}Ga was gained from a ^{68}Ge/^{68}Ga generator (IGG; Eckert & Ziegler, Berlin, Germany).

2.2. Fungal strains and preparation of A.f. cultures

Fungal strains used for in vitro studies were *A.f.* wild-type ATCC46645 (American Type Culture Collection) cultured at 37°C in *Aspergillus* minimal medium containing 1% glucose as the carbon source, 20 mM glutamine as the nitrogen source, salts and trace elements, as described previously [19]. Iron-sufficient media contained 30 mM $FeSO_4$. For preparation of iron-deficient media, iron addition was omitted. Iron-deficient conditions were verified by detection of extracellular siderophore production, which is suppressed by iron.

2.3. Radiolabelling

^{68}Ga was eluted from a ^{68}Ge/^{68}Ga generator using 0.1N HCl (Fluka, Buchs, Switzerland). Varying amounts (10–40 μg) of desferri-siderophores dissolved in water (1 μg/μl) were mixed with 30–80 μl of sodium acetate (155 mg/ml in water) and 300 μl of generator eluate (10–150 MBq of $^{68}GaCl_3$). Reaction mixtures (pH 3–4) were incubated at varying temperatures (RT–80°C) for less than 30 min. After the reaction, 100 μl of sodium acetate was added to increase the pH to 6–7. Radiochemical purity (RCP) of labelled siderophores was analyzed on reverse-phase high-performance liquid chromatography (RP-HPLC) or using instant thin-layer chromatography on silica gel impregnated glass fibres (ITLC-SG).

2.4. HPLC and TLC

For determination of radiochemical purity of radiolabelled siderophores, a RP-HPLC gradient method was used, as described previously [18]. ITLC-SG (Pall Corporation, East Hills, NY, USA) using 0.1 M sodium citrate (pH=5) as a mobile phase was used for rapid estimation of the product quality. The retention factor (Rf) of labelled siderophores was 0–0.2 and Rf of free ^{68}Ga was 0.8-1.

2.5. In vitro characterization of selected siderophores

2.5.1. Log P

^{68}Ga-labelled siderophore in 0.5 ml phosphate buffered saline was added to 0.5 ml octanol in an Eppendorf tube. The tube was vigorously vortexed over a period of 15 min. An aliquot of both the aqueous and the octanol layers was