Multivalent Siderophore DOTAM Conjugates as Theranostics for Imaging and Treatment of Bacterial Infections

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Abstract: Bacterial infections are a major cause of morbidity and mortality worldwide. In addition to novel treatment options, there is a strong need to better diagnose infections at deep body sites through noninvasive molecular imaging methods. Here, we describe the synthesis and characterization of probes based on siderophore conjugates with catechol moieties and a central DOTAM scaffold. The probes can accommodate a metal ion as well as an antibiotic moiety and are therefore suited for theranostic purposes. The translocation of the conjugates across the outer and the inner cell membrane of *E. coli* was proven by growth recovery experiments with enterobactin-deficient strains, by the antibacterial activity of conjugates with ampicillin, and by confocal imaging using a fluorogen-activating protein-malachite green system adapted to *E. coli*. A first application of the probes for in vivo imaging is reported with a Cy5.5 conjugate in mice infected with *P. aeruginosa*.

Infections caused by pathogenic bacteria represent a major health threat that is expected to increase further in the future,^[1] fueled by the facilitated spread of pathogens through international mobility, and an increase of modern medical procedures that disrupt physical biological barriers and the immune system. The fact that a growing proportion of multidrug resistant pathogens meet a declining pipeline of novel antibiotic drugs raises serious concerns. There is also a lack of methods to detect infections at sites that are unknown or inaccessible for sampling; this concerns in particular biomaterial-associated infections or endocarditis. For this purpose, noninvasive imaging methods that can detect early stage-infections are highly desirable, as they would enable timely interventions before local manifestations (e.g. through biofilm formation) or a systemic spread of the infection.^[2] The positron emission tomography (PET) agent 2-[¹⁸F]-fluorodeoxyglucose (¹⁸F-FDG) is in clinical use, but cannot differentiate between sterile inflammation and bacterial infections. To address this issue, several probes for bacterial imaging have recently been published that are based on fibrinogen,^[3] maltodextrin,^[4] sorbitol,^[5] vancomycin, or substrates for micrococcal nuclease (MN) and for β -lactamase in *M. tuberculosis*.

In this paper, we report the targeting of the bacterial iron transport machinery to monitor and treat infections. To satisfy their iron demand, bacteria biosynthesize and secrete siderophores, small molecules with a high affinity for iron that capture the metal from the environment, followed by an active uptake of the iron-siderophore complex.^[6] Multiple transporters enable bacteria to not only import endogenous siderophores, but also so-called xeno-siderophores, thereby assuring an supply even in the absence of the endogenous molecules.^[6b] Various groups have exploited this property for the design of actively transported antibacterial conjugates of an antibiotic coupled to an iron-binding motif. Achieving reliable efficacy towards adapted bacterial populations is seen as a major challenge of the approach for treatment^[7] and imaging^[8] applications. We searched for a small molecule scaffold that enables bacterial imaging and treatment (i.e. theranostic)^[9] applications at the same time by accommodating (i) an iron binding motif, (ii) variable imaging modalities and (iii) an optional effector antibiotic. The tetrapodal 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic amide (DOTAM) moiety was selected,^[10] because it fulfills all functional requirements and has advantages like a straightforward synthetic access, high solubility, low toxicity and proven biocompatibility.^[11] The scaffold is widely applied for the chelation of metals, a property that is utilized in Ln-based magnetic resonance imaging, ⁶⁸Ga- or ⁶⁴Cu-based nuclear imaging, or ⁹⁰Y-based radiotherapy.^[12]

For siderophore-based bacterial imaging, three of the four arms branching from the DOTAM core were functionalized with catechol moieties for iron binding, while the fourth arm was coupled to a fluorophore through various linkers (Figure 1a). The imaging probes, antibiotics and control substances **1-13** were prepared in 3-8 steps from cyclen (Scheme 1 and Supplementary Schemes 1-7). The iron binding properties of the compounds were characterized by a colorimetric titration assay based on chrome azurol S (CAS).^[13] Whereas **1** bound one equivalent of ferric iron, no binding was observed for **2**, a complex that lacked free hydroxyl groups for iron binding (Figure 1b). This suggests that ferric iron was indeed complexed by the catechol groups, and not by the DOTAM moiety. The comparison with **3** demonstrates that the modification of the 4th arm by a GABA linker had no influence on binding. To probe whether a metal complexed to DOTAM would interfere with iron binding, the Eu³⁺-containing complex **4** was titrated into an Fe-HDTMA-CAS solution. Again one equivalent of ferric iron was found to bind to **4**, indicating that the scaffold can accommodate ferric iron and Eu³⁺ simultaneously. Next, the ability of DOTAM conjugates to label medically important bacterial pathogens of the ESKAPE panel, comprising the Gram-negative species *A. baumannii*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and the Gram-positive species *E. faecium* and *S. aureus*,^[14] was probed using **5**, a conjugate with a Bodipy fluorophore. All ESKAPE pathogens were labeled by **5** according to confocal microscopy and flow cytometry experiments (Figures 1 and S1). However, we observed a varying efficiency of staining between strains, and only a subpopulation of a given strain was labeled. This might be attributed to different growth phases of the respective cells, but the molecular mechanism behind this remains unknown at this stage.

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First, the internalization of **1** and **6** into *E. coli* and *P. aeruginosa* was assessed by growth recovery assays with the mutants *E. coli* $\Delta pvdA$ and *P. aeruginosa* $\Delta pvd/\Delta pch$. These strains cannot biosynthesize endogenous siderophores and therefore require the presence of exogenous siderophores to grow under iron-limiting conditions. Indeed, the growth of the *E. coli* and *P. aeruginosa* mutants was restored by the addition of the respective endogenous siderophores enterobactin and pyoverdinin (Figure S2). Notably, DOTAM-based siderophore mimics **1** (carrying free catechols) and **6** (carrying acetylated catechols) also promoted growth of the mutant strains. In contrast, **2** (carrying benzylated catechols) failed to recover growth of siderophore-deficient strains. The experiments provide evidence that the DOTAM-catecholates are indeed capable to deliver iron into Gram-negative bacteria through internalization. We applied acetylated catechols, because the use of an acetyl prodrug that is activated *in situ* was reported to be favourable to prevent a permanent inactivation of catechols by enzymatic methylation.^{[7], [14]} In order to verify whether **6** can be deacetylated and complexed with iron, the relative abundances of DOTAM catecholates carrying 6 or 0 acetyl groups as well as the iron-complexed, deacetylated species **7** were determined by LC/MS/MS (Figures 2c, S2 and Table S1).

Figure 1. Binding properties of siderophore conjugates. a) General structure of conjugates. b) Iron binding assays using CAS with **1-4** and enterobactin. c and d) Labelling of *P. aeruginosa* and *E. coli* with DOTAM-Bodipy conjugate or free Bodipy monitored by confocal microscopy (c) and flow cytometry (d).

Table 1. Growth inhibition activities (IC₅₀) of DOTAM conjugates and free ampicillin in μM .

Compound	<i>E. coli</i>	Δ entA	Δ entB	Δ fepA
8	30.2	2.1	3.3	7.2
9	25.0	1.9	2.1	>50
Ampicillin	4.3	2.1	6.2	3.8

Figure 2. Growth recovery experiments. a) Growth of *E. coli* BW25113 (wt) and the enterobactin-deficient strain (Δ entA) was assessed 48h after compound addition. b) Growth of the *P. aeruginosa* strain PAO1 (wt) and the pyoverdine/pyochelin-deficient strains (Δ pvdF/ Δ pvdA) was assessed 24h after compound addition. All values are OD₆₀₀ measurements, $n = 2$, $\pm$ SEM. c) Relative quantification of deacetylation and iron complexation of **6**. Compound **6** was incubated in LB medium at 10 μ M final concentration with (black bars) or without *E. coli* Δ entB (grey bars), extracted at the indicated time points and quantified by LC-MS/MS. Area under curve (AUC) values represent the abundance of the monitored molecule and are shown for **6** (contains six acetyl groups: 6Ac, left), **1** in complex with iron (contains zero acetyl groups: 0Ac + Fe, middle) and **1** (contains zero acetyl groups: 0Ac, right). Ac: Acetyl; EB: Enterobactin; PVD: Pyoverdine; Fe: Iron.

We indeed observed full deacetylation and Fe-complexation; to our surprise, deacetylation occurred independent of bacterial hydrolases, as equal amounts of **1** were formed upon incubation in plain medium or with *E. coli* Δ entB (Figure 2c). Next, the β -lactam antibiotic ampicillin was coupled to the DOTAM core by a covalent, non-cleavable linker in 6 steps to give **8** with an overall yield of 15 % (Scheme S5). Conjugate **8** inhibited the growth of the enterobactin-deficient strains *E. coli* Δ entA, *E. coli* Δ entB and the enterobactin receptor-deficient strain *E. coli* Δ fepA with IC₅₀'s comparable to free ampicillin (Table 1). The enhanced activity of **8** on the enterobactin-deficient mutants compared to the wild type implies that the DOTAM core is accepted as an iron-carrier, while the lack of activity on the wild type may reflect a saturation of *E. coli*'s iron demand by its endogenous siderophore. The activity against FepA-deficient mutants demonstrates that DOTAM may also enter the bacterial cell through different transporters; this is seen as an advantage, as it suggests that resistance cannot be induced by mutations or downregulation of a single target. The addition of **9** to yield **9** slightly improved the IC₅₀ for *E. coli* Δ entA and *E. coli* Δ entB, but decreased activity on *E. coli* wild type Δ fepA. As the targets of ampicillin are located in the periplasmic space, the antibiotic activity indicates a translocation of the conjugate across the outer membrane.

The question whether the DOTAM could pass the inner membrane was also investigated by a conjugate with the fluorogenic dye malachite green (MG). MG itself is not fluorescent, binding to the fluorogen activating protein (FAP), a 25 kDa single-chain antibody fragment, hinders rotation of the aromatic rings, thereby strongly enhancing MG's fluorescence (Figures 3a and S4).^[15] While a first application of the FAP/MG system to high resolution imaging of prokaryotic cells was recently reported,^[16] we aimed at applying MG/FAP to study translocation efficiencies of molecular scaffolds designed to transport cargos in bacteria. For this purpose, the sequence of variant FAP6.2 was cloned into pET23b expression vector and recombinantly expressed in *E. coli* Origami™ B(DE3). FAP6.2 expression was verified on Western Blot and by monitoring

MG-fluorescence upon incubation with purified FAP6.2 (Figure S4). While the incubation of *E. coli* FAP6.2 with the MG-coupled, benzyl-protected DOTAM conjugate **10** did not induce fluorescence, incubation with **11** led to a time-dependent increase of fluorescence (Figure 3b). After 16h of incubation, the MG-dependent fluorescence of the bacteria was further analyzed by flow cytometry and confocal microscopy (Figures 3b and S5). To exclude that an antibacterial effect of **11** caused the delayed uptake kinetics, bacteria were afterwards incubated with propidium iodide, a dye specifically labeling dead cells, and examined by confocal microscopy (Figure S6). We did not observe an enhanced amount of dead cells due to treatment with **11**, underlining the validity of uptake into live cells. Because the observed fluorescence required the binding of the intracellular FAP to DOTAM-conjugated MG, the findings provide

Figure 3. a) Principle illustrating the application of FAP system for uptake visualization of malachite green (MG)-carrier conjugates. b) **10** or **11** were added at a final concentration of 10 μ M to *E. coli* FAP6.2, and fluorescence was recorded at λ_{EX} 610nm/ λ_{EM} 665nm for 16 h. **11** was translocated to the cytoplasm, whereas **10** was not. c) Confocal microscopy of *E. coli* FAP6.2 upon incubation with 10 μ M **10** or **11** for 16 h. BF: brightfield. Scale bars 11 μ m.

evidence that **11** had indeed been translocated into the cytosol of *E. coli*. Encouraged by the cellular imaging data, the ability of DOTAM siderophore conjugates to monitor bacterial infections *in vivo* was probed by **12**, a conjugate that carried the near infrared dye Cy5.5 that is compatible with optical imaging of small animals. Interferon- β luciferase reporter mice were infected subcutaneously with a nonlethal dose of *P. aeruginosa*, or with a solvent control. Following the injection of **12** (20 μ g/kg) into the tail vein, fluorescence was monitored over 24 hours (Figures 4a and S7). A second negative control group was infected with *P. aeruginosa*, and then treated with the DOTA conjugate **13** that was devoid of catechol moieties.

Figure 4. Small animal model for diagnosis of bacterial infections by siderophore conjugates. a) A suspension of bacteria (*P. aeruginosa* PAO1) was injected s.c. left and right of the back of interferon- β luciferase reporter mice at the positions indicated by dashed white ovals (Bac), followed by an i.v. injection of **12** or the control **13** in the tail vein. Uninfected animals (Ctr) were treated likewise. Fluorescence images were recorded at different time points. b) A luciferin solution was injected i.p., and the host immune response-induced bioluminescence of the animals was recorded.

The fluorescence intensity of **12** was highest at the site of bacterial injection at the 0.6h time point and decreased gradually; a clear signal was still visible after 5h. In contrast, infected mice treated with **13** exhibited weaker signals. Also uninfected mice treated with **12** had weaker intensities than the infected group. To visualize the site of infection by an independent method, type I interferon (IFN) production as part of the immune response to bacterial infections was localized by bioluminescent imaging 5h post injection, accounting for the time lag associated with IFN induction. A strong bioluminescent signal was co-localized with the fluorescence induced by **12**, thereby demonstrating that **12** had not dissipated across the body, and that **12** correctly labeled the site of infection (Figure 4b). Thus, a first proof of concept for the imaging of bacterial infections based on siderophore targeting could be achieved.

In summary, siderophore conjugates based on a DOTAM scaffold were characterized as multifunctional molecules that are applicable for the diagnosis as well as for the treatment of bacterial infections. Their ability to cross the Gram-negative cell wall was demonstrated by microbiological experiments as well as by an adaptation of the FAP/MG systems to *E. coli*, which encourages the synthesis of antibiotic conjugates. While the penicillin conjugates **8** and **9** provided a first proof of the general concept of the DOTAM scaffold as a carrier of antibiotics, we attempt to improve antibacterial activities by employing more potent effector molecules in next generation conjugates. Additional future studies will be directed to analogs with metal-bound DOTAM cores that may be suited for the detection of infections.

Keywords: antibiotics • imaging • drug delivery • drug conjugates • siderophores

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