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Citation for the original published paper (version of record):

Hakobyan, S., Rzhapishevska, O., Björn, E., Boily, J-F., Ramstedt, M. (2016)
Influence of Chelation Strength and Bacterial Uptake of Gallium Salicylidene Acylhydrazide on
Biofilm Formation and Virulence by *Pseudomonas aeruginosa*.
Journal of Inorganic Biochemistry, 160: 24-32
<http://dx.doi.org/10.1016/j.jinorgbio.2016.04.010>

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Influence of chelation strength and bacterial uptake of gallium salicylidene acylhydrazide on biofilm formation and virulence of *Pseudomonas aeruginosa*

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Final version of manuscript published as an article in

Journal of Inorganic Biochemistry in 2016

DOI:10.1016/j.jinorgbio.2016.04.010

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Abstract

Development of antibiotic resistance in bacteria causes major challenges for our society and has prompted a great need for new and alternative treatment methods for infection. One promising approach is to target bacterial virulence using for example salicylidene acylhydrazides (hydrazones). Hydrazones coordinate metal ions such as Fe(III) and Ga(III) through a five-membered and a six-membered chelation ring. One suggested mode of action is *via* restricting bacterial Fe uptake. Thus, it was hypothesized that the chelating strength of these substances could be used to predict their biological activity on bacterial cells. This was investigated by comparing Ga chelation strength of two hydrazone complexes, as well as bacterial Ga uptake, biofilm formation, and virulence in the form of production and secretion of a toxin (ExoS) by *Pseudomonas aeruginosa*. Equilibrium constants for deprotonation and Ga(III) binding of the hydrazone N'-(5-chloro-2-hydroxy-3-methylbenzylidene)-2,4-dihydroxybenzhydrazide (ME0329), with anti-virulence effect against *P. aeruginosa*, were determined and compared to bacterial siderophores and the previously described Ga(III) 2-oxo-2-[N-(2,4,6-trihydroxy-benzylidene)-hydrazino]-acetamide (Ga-ME0163) and Ga-citrate complexes. In comparison with these two complexes, it was shown that the uptake of Ga(III) was higher from the Ga-ME0329 complex. The results further show that the Ga-ME0329 complex reduced ExoS expression and secretion to a higher extent than Ga-citrate, Ga-ME0163 or the non-coordinated hydrazone. However, the effect against biofilm formation by *P. aeruginosa*, by the ME0329 complex, was similar to Ga-citrate and lower than what has been reported for Ga-ME0163.

Keywords: Gallium; equilibrium constants; virulence; biofilm; bacteria; UV-vis

1. Introduction

The development of antibiotic resistance in bacteria is leading to clinical situations where bacterial infections in patients can no longer be treated successfully. This is a major challenge for society and there is a great need for new and alternative treatment methods,

for example in the form of new substance classes that target other pathways in bacteria than traditional antibiotics do. Such drugs would give the possibility to treat pathogens that have already developed multi-resistance against traditional antibiotics, and thus enable eradication of infections. Several examples for alternative pathways that could be targeted have been given, including Fe acquisition pathways, virulence and biofilm formation [1, 2].

A substance group that targets virulence in several Gram-negative bacteria is salicylidene acylhydrazide, here called hydrazones. This class of substances targets the type three secretion system (T3SS) that delivers bacterial toxins into a host cell [3-5]. Secretion systems are only one virulence factor among many, but they are interesting as drug targets as disrupting secretion systems would “disarm” bacteria without killing them. Thus, the evolutionary pressure to develop resistance would be expected to be lower than for biocidal compounds [6, 7]. The exact target for the hydrazones is yet not fully identified although recent studies indicate that this class of compounds interacts with multiple proteins [8, 9]. In addition, the compounds efficiently chelate Fe(III) and this has also been hypothesized as a possible mode of action [10, 11]. The whole group of compounds exhibits a chelating motif at the center of the molecule that can coordinate metal ions through a five-membered and a six-membered ring consisting of a deprotonated phenolic group, an azomethine nitrogen and a carbonyl (Figure 1) [12]. Thus, it could be hypothesized that the chelating strength of substances in this group of compounds could be used to predict their biological activity in bacterial cells.

In previous studies we have investigated the synergistic effects of a hydrazone (2-oxo-2-[N-(2,4,6-trihydroxy-benzylidene)-hydrazino]-acetamide, also called ME0163) with the Fe(III) antagonist Ga(III) on *Pseudomonas aeruginosa* [12, 13]. We found that it exhibited a chelation strength to Ga(III) similar to that of EDTA and that, as a complex, the Ga hydrazone (Ga-ME0163) reduced biofilm formation as well as secretion of the bacterial toxin ExoS secreted through the T3SS in *P. aeruginosa*. However, the inhibitory effect on ExoS expression and secretion was similar to that of Ga-citrate and ME0163 did not exhibit any

significant effect on ExoS secretion by itself. Following these results we hypothesized that a hydrazone with effect on ExoS secretion should become further enhanced through complexation with Ga(III) and that this effect would be correlated to the metal chelation strength of the ligand. To test this hypothesis we selected a hydrazone, *N*-(5-chloro-2-hydroxy-3-methylbenzylidene)-2,4-dihydroxybenzhydrazide (INP0341, or ME0329), that previously has been described to have a good antibacterial effect against other Gram-negative bacteria [14, 15], and that showed to have an effect against the T3SS in *P. aeruginosa* in preliminary experiments. We used *P. aeruginosa* as a relevant model organism in this context, since it is an opportunistic pathogen giving rise to a range of different infections in humans. For example, it is found in eye infections associated to contact lenses, as well as hospital-acquired infections in airways, urinary tract, blood stream and burn wounds [16]. It is also a well-studied model organism for biofilm formation and virulence and has several secretion systems [17, 18].

In this work we investigate to what extent metal chelation can be used to predict Ga(III) uptake into bacteria from Ga(III)-hydrazone complexes. We further show how biofilm formation and secretion of ExoS by *P. aeruginosa* differ between hydrazone complexes, and that synergistic effects are observed between Ga(III) and the hydrazone in some cases.

2. Materials and methods

2.1. Chemicals

Chemicals were purchased from Sigma-Aldrich and used without purification unless stated otherwise. ME0329 (INP0341) [14] was obtained from the group of Mikael Elofsson, Umeå University, ME0163 was synthesized as previously described [5]. All solutions were prepared from deionized and boiled water (resistance = 18.2 MΩ) at an ionic strength of 0.1 M NaCl (Merck p.a., dried at 453 K) as reported in a previous study [13]. Hydrazone stock solutions were made by dissolving dry hydrazone into a well-determined volume of ionic medium.

2.2. UV-VIS spectrophotometric titrations

Equilibrium constants for ME0329 deprotonation and Ga-hydrazone binding were determined by UV-VIS spectrophotometric titrations, as described in a previous study [13]. Because of the low solubility of the hydrazone and the sensitivity range in the UV-VIS spectrophotometer, very dilute solutions of ligand (L), $[L] = 0.04\text{-}0.1\text{ mM}$ and $[Ga] = 0.05\text{-}0.07\text{mM}$, were used.

2.3. Speciation calculations

Extraction of equilibrium constants from UV-VIS spectrophotometric spectra was carried out as detailed in Boily and Suleimenov [19]. Briefly the matrix form of the Beer-Lambert law ($\mathbf{A}=\epsilon\mathbf{C}$) was solved for molar absorption coefficients (ϵ_n) of n number of species by evaluation of the concentration matrix ($\mathbf{C}$) from the aqueous speciation calculations. Searches for optimal equilibration constants were made by minimizing the sum-of-squares of the deviation of the experimental absorption matrix ($\mathbf{A}_{\text{exp}}$) from its calculated counterpart ($\mathbf{A}_{\text{calc}}$), using the objective function $\Sigma(\mathbf{A}_{\text{calc}} - \mathbf{A}_{\text{exp}})^2$ normalized for the number of degrees of freedom. The matrix $\mathbf{A}_{\text{calc}}$ was obtained from $\mathbf{A}=\epsilon\mathbf{C}$ using a matrix rotation approach [19], starting from the results of a Singular Value Decomposition (SVD) of $\mathbf{A}_{\text{exp}}$. Inspection of the SVD results also determined the dimensionality of $\mathbf{A}_{\text{exp}}$, and consequently of the number of aqueous species responsible for the variance in the spectrophotometric data.

Aqueous speciation calculations were made by numerically solving for mass action and balance equations.

Mass action equations are expressed as:

$$\text{where } K_a \cdot [\text{H}_4\text{L}^0]^r = [\text{H}^+]^p \cdot [\text{H}_{4-r-p}\text{L}_r^{-p}]$$

and for Ga(III)-H₄L with :

where $[\text{Ga}_q(\text{H})_p(\text{H}_4\text{L})_r]^{3q+p} = \beta_{pqr} \cdot [\text{Ga}^{3+}]^q \cdot [\text{H}_4\text{L}^0]^r \cdot [\text{H}^+]^p$.

Mass balance equations are:

$$[\text{Ga}]_{\text{Tot}} = \sum_0^p \sum_0^q \sum_0^r q [\text{Ga}_q(\text{H})_p(\text{H}_4\text{L})_r]^{3q+p} \quad (3)$$

$$[\text{L}]_{\text{Tot}} = \sum_0^p \sum_0^q \sum_0^r r [\text{Ga}_q(\text{H})_p(\text{H}_4\text{L})_r]^{3q+p} \quad (4)$$

$$[\text{H}]_{\text{Tot}} = \sum_0^p \sum_0^q \sum_0^r p [\text{Ga}_q(\text{H})_p(\text{H}_4\text{L})_r]^{3q+p} + [\text{H}^+] - K_w^{-1} [\text{H}^+]^{-1} \quad (5)$$

where p , q and r are stoichiometric coefficients.

As all experiments were carried out in a constant ionic medium, chloride and sodium ion mass balances were not considered in the calculations, nor were activity coefficients used.

After determining molar absorption coefficients and stability constants for ME0329, these values were used in the evaluation of Ga-ME0329 complexes. Moreover, gallium-oxyhydroxide precipitation possibilities were taken into account in the modeling procedure by determining the total gallium solubility in the ME0329-bearing system.

2.4. Molecular modeling

Partition coefficients (log P) for ME0329 and ME0163 hydrazones were calculated by MarvinSketch 6.1.3 [20] using four methods available in the software: VG[21], KLOP[22], PHYS (PhysProp© database) and weighted (1(VG):1(KLOP):1(PHYS):0(weighted)); all in 0.1 M electrolyte concentration and considering tautomerization/resonance (all shown in Supplementary Table S1). Theoretical pK_a values were also estimated by MarvinSketch 6.1.3 using two methods: macro and micro (all shown in Supplementary Table S1).

Geometry optimization calculations of the neutrally-charged $\text{Ga}(\text{OH})_2$ -ME0329 complexes were carried out at the B3LYP/6-31G level of theory [23]. Two water molecules were used to

complete the octahedral coordination shell of Ga(III). Calculations were carried out using Gaussian 09 [24].

2.5. Ga uptake

Ga uptake by *P. aeruginosa* from media with Ga-citrate, Ga-ME0163 or Ga-ME0329 was performed as previously described [25]. Briefly, PAO1 was grown to an optical density (at 600 nm, OD₆₀₀) of 1.1 in Fe-depleted Iso-Sensitest 20%. Bacteria were collected by centrifugation and washed twice with PBS. The washed bacteria were added to obtain an OD₆₀₀ of 1.1 in iron-depleted 20% Iso-Sensitest media with 25 µM of one of the following: Ga-citrate, Ga-ME0329, Ga-ME0163, ME0329 or ME0163. The Ga hydrazone complexes (Ga-ME0163 or Ga-ME0329) were prepared by mixing Ga-citrate solution with the ligand in 1:1 ratio. The number of viable counts in a reference bacterial suspension was determined to enable normalization to number of cells. The data presented are from two experiments performed on different batches of growth medium with two to three replica each.

After 1 h of incubation at 37 °C (with shaking), 2 ml of the medium was centrifuged. Thereafter bacteria were washed twice in 2 ml PBS, and re-suspended in 1 ml 0.1 % SDS + 1 mM NaOH. Bacteria were pipetted and vortexed until they had dissolved. The resulting liquid was filtered through a 0.2 µm filter.

Determination of Ga-concentrations in dilutions of the samples was conducted with an Elan DRC-e ICP-MS (Perkin Elmer SCIEX, Ontario, Canada). Gallium was quantified by external calibration with matrix matched standards, using the ⁷¹Ga isotope with indium (¹¹⁵In) as internal standard.

The amount of Ga present at the surface of bacteria was determined using cryo-X-ray photoelectron spectroscopy of bacterial cells [26] previously exposed to 25 µM Ga-ME0329 for 1 h (the same procedure as for the Ga uptake), followed by washing with PBS centrifugation and freezing of the bacterial pellet as droplets into liquid nitrogen inside

centrifuge tubes. The centrifuge tubes with frozen droplets were stored overnight at -80 °C and moved to the XPS instrument immersed in liquid nitrogen the following day. The suspension droplets were handled and analyzed frozen (without thawing) at cryogenic temperatures (-160 °C). XPS spectra were collected using a Kratos Axis Ultra DLD spectrometer with a monochromated Al K α source operated at 150 W. Analyzer pass energy of 160 eV was used for acquiring survey spectra and 20 eV for acquiring spectra of individual photoelectron lines. The analysis area was 0.3 × 0.7 mm. The spectrometer charge neutralizing system was used to compensate for sample charging during the measurement, and the binding energy scale was referenced to the C1s aliphatic carbon peak at 285.0 eV. This analysis gives the near surface (< 10 nm) chemical composition of the bacterial surface.

2.6. *Bacterial growth and biofilm formation*

P. aeruginosa, PAO1, was cultured on blood agar plates. Iron-free medium ISO-SENSITEST broth (20%) was prepared according to Rzhepishevskaya et al. [12] and trace elements were adjusted to following final concentration: 0.03 mM MgSO₄; 0.08 μ M ZnCl₂; 0.01 μ M CuSO₄; 0.05 μ M MnCl₂; 0.25 μ M CaCl₂. Effect of Ga complexes on bacterial growth was investigated at 37 °C in liquid culture in multi-well plates (without shaking). Growth was measured as culture absorbance at 600 nm (OD₆₀₀) after 9 h. Inhibition of *P. aeruginosa* biofilm formation was assessed by the crystal violet staining assay [27, 28]. Briefly, 200 μ l of iron-free ISO-SENSITEST broth containing Ga-citrate, ME0329, or Ga-ME0329, was inoculated with an overnight culture of *P. aeruginosa* PAO1 (approximately 5×10⁵ - 1×10⁶ cells) and incubated at 37 °C. Thereafter the suspension was discarded, the remaining biofilm gently rinsed, a crystal violet solution (0.1%) added to each well and left to incubate for 10 min. Thereafter the multi-well plates were rinsed with phosphate buffered saline (PBS) and air dried. Crystal violet was dissolved in 33% acetic acid and absorbance of the solutions was measured at 595 nm.

2.7. Expression and secretion of ExoS protein

T3SS was induced by culturing bacteria in media with low Ca^{2+} availability (TSB broth with EGTA and 5 mM MgCl_2) as previously described [12]. *P. aeruginosa* PAO1 was grown to OD_{600} of 1.2 for all conditions (Fig. 8d). Thereafter bacteria were pelleted and the culture supernatant was used for analysis of secreted proteins. The proteins were precipitated using 10% trichloroacetic acid/acetone and resuspended in loading buffer for electrophoresis [29-31]. Aliquots of the culture containing both bacteria and the supernatant were collected and mixed with the loading buffer to analyze total (expressed and secreted) protein. Protein fractions were separated on BioRad ready-made 12% gels. ExoS protein, a marker of T3SS expression and secretion, was detected with Western blot using ExoS antibodies. To make sure that equal amounts of sample were loaded, sample suspensions with equal absorbance at 600 nm were used (Fig. 8d), and Coomassie stained polyacrylamide gel and Western blot were prepared in parallel (Fig. 8c). All experiments were performed in triplicate.

3. Results and discussions

3.1. Deprotonation of ME0329

Determination of deprotonation constants of ligands, as well as their metal complexation, is essential to enable a better understanding of the connection between the metal-binding strength of complexes and their biological activity. Thus, in order to compare previous data for the Ga-ME0163 complex [13] with the Ga-ME0329 complex, equilibrium constants for the ligand ME0329, as well as its Ga(III) complex were determined. In Figure 1 the potential Brønstedt-acid protonation sites for the ligand are identified. Theoretical pK_a values for these protonation sites were estimated by MarvinSketch 6.1.3 and were used to interpret the experimental data (Table 1). Spectrophotometric titrations showed that both aromatic rings were characterized by strong $\pi\text{-}\pi^*$ transitions of *p*-orbitals as well as charge-transfer-to-solvent (CTTS) transitions of the lone pairs hydrogen-bonded to solvent water molecules. The wavelength of the absorption maximum (λ_{max}) of conjugated molecules can be calculated empirically using Woodward-Fieser rules [32] (Table 2). These calculations showed that, in

alkaline solution, the absorption bands of the two aromatic rings overlap and give rise to the peak at 388 nm with a shoulder at 339 nm (Fig. 2). Increasing solution acidity lowers the level of electron delocalization and hence shifts the absorption maxima to a lower wavelength during each protonation step. Consequently, leading to separation of the absorption bands of the two phenyl-rings both from each other, as well as from the $n-\pi^*$ transition displayed at 402 nm in the fully protonated ligand. The difference between calculated and experimental data could be due to CTTS effects, as well as the phenyl rings being part of a larger conjugated molecule (Table 2).

A SVD-based dimensional analysis of the UV-VIS spectra points to three deprotonation steps in ME0329 with contribution of four linearly-independent components. Extraction of the stability constants and molar absorption coefficients of these species gave equilibrium constants $pK_{a1}=7.4$, $pK_{a2}=8.9$ and $pK_{a3}=10.0$ (Table 1).

These values are comparable to predicted values, and suggest that the first two constants correspond to the deprotonation of phenolic groups in *ortho*-positions, and the third pK_{a3} relates to the *para*-phenolic group. According to the theoretical calculations using MarvinSketch, the *ortho*-position of the 2,4-dihydrobenzohydrazide ring is the first deprotonated site (Fig. 1). The deprotonation constant for the N-NH bridge was not expected to be possible to extract from the experimental data as it is expected at $pH > 13$.

We note that these constants are in line with those of the related ME0163 hydrazone ligand reported in our previous study [13] (Table 1). Differences in proton affinity between these two substances can be explained by the presence of different substituents of the aromatic rings. For example, the higher pK_{a2} value of ME0329 should consequently arise from the chlorine atom with positive mesomeric effect, as well as the alkyl group with an electron releasing inductive effect which is expected to increase the electron density on the oxygen and thus stabilize the O-H bond.

3.2. Ga-ME0329 complexation

Ga-ME0329 binding was studied in solutions prepared with ratios L:Ga= 1:1.5 and 1:1.8. The acidity constants and molar absorption coefficients of ME0329 detailed in the previous section, were used in the analysis of the spectrophotometric data to study Ga-ME0329 binding. SVD-analysis of the resulting spectra extracted seven orthogonal components. Excluding contributions from any of the soluble gallium hydroxide complexes in these solutions, and considering that four ME0329 species are formed in the studied pH range, our SVD-based analysis provides evidence that three additional Ga-ME0329 species are needed to reproduce the variance of the data.

The most plausible species combination was searched by a systematic search of $\text{Ga}_q(\text{H})_p(\text{H}_4\text{L})_r$ species with varied p, q, r coefficients. The combination of $[\text{Ga}(\text{OH})_2\text{H}_3\text{L}]^0$ ($p, q, r = -3, 1, 1$), $[\text{Ga}(\text{OH})_2\text{H}_2\text{L}]^-$ ($p, q, r = -4, 1, 1$) and $[\text{Ga}(\text{OH})_2\text{HL}]^{2-}$ ($p, q, r = -5, 1, 1$) species showed the best-fit. Therefore the solutions only consisted of 1:1 Ga-ME0329 complexes of different deprotonation steps. As all three molar absorption coefficients were shifted with respect to each other (Fig. 3) they are likely from different deprotonation steps of the ligand. Based on the deprotonation steps of the pure ligand system and the hydrolysis of Ga(III) in the absence of ligand, the assignment of $(-3, 1, 1)$, $(-4, 1, 1)$ and $(-5, 1, 1)$ best correspond to the $[\text{Ga}(\text{OH})_2\text{H}_3\text{L}]^0$, $[\text{Ga}(\text{OH})_2\text{H}_2\text{L}]^-$ and $[\text{Ga}(\text{OH})_2\text{HL}]^{2-}$ species respectively. The equilibrium reactions for the formation of these three complexes can be presented as:

The log β values are presented in Table 1.

A distribution diagram, constructed using these formation constants, shows that a mixture of the $[\text{Ga}(\text{OH})_2\text{H}_3\text{L}]^0$ and $[\text{Ga}(\text{OH})_2\text{H}_2\text{L}]^-$ complexes exist in the solution at physiological pH

(Fig. 4). We also hypothesize that Ga(III) binds in the same way as was shown for the ligand ME0163, namely in the chelating motive at the center of the ligand through the azomethine nitrogen, the carbonyl and one phenolic group, forming both a 5-, and a 6-membered chelate [12]. These modes of coordination were assessed further by DFT calculations of the neutrally-charged $[\text{Ga}(\text{OH})_2\text{H}_3\text{L}]^0$ species. Calculations considering various starting geometries, and dispositions of OH and H₂O around Ga(III) provided support for the greater stability of the configuration and bond distances shown in Figure 5.

3.3. *Binding strength of Ga-ME0329*

The equilibrium concentration of free gallium ions in the presence of ligand can be used as an indicator for the chelating strength of ligands. Higher pGa ($-\log[\text{Ga}^{3+}]=\text{pGa}$), i.e. low free metal ion concentrations, illustrates strong complex formation. In order to compare the binding strength of Ga(III) ions to the ME0329 hydrazone with other similar ligands, we calculated free amount of gallium ions in the presence of ligand at 1 μM Ga(III), 10 μM ligand and pH 7.4, using the obtained equilibrium constants above as well as constants in the literature. The results show that the pGa of ME0329 (22.9) is higher than pGa of the chelators such as EDTA (22.6), citrate (19.3) and hydrazone ME0163 (21.3) described in our previous study [13]. At the same time it is lower than the pGa of bacterial siderophores, such as DFOB (25.1), which indicates that the Ga(III)-ME0329 complex will be stable in most biological media, and that Ga(III) ions will be in a bioavailable form enabling bacterial siderophores to take up Ga from the hydrazone and transfer it into the bacterial cells through the Fe(III)- uptake system of the bacteria[33]. However, since the Fe(III) chelation of most siderophores (and metal chelators) will be more favorable [36] (pFe for citrate = 21.7, DFOB= 27.5, EDTA 26.1) it is expected that the biological effect and uptake of Ga(III) will be reduced in the presence of Fe(III).

3.4. Ga uptake into bacteria

To experimentally investigate how ligand complexation of hydrazones influenced gallium uptake into *P. aeruginosa* PAO1 we investigated the cellular content of gallium after exposure of 25 μM of one of three Ga(III) complexes during 1 h (Fig. 6). From these uptake studies it was concluded that the presence of the Ga-ME0329 complex resulted in a higher accumulation of Ga intracellularly compared to the previously investigated Ga-ME0163 complex as well as Ga-citrate. Some variation in uptake from the Ga-ME0329 complex was seen in different repeats of the experiments. To investigate if this effect was originating from surface deposition of the complexes in outer layers of the bacterial cell wall, XPS analyses were performed of intact bacterial cells [26, 34]. No Ga could be detected using XPS which suggests that the Ga observed was intracellular or present in concentrations too low to detect using XPS (data not shown). We cannot completely rule out surface accumulation as precipitation reactions can be difficult to reproduce and the relatively large variation in the uptake of Ga-ME0329 indicate some degree of variability between repeats. However, the XPS data indicate that the majority of the Ga should be taken up into the bacterial cell.

The increased uptake of Ga in presence of ME0329 is hypothesized to be due to the physicochemical properties of the Ga-ME0329 complex. The ligand is a good chelator that will keep Ga(III) from forming Ga hydroxide and deliver it to the Fe(III) uptake system of bacteria. The ME0329 is a slightly stronger Ga(III) chelator than the ME0163. However, the complex with ME0329 is the only complex of the three tested that is neutral at physiological pH. Thus, this complex should have a much higher possibility to diffuse through cell membranes and enter into the bacterial cell [35] than Ga-citrate or Ga-ME0163 (that both have negative charge at physiological pH). Furthermore, the difference in lipophilicity between the two hydrazones suggests that the ME0329 complex is more hydrophobic (Table 1 and S1). Consequently, we hypothesize that the Ga-ME0329 complex should be able to deliver Ga(III) into the bacterial cell both through the Fe(III) transport systems of the bacteria (similar to Ga-citrate and Ga-ME0163), as well as through interaction and diffusion through

the cell membrane. Thereby presenting an explanation for the higher uptake of Ga-ME0329 in comparison to Ga-citrate and Ga-ME0163.

3.5. *Growth and Biofilm Formation*

In the presence of Ga-citrate and Ga-ME0329 there was a decreased growth after 9 h compared to control (Fig. 7). This decrease in growth also led to a decreased biofilm formation. Intriguingly, the biological effects of Ga-ME0329 and Ga-citrate were similar despite that much more Ga was taken up from Ga-ME0329.

Compared to our previous study of Ga-citrate and Ga-ME0163 [12], the growth-inhibiting effect observed for Ga-citrate was more pronounced. This, most likely, reflects small differences in Fe(III) content of the medium used between the two studies. Great care was taken to use Fe-depleted medium, however, the process of depletion, as well as handling of the medium, may result in small variations in Fe(III) content between medium batches. It has previously been reported that the Fe(III) content of media can have an effect on the level of the observed biological effects from Ga(III) [12]. The main reason being that the formation of siderophore complexes with Fe(III) is thermodynamically more favorable compared to formation of the corresponding Ga(III) complexes, in presence of both metal ions (shown by the pFe and pGa calculations above).

3.6. *ExoS expression and secretion*

To investigate possible synergistic effects between Ga and ME0329 with respect to release of bacterial toxin, secretion of ExoS into the media was studied in presence of Ga-citrate, Ga-ME0329 and only ME0329, at 25 and 50 μ M (Fig. 8a, protein loading control in Fig. 8c). It was observed that the intensity of the secreted ExoS band in the Western blot was lowered for all three conditions compared to control, and the most pronounced effect was seen in the presence of Ga-ME0329 (Fig. 8a). Next, total ExoS (expressed and secreted) was investigated through the levels of ExoS associated with both cells and media (Fig. 8b). Again, compared to the control, the levels of ExoS were lower for all three conditions and

most reduced in the presence of the Ga-ME0329 complex (Fig. 8b). These results indicate that there is a synergistic effect between Ga ions and ME0329 with respect to reducing both expression and secretion of the T3SS. The ExoS inhibition appears to be specific and not a consequence of growth inhibition, as the bacteria used were harvested at the same optical density for all experimental conditions and exhibited equal amounts of total protein (Fig. 8c,d).

3.7. *The role of metal chelation and uptake on biological effects of hydrazones*

In this study we have investigated the hypothesis that gallium-chelation strength of two hydrazone compounds would be related to their biological effect on *P. aeruginosa*. Furthermore, we investigated if the anti-virulence effect of hydrazones, with respect to ExoS secretion through the T3SS, could be enhanced through hydrazone complexation with Ga(III).

Interestingly, the uptake of Ga(III) into bacterial cells was shown to be higher from the hydrazone with higher affinity for Ga(III) (Ga-ME0329), than for Ga-citrate or Ga-ME0163. However, the Ga-ME0329 complex showed to have additional properties that also would enhance its diffusion across cell membranes, thus, it can be hypothesized that this complex could enable two uptake routes for Ga(III) into bacteria. The biological effect of the Ga-ME0329 complex was shown to be slightly different from the earlier studied Ga-ME0163 complex. The former displayed a pronounced uptake of Ga(III) and a synergistic effect with Ga(III) on ExoS secretion, but a similar effect to Ga-citrate against biofilm formation. The latter displayed synergistic effects with Ga(III) against biofilm formation, but did not show synergistic effect with respect to decreasing ExoS secretion [12]. This intriguing finding suggests that apparently similar Ga complexes can potentially induce very different biological responses in bacterial cells. These contrasting effects are possibly connected to the levels of intracellular Fe and the signaling pathways connected to Fe acquisition and metabolism of the bacteria, as a larger uptake of the Fe(III)-antagonist, Ga(III), was observed in presence of

Ga-ME0329. Ga has been shown to interfere with Fe metabolism and hence perturb multiple metabolic pathways in *P. aeruginosa* [25, 37]. It may be speculated that Ga-related changes in metabolic status influence expression of T3SS, since the regulation of T3SS has been shown to also be subjected to metabolite control [38]. More specific mechanism of T3SS inhibition by Ga may be connected to T3SS control by IscR, a Fe containing transcription factor, shown to be important in *Yersinia pseudotuberculosis* and by analogy probably also in *P. aeruginosa*, since this transcription factor is well conserved between bacteria [39]. This could explain why the inhibitory effect on virulence was enhanced in presence of the Ga-ME0329 complex. One could further hypothesize that synergistic effects between Ga(III) and ME0329 are a result of both of them targeting the Fe(III) acquisition system. However, the observed differences between the two Ga hydrazone complexes would suggest that hydrazones also can have additional targets since two hydrazones with relatively similar affinity for Ga(III) display such differences in biological response. Alternatively, the biological response differs depending on the intracellular Fe(III) (and Ga(III)) levels.

3.8. Conclusions

By determining previously unknown equilibrium constants of a Ga-hydrazone complex with enhanced effect against T3SS in *P. aeruginosa*, we have been able to draw comparisons with a previously determined Ga hydrazone complex. We have also shown that the biological responses to these complexes, such as Ga uptake, anti-virulence and anti-biofilm effects, are very complex and may result from the metal chelating capacity of the substances, their physicochemical properties, as well as interactions with other biological targets. Our study also shows that synergies between the Fe(III)-mimetic, Ga(III), and hydrazones can be obtained in Ga hydrazone complexes and that these complexes thereby can enhance the targeting of virulence in the form of secretion from the T3SS, as well as biofilm formation by *P. aeruginosa*. We have not, however, been able to show that both these targets can be met through the use of only one Ga hydrazone complex. Thus, this study suggests that the interaction of Ga hydrazone complexes with the bacterial cell is complex and either targets

more than just the Fe acquisition, or that the Fe regulation and metabolism in *P. aeruginosa* gives rise to very different phenotypes depending on Fe(III) (or Ga(III)) levels in the cell. As such, this study contributes to future developments of novel drug types targeting the Fe metabolism in bacteria, as it illustrates how several physicochemical parameters of a substance can co-influence its resulting biological effect.

Abbreviations

ME0329	N'-(5-chloro-2-hydroxy-3-methylbenzylidene)- 2,4-dihydroxybenzhydrazide
ME0163	2-oxo-2-[N-(2,4,6-trihydroxy-benzylidene)-hydrazino]-acetamide
L	ligand
T3SS	type three secretion system
EDTA	Ethylenediaminetetraacetic acid
K_a	the acid dissolution/deprotonation constant
β	overall formation constant
β_{pqr}	overall formation constant for reaction of reactants with stoichiometric coefficients p, q and r
SOS	sum of squares of errors
SVD	singular value decomposition
CTTS	charge-transfer-to-solvent
log P	partition coefficients
DFT	density functional theory

Acknowledgements

This research was funded by the Swedish Research Council (#2011- 3504 for M. Ramstedt; #2012-2976 for J.-F. Boily). Mikael Elofsson, Department of Chemistry, Umeå University, Sweden, is greatly acknowledged for providing the ME0329 ligand.

Supplementary material

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.jinorgbio.2016.04.010>. These data include MOL files and InChiKeys of the most important compounds described in this article, as well as Table S1.

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Tables

Table 1. Experimental and theoretical (Macro mode) equilibrium constants for $\text{Ga}_q(\text{H})_p(\text{H}_4\text{L})_r$ describing the deprotonation of ligands ME0329, ME0163 and Ga-ME0329, Ga-ME0163 complexes in solution at 25 °C and $I=100$ mM, as well as estimated log P for neutral ligands (using VG model) [13].

System	p,q,r	Product	Log β		pK _a		Log P	
					ME		ME	ME
			ME 0329	ME 0163	exp	theory	exp	theory
Ligand	1,0,1	H_3L^- (pK _{a1})	-7.4	-6.8	7.4	7.8	6.8	4.0
	2,0,1	H_2L^{2-} (pK _{a2})	-16.3	-14.5	8.9	8.6	7.7	
	3,0,1	HL^{3-} (pK _{a3})	-26.3	-25.1	10.0	9.4	10.6	
	4,0,1	L^{4-} (pK _{a4})				13.9		
Ga-Ligand	-3,1,1	$[\text{Ga}(\text{OH})_2\text{H}_3\text{L}]^0$	27.1					
		$[\text{Ga}(\text{OH})_2\text{H}_3\text{L}]^0$		32.8				
	-4,1,1	$[\text{Ga}(\text{OH})_2\text{H}_2\text{L}]^-$	18.7					
		$[\text{Ga}(\text{OH})_3\text{H}_3\text{L}]^-$		29.4				
	-5,1,1	$[\text{Ga}(\text{OH})_2\text{HL}]^{2-}$	9.1					
	-6,1,1	$[\text{Ga}(\text{OH})_3\text{HL}]^{3-}$		16.1				

Table 2. The wavelengths of maximum absorption of ME0329 ligand species using Woodward-Fieser rule

Species	λ_{max} of Ring 1/Ring 2 (nm)		Substituted effect in Ring 1 /Ring2
	theory	exp
375 /300	388/339	256(ar)+11(<i>o</i> -O ⁻)+78(<i>p</i> -O ⁻)+30(C=O) / 256(ar)+11(<i>o</i> -O ⁻)+0(<i>m</i> -Cl)+3(CH ₃)+30(C=N)
322 /300	368/327	256(ar)+11(<i>o</i> -O ⁻)+25(<i>p</i> -OH)+30(C=O) / 256(ar)+11(<i>o</i> -O ⁻)+0(<i>m</i> -Cl)+3(CH ₃)+30(C=N)
322 /296	360/299	256(ar)+11(<i>o</i> -O ⁻)+25(<i>p</i> -OH)+30(C=O) / 256(ar)+7(<i>o</i> -OH)+0(<i>m</i> -Cl)+3(CH ₃)+30(C=N)
318 /296	342/300	256(ar)+7(<i>o</i> -OH)+25(<i>p</i> -OH)+30(C=O) / 256(ar)+7(<i>o</i> -OH)+0(<i>m</i> -Cl)+3(CH ₃)+30(C=N)

Graphical abstract

Figures

Fig 1. The chemical structure of ME0329 (INP0341). Arrows show acidic sites.

Fig 2. Spectrophotometric titrations of ME0329 in 0.1 M NaCl at 25 °C in the pH 10.2-7.3 range. a) $[L] = 0.04$ mM and b) $[L] = 0.05$ mM). c) Molar absorption coefficients of the species responsible for the data and related with the pKa values on Table 1.

Fig 3. Spectrophotometric titrations of Ga-ME0329 in 0.1 M NaCl at 25 °C in the pH 10.2-7.3 range. a) L:Ga= 1:1.5 and b) L:Ga=1:1.8. c) Molar absorption coefficients of the species responsible for the data and related with the pKa values of Table 1 (subsystem species are shown with dotted lines for comparison).

Fig 4. Distribution of ME0329 Ligand species for 0.07 mM Ga(III) and 0.04 mM Ligand (L). Black line represents $[\text{Ga}(\text{OH})_2\text{H}_3\text{L}]^0$ (-3,1,1), yellow line $[\text{Ga}(\text{OH})_2\text{H}_2\text{L}]^-$ (-4,1,1) and purple line $[\text{Ga}(\text{OH})_2\text{HL}]^{2-}$ (-5,1,1).

Fig 5. Comparison of distances obtained from DFT calculations for the Ga-ME0329 and the Ga-ME0163 complexes [12].

Fig 6 Ga uptake by *P. aeruginosa* PAO1 in presence of ME0163, ME0329, Ga-citrate (Ga cit), Ga-ME0163 and Ga-ME0329 complexes at 25 µM concentration in 20% Fe-free iso-sensitest medium. The bars represent repeated experiments performed with different batches of medium and different bacterial cultures. The inset shows the ratio between the Ga complexes and Ga-citrate from both repeats.

Fig. 7 Growth and biofilm formation of *P. aeruginosa* PAO1 after 9 h in presence of ME0329, Ga-citrate and Ga-ME0329 complexes in 20% iso-sensitest medium. (From left) Black bar: control; red bar: 50μM; green bar: 25 μM; purple bar: 12,5 μM; and blue bar: 6,25 μM.

Fig 8. Effect of Ga-citrate, ME0329 and Ga-ME0329 on secretion and expression of ExoS toxin of *P. aeruginosa*. a) Secretion of ExoS to the growth medium at different concentrations of Ga-citrate and Ga-ME0329. b) Expression of ExoS by *P. aeruginosa* cells in presence of 25μM of Ga-citrate or Ga-ME0329. c) Coomassie staining of SDS (for loading control); * each well loaded with equivalent of 4.8×10^6 bacterial cells; ** each well loaded with equivalent of 2.4×10^6 bacterial cells. d) Growth of *P. aeruginosa* PAO1 in low-calcium TSB medium in absence (control, grey diamonds) or presence of Ga-citrate (grey squares), ME0329 (black triangles) and Ga-ME0329 (grey crosses), all in 25μM concentration; horizontal dashed line marks sampling points for ExoS.

Supporting information

Table S1. Theoretical estimates of log P and pK_a using the MarvinSketch 6.1.3 software

logP estimate / method	ME0329	ME0163
VG	4.0	-0.3
KLOP	3.6	-0.9
PHYS	3.6	-0.7
Weighted	3.8	-0.7
pK_a estimate - macro mode		
pK _{a1}	7.8	8.5
pK _{a2}	8.6	9.7
pK _{a3}	9.4	10.7
pK _{a4}	13.9	11.3
pK_a estimate - micro mode		
pK _{a1}	8.0	8.9
pK _{a2}	8.5	8.9
pK _{a3}	8.6	9.4
pK _{a4}	10.0	10.9