Antimicrobial activity of an iron triple helicate

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Abstract

The prevalence of antibiotic resistance has resulted in the need for new approaches to be developed to combat previously easily treatable infections. Here we report the investigation into the potential of a synthetic metallomolecule, $[Fe_2L_3]^{4+}$ as an antibacterial agent. This molecule has previously been shown to bind DNA in vitro in the major groove and coil DNA. The work described here shows that the iron triple helicate is cytotoxic to *Bacillus subtilis* (MIC = 32 mg/ml) and *Escherichia coli* (MIC = 64 mg/ml) via a bactericidal mode of action. We also demonstrate that the helicate binds bacterial DNA in vivo and, strikingly, that it kills *B. subtilis* cells very rapidly (within minutes). At sublethal concentrations, upregulation of two stress proteins in *B. subtilis* was observed. This work introduces a promising lead compound for a new class of antibiotics.

Keywords

metallomolecule, helicate, DNA binding, major groove binder, antibiotic, antimicrobial

1. Introduction

Since their discovery in the 20th century, antibiotics have played an extremely important role in human health care. However, the widespread use of antibiotics has also resulted in the emergence of resistant bacteria, and in combination with a substantive drop in the number of new antimicrobials coming onto the market, it is clear that there is a major global problem. It is thus important that we continue to develop novel types of antimicrobial agents.

One target that is of interest for the development of novel classes of antibiotics is DNA. Most of the known compounds that target DNA interact in different ways, through e.g. covalent binding, intercalation, or non-covalent binding in the minor groove [1-3]. Such DNA-binding compounds can inhibit a variety of processes including DNA synthesis and repair through inhibition of enzymes such as toposiomerases, helicases or gyrases. DNA-binding compounds are particularly known for their anti-tumour activity, but several of these also have antibacterial activity [1].

Building on this DNA-drug interaction premise, molecules can be designed that bind in specific manners to B-DNA. An example of this is an iron triple helicate (denoted as $[Fe_2L_3]^{4+}$; Fig. 1), which binds strongly to DNA [4]. $[Fe_2L_3]^{4+}$ is synthesised as a mixture of two enantiomers (M and P, denoting left handed or minus, and right handed or plus helical twists) which have different structural effects on DNA [5]. The M enantiomer binds to the major groove and strongly induces coiling of DNA, while the P enantiomer binding site(s) of the P enantiomer is more ambiguous and it has less pronounced coiling

effects. Intriguingly, the M enantiomer can also bind to or induce DNA junction structures [6]. The main aim of the work reported here was to establish whether $[Fe_2L_3]^{4+}$ has antimicrobial activity and whether its potential target (DNA) is reached.

[Figure 1 near here]

2. Materials and Methods

2.1 Strains and growth media

Bacillus subtilis 168 has been described previously [7]. *Escherichia coli* GM2163 was obtained from New England Biolabs (Hitchin, UK). Bacteria were grown in Luria Broth (LB) containing 10 g/L tryptone (Becton Dickinson, Sparks, USA), 5 g/L yeast extract (Becton Dickinson), and 10 g/L NaCl (Fisher Scientific, Loughborough, UK), or in Richards-Bolhuis (RB) medium: 20 g/L glucose (Merck KGaA, Darmstadt, Germany), 10 mM ammonium sulphate (Fisher Scientific), 20 μM magnesium chloride (Merck KGaA), 7 μM calcium chloride (Merck KGaA), 0.5 μM manganese(II) chloride (Sigma-Aldrich, Gillingham, UK), 0.02 μM thiamine (Sigma-Aldrich), 0.01 μM zinc(II) chloride (Merck KGaA), 0.05 μM iron(III) chloride (Sigma-Aldrich), 0.02 mg/ml tryptophan (Sigma-Aldrich). After mixing the medium components, 0.1% casamino acids (Becton Dickinson) were added and after further mixing 10 mM potassium phosphate (Merck KGaA) pH 7 buffer was added. RB-starvation medium contained the same ingredients, except that the casamino acids were replaced by a mixture of 18 amino acids at 20 mg/L (all amino acids except methionine and cysteine).

2.2 $[Fe_2L_3]^{4+}$ synthesis

$[Fe_2L_3][Cl_4]$ was synthesised as described [8]. Purity was confirmed by solution state NMR and partial microanalytical (C, H, N) data.

2.3 Protocol for determining minimal inhibitory concentration and minimal bactericidal concentration.

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were established using a standard macro broth dilution method in RB medium with the appropriate controls and conducted in triplicate [9].

2.4 Time-dependent uptake of $[Fe_2L_3]^{4+}$ in B. subtilis

B. subtilis was grown overnight in LB medium and then diluted to 5×10^5 cells/ml in RB medium. Different concentrations of $[Fe_2L_3]^{4+}$ dissolved in water (maximum volume 40 μl) were then added to 1 ml of cells and incubated at room temperature for different lengths of time. Cells were collected by centrifugation, washed with fresh RB medium, and resuspended in LB medium. Subsequently, cells were plated on LB agar plates and counted. This experiment was conducted in triplicate.

2.5 Binding of $[Fe_2L_3]^{4+}$ to chromosomal DNA

B. subtilis was grown overnight in RB medium. Next, the culture was incubated for 1 hour in the presence or absence of 100 μM $[Fe_2L_3]^{4+}$ at room temperature. Cells were then collected by centrifugation, washed with fresh RB medium, and chromosomal DNA was isolated as described [10]. Chromosomal DNA was resuspended in 10 mM Tris-HCl pH 8 buffer, and the absorbance at 575 nm (absorbance maximum of $[Fe_2L_3]^{4+}$) was determined. This experiment was conducted twice.

2.6 Analysis of protein synthesis

B. subtilis cells were grown at 37 °C until an optical density at 600 nm of 0.5 in LB medium. After this, cells were collected by centrifugation and resuspended in RB-starvation medium. 1 ml aliquots were incubated for 30 minutes at 37 °C, after which different concentrations of $[Fe_2L_3]^{4+}$ were added to each sample. Cells were then incubated for a further 30 minutes, after which they were labelled for 10 minutes with 30 μCi ^{35}S-methionine. Reactions were stopped by the addition of 20 μl of 2 mg/ml of non-radioactive methionine and placing the samples on ice. Next, cells were collected by centrifugation, the cell pellets were resuspended in SDS-PAGE loading buffer, and samples were analysed by SDS-PAGE and fluorography as described [11]. The gel was exposed to an X-ray film (Fujifilm, Bedfordshire, UK) and also analysed by phosphor imaging using a Fuji FLA-5000 image reader and Aida-image analyser software (Raytest Isotopenmessgeraete, Staubenhard, Germany). This experiment was repeated twice.

3. Results and Discussion

Initial experiments on the antimicrobial activity of $[Fe_2L_3]^{4+}$ gave variable results because the helicate precipitated out of solution. It became apparent that the high concentrations of anions in LB medium, which can bind electrostatically to the highly positively charged helicate, were the cause. For that reason a modified medium (RB medium) was developed that still allowed for rapid cell growth but in which $[Fe_2L_3]^{4+}$ remained soluble. The primary modification to standard minimal media was the minimization of negatively charged molecules. Growth experiments showed that the modified RB medium performed as well as a standard minimal medium (not shown).

To test the antimicrobial activity of $[Fe_2L_3]^{4+}$ we analysed its cytotoxicity on the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *B. subtilis* in RB medium. *B. subtilis* was found to be more susceptible to $[Fe_2L_3]^{4+}$ than *E. coli*, with an MIC for *B. subtilis* of 32 mg/L (MBC = 64 mg/L) and an MIC for *E. coli* of 64 mg/L (MBC = 128 mg/L). There is only one dilution step difference between the MIC and MBC for both organisms, which indicates that the action of the helicate is bactericidal rather than bacteriostatic.

As the action of the helicate is primarily bactericidal, we analysed the kinetics of killing by determining the surviving fraction of *B. subtilis* cells after incubation with $[Fe_2L_3]^{4+}$. As shown in Fig. 2, the rate of killing is fast with a concentration at the MIC (25 μM; 32 mg/L), completely inhibiting growth within 2 minutes (the shortest time that could be reliably measured). The sublethal concentrations of $[Fe_2L_3]^{4+}$ (5 μM and 15 μM) also showed considerable cytotoxicity (e.g. ~99% ± 0.5% cell death seen after 10 minutes incubation with 15 μM helicate). At sublethal concentrations the killing efficiency is greater with longer periods of incubation with the helicate. For instance, at 5 μM (5-fold less then the MIC), only 26% of cells are killed after 2 minutes, but more then half the cells are killed after 10 minutes incubation.

[Figure 2 near here]

To determine whether $[Fe_2L_3]^{4+}$ reaches its putative target, chromosomal DNA was isolated from *B. subtilis* cells incubated with or without the helicate. $[Fe_2L_3]^{4+}$ has a strong purple colour with a peak absorbance of 575 nm, providing a simple means to determine whether the helicate binds DNA. The average absorbance signal at 575 nm (A_{575}) of the DNA of the control samples without $[Fe_2L_3]^{4+}$was 0.085, while DNA isolated from cells incubated with 100 μM $[Fe_2L_3]^{4+}$ was clearly purple and had an A_{575} of 0.312. This demonstrated that the helicate does reach and bind to chromosomal DNA within cells. It should be noted that this does not necessarily mean that DNA is the only target or is the primary cause of death. A highly charged molecule could bind to multiple targets as is the case, e.g. for the anticancer drug *cis*-platin, for which an estimated 5% of the administered drug binds to its intended target (DNA) whilst the rest binds elsewhere with the cell – primarily to ribosomes [12]. Notably, as shown above the rate of killing is extremely fast, and it seems therefore unlikely that DNA binding is the only mechanism through which bacteria are killed. Notwithstanding this, the result proves that the molecules do in fact reach and bind to their intended target – chromosomal DNA.

To analyse the effects of the helicate on protein synthesis in *B. subtilis* cells, a pulse-labelling experiment was performed. For this purpose cells were treated with the helicate for 30 minutes after which cells were labelled with ^{35}S-methionine, and proteins were visualised by SDS-PAGE and fluorography. As shown in Fig. 3, the amount of protein synthesised in cells is reduced with increasing concentrations of helicate. The results from the gel (Fig. 3A) were confirmed by measuring the total amount of radioactivity in each lane with a phosphor imager (Fig 3B). Note that there is still protein synthesis at a

helicate concentration above the MIC (~24% at 40 μM), but the number of cells used here is about 500-1000 fold higher than the number of cells used in the experiments to determine the MIC or the rate of killing. Strikingly, some proteins appear in greater abundance after treatment with the helicate, most notably small proteins around 6-9 and 16 kDa (indicated with arrows). These are probably stress proteins encoded by genes that are upregulated in response to uptake of the helicate. At the highest concentration of 80 μM no bands were visible indicating a complete inhibition of protein synthesis, although it is also conceivable that the uptake of ^{35}S-methionine is affected at that concentration.

[Figure 3 near here]

The work reported herein provides the first evidence of efficacy in both Gram-positive and Gram-negative strains of bacteria with the synthetic metallomolecule $[Fe_2L_3]^{4+}$. The MIC and MBC in both bacteria, whilst clearly showing cytotoxicity of a bactericidal nature, are not low enough to be comparable with most leading antibiotic working concentrations (often in the order of 1 μM). However, the major attraction of this molecule as a lead compound is that because of the novel nature of (one of) its mode(s) of action in targeting DNA directly in the major groove, it may be effective on bacterial strains currently resistant to other antibiotics. A library of similar compounds has recently been synthesised [13], some of which may show a greater degree of bacterial cytotoxicity. One of the more surprising results reported here is the very fast action time of $[Fe_2L_3]^{4+}$ in killing bacteria – less than 2 minutes at the MIC. This could prove very useful in treating nosocomial infections where time is an important factor in the choice of

treatment of patients. The presence of upregulated stress proteins by cells incubated with sublethal concentrations of $[Fe_2L_3]^{4+}$ is evidence that the cells do indeed recognise the presence of a hostile molecule, or at least are responding to the DNA damage and any other target-associated damage within the cell

In conclusion, a promising synthetic lead compound, $[Fe_2L_3]^{4+}$, has been shown to be cytotoxic to *B. subtilis* and *E. coli*, acting very rapidly in a bactericidal manner and reaching its target molecule DNA.

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Figure legends

Figure 1

Structure of the triple helicate $[Fe_2L_3]^{4+}$. Adapted from PDB file 2ET0.

Figure 2

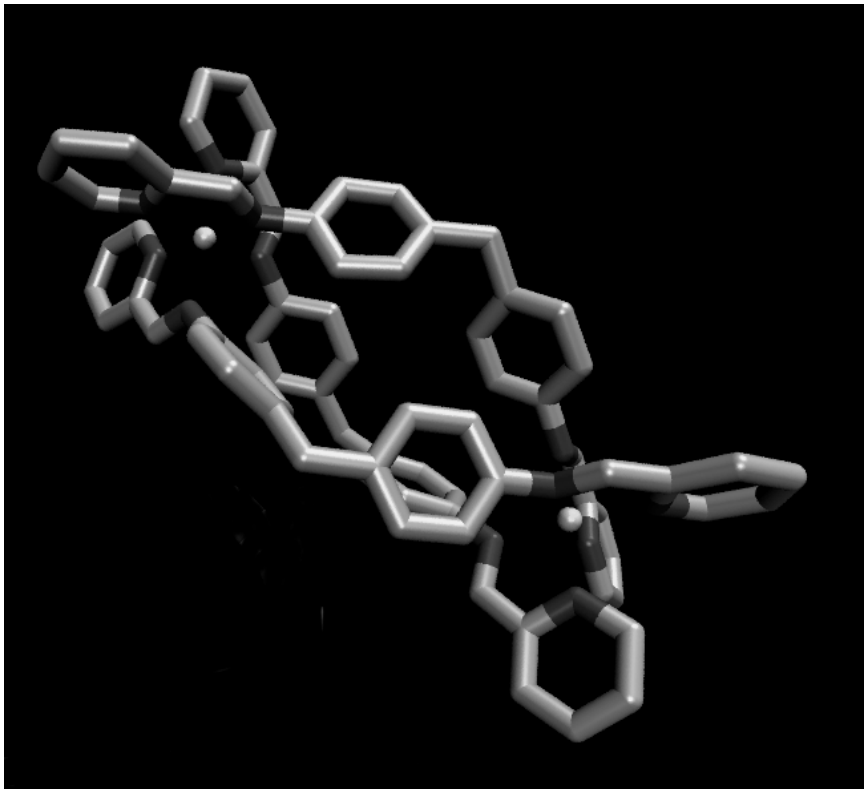
The percentage of surviving cells after 2 minutes (open bars) or 10 minutes (closed bars) incubation with $[Fe_2L_3]^{4+}$.

Figure 3

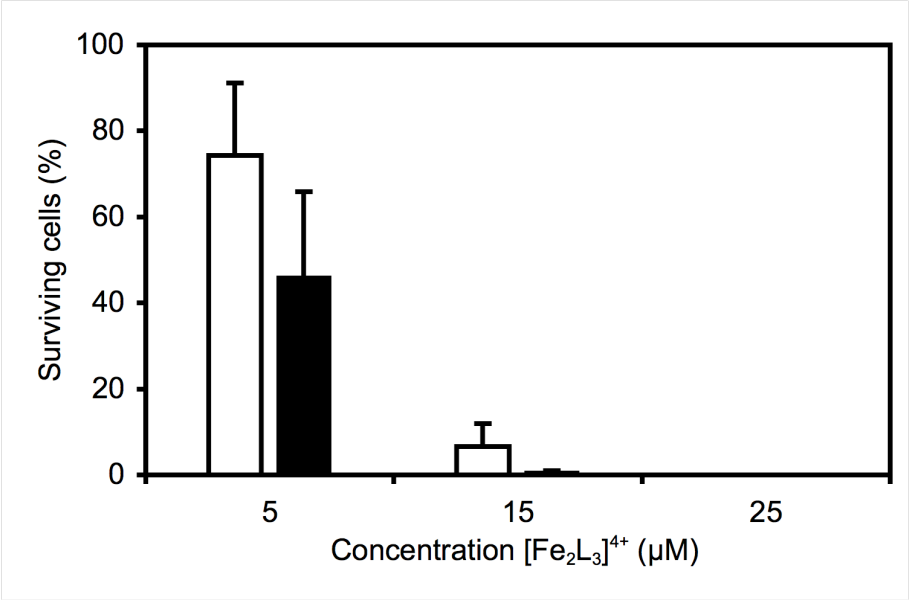
Protein synthesis by *B. subtilis* after treatment with different concentrations of $[Fe_2L_3]^{4+}$. (A), SDS-PAGE of proteins synthesised. The arrows indicate proteins encoded by upregulated genes, at 6-9 kDa and 16kDa. M: molecular weight marker (kDa). (B), total amount of protein synthesised determined by measuring total amount of radioactivity from the SDS-PAGE gel using phosphor imaging. Radioactivity was corrected for the background.

Figures

Figure 1



1 Figure 2



2

3

4

5

6

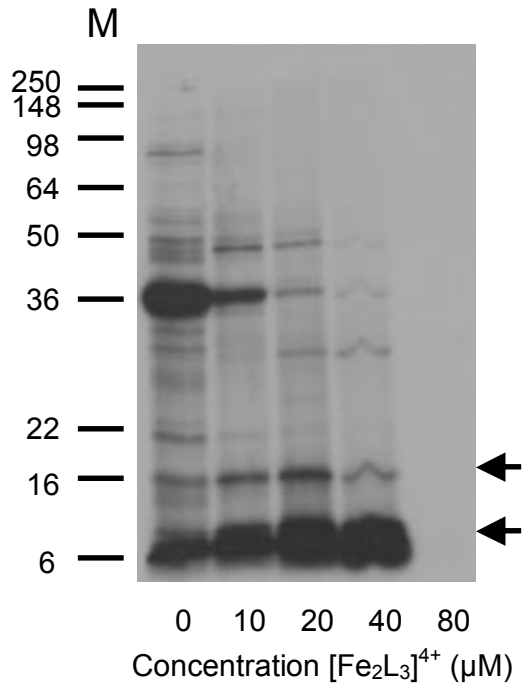
7

8

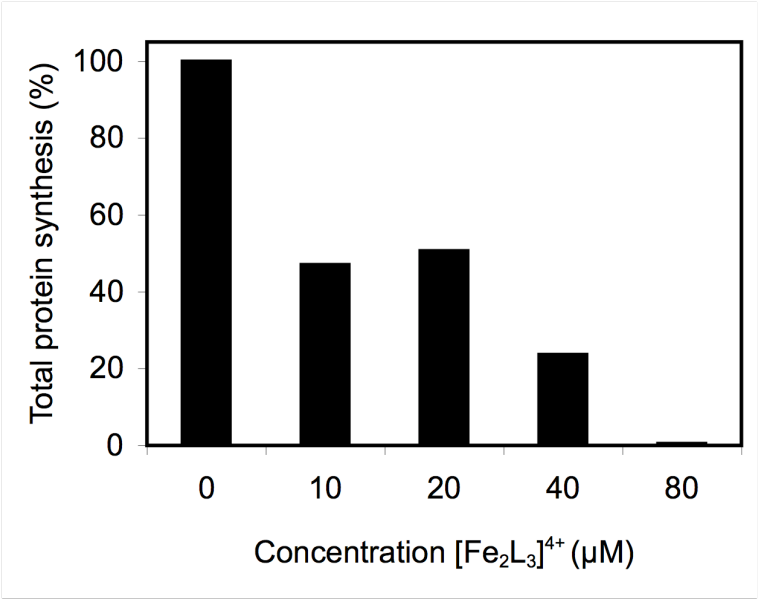
9

Figure 3

A


M
250
148
98
64
50
36
22
16
6
0
10
20
40
80
Concentration $[Fe_2L_3]^{4+}$ (μM)

B


Total protein synthesis (%)
100
80
60
40
20
0
0
10
20
40
80
Concentration $[Fe_2L_3]^{4+}$ (μM)