Galbofloxacin: a xenometal-antibiotic with potent in vitro and in vivo efficacy against S. aureus†

Apurva Pandey, Dariusz Śmiłowicz and Eszter Boros*†

Siderophore-antibiotic drug conjugates are considered potent tools to deliver and potentiate the antibacterial activity of antibiotics, but only few have seen preclinical and clinical success. Here, we introduce the gallium(s) complex of a ciprofloxacin-functionalized linear desferrichrome, Galbofloxacin, with a cleavable serine linker as a potent therapeutic for S. aureus bacterial infections. We employed characterization using in vitro inhibitory assays, radiochemical, tracer-based uptake and pharmacokinetic assessment of our lead compound, culminating in in vivo efficacy studies in a soft tissue model of infection. Galbofloxacin exhibits a minimum inhibition concentration of (MIC₉₈) 93 nM in wt S. aureus, exceeding the potency of the parent antibiotic ciprofloxacin (0.9 μM). Galbofloxacin is a protease substrate that can release the antibiotic payload in the bacterial cytoplasm. Radiochemical experiments with wt bacterial strains reveal that ⁶⁷Galbofloxacin is taken up efficiently using siderophore mediated, active uptake. Biodistribution of ⁶⁷Galbofloxacin in a mouse model of intramuscular S. aureus infection revealed renal clearance and enhanced uptake in infected muscle when compared to ⁶⁷Ga-citrinate, which showed no selectivity. A subsequent in vivo drug therapy study reveals efficient reduction in S. aureus infection burden and sustained survival with Galbofloxacin for 7 days. Ciprofloxacin had no treatment efficacy at identical molecular dose (9.3 μmol kg⁻¹) and resulted in death of all study animals in <24 hours. Taken together, the favorable bacterial growth inhibitory, pharmacokinetic and in vivo efficacy properties qualify Galbofloxacin as the first rationally designed Ga-coordination complex for the management of S. aureus bacterial infections.

Introduction

Infectious diseases are one of the leading causes of death worldwide. Among infective agents, the Gram-positive pathogen Staphylococcus aureus (S. aureus) can trigger a variety of pathologies, such as skin and soft tissue infections, endocarditis, osteomyelitis, lethal pneumonia, and causes the majority of hospital acquired bacterial infections and deaths in the United States. Depending on the susceptibility of pathogen towards currently used antibiotics, S. aureus can be divided into MSSA (methicillin susceptible S. aureus) and MRSA (methicillin resistant S. aureus). In recent decades, the opioid epidemic and the over-preservation of antibiotics, has resulted in rapid emergence of drug resistant S. aureus worldwide. The incidence of infection requiring hospital care has increased, yet clinical treatment of MRSA has become more challenging. The emergence of bacteria with resistance to currently used antibiotics, combined with the paucity of new compounds in the pipeline necessitates the development of targeted novel antibiotics with new mechanisms of action.

Bacterial virulence is closely associated with nutrient acquisition, which is essential for bacterial growth and proliferation. During an infection, iron – an indispensable micronutrient, essential for a plethora of cellular reactions in both humans and pathogens, is restricted for bacterial uptake as the available iron is tightly sequestered by host proteins such as – hemoglobin, transferrin and ferritin. Bacteria rely on scavenging essential transition metal ions from their host, specifically iron in the form of ferrous/ferrie (Fe²⁺/Fe³⁺). The low solubility of Fe(OH)₃ (Kₚ = 6.3 × 10⁻³⁸) at pH 7.4 results in an insufficient quantity of iron for bacteria to grow. Given the essential role of iron (Fe) in bacterial physiology and pathogenicity, and the ability of bacteria to actively transport essential nutrient suggests that the iron uptake and metabolism pathway can be hijacked for development of new theranostic agents that can circumvent the membrane permeability barrier of bacteria. Consequently, to maintain the Fe homeostasis and counteract iron limitation, bacteria have developed iron uptake strategies, by producing low molecular weight, water soluble iron chelators called siderophores.

Siderophore-antibiotic conjugates are a compound class with a long history of efficacy against various bacterial strains.
These conjugates act as Trojan horses for delivery of antibiotics across the bacterial membrane, taking advantage of the Fe assimilation pathway in bacteria.\textsuperscript{16–18} In 2019, the FDA (Food and Drug Administration) approved cefiderocol (Fig. 2), a siderophore based antibiotic conjugate for treatment of complicated urinary tract infections (cUTI).\textsuperscript{17} The conjugate consists of a bidentate catechol siderophore moiety that promotes the formation of chelated complexes with ferric iron and thereby facilitates siderophore-like transport across the outer bacterial membrane and a beta lactam-based cephalosporin drug counterpart.\textsuperscript{19} The conjugate is active against carbapenem-resistant Enterobacteriaceae (CRE) and extended-spectrum beta-lactamases (ESBLs) producing bacteria.\textsuperscript{19,20} However, the bidentate coordinating donor site limits recognition by a large fraction of Fe-siderophore transport proteins, especially those accessing the cytoplasm.\textsuperscript{21,22}

While the coordinating moiety is not essential for periplasm targeting antibiotics such as cefiderocol, it could significantly limit siderophore-mediated delivery of antibiotics that interact with a cytoplasmic target. In this regard, a more complete, tetra- or even hexacoordinate, siderophore-like motifs are desired, as they are more efficiently recognized and able to be transported to the cytoplasm (Fig. 1). However, the successful demonstration of \textit{in vivo} potency of other hexadentate siderophore antibiotic conjugates remains scarce.\textsuperscript{23,24} Two primary reasons emerge as the possible cause for this significant knowledge and data gap: (1) a common hypothesis for the lack of translatability of these constructs due to their need for an iron depleted environment for efficacy and (2) a sensitivity to premature proteolytic degradation of hexadentate siderophores in mammalian plasma.

Previously, we introduced the Ga\textsuperscript{3+} complex of the hexadentate, ornithine-derived tripeptide siderophore linear desferrichrome (LDFC) linked to the FDA approved antibiotic, ciprofloxacin \textit{via} a beta-alanine linker, Ga-D\textsubscript{2}.\textsuperscript{25} We showed that the complexation with the xenometal Ga(m) produces enhanced potency when compared with the apo-(metal free) or endogenous metal ion (Fe)-bound siderophore conjugate.\textsuperscript{25} Our work was inspired by the structure of the natural product and antibiotic albomycin (Fig. 2) and the recent clinical success of Ga(NO\textsubscript{3})\textsubscript{3} (ref. 26–29) in a phase 1 trial for treatment of \textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa}) lung infections in adults with cystic fibrosis (CF).\textsuperscript{28} Ga(NO\textsubscript{3})\textsubscript{3} imparted significant therapeutic effects.\textsuperscript{30} However, the drug was dosed at a high concentration of 200 mg per m\textsuperscript{2} per day for 5 days, so we posited that delivery of the Ga cargo with a siderophore-conjugate could significantly reduce dosing needs. Indeed, our first-generation construct Ga-D\textsubscript{2} (Fig. 2) demonstrated good, broad spectrum potency in \textit{E. coli}, \textit{S. aureus}, \textit{P. aeruginosa} and \textit{Klebsiella pneumoniae} (\textit{K. pneumoniae}) with a minimum inhibitory

![Fig. 1](image1.png) Proposed transport internalization pathway for ferrichrome based Galbofloxacin in \textit{wt S. aureus} using active transmembrane transport.

![Fig. 2](image2.png) Chemical structures of (top panel) structurally related hydroxamate siderophore-antibiotic conjugates synthesized and investigated previously, as well as subject of studies in this work: D\textsubscript{2}, D\textsubscript{5}, albomycin (Fe-D\textsubscript{3}), a “Trojan horse” conjugate produced by Streptomyces. (Bottom panel) Enterobactin–ciprofloxacin, mixed ligand biscatecholate–monohydroxamate siderophore–carbacephalosporin conjugate, 1b and cefiderocol, FDA approved siderophore based “Trojan horse” antibiotic. Black: metal ion, red: siderophore, green: linker, blue: antibiotic.
concentration for 90% inhibition of growth (MIC_{90}) of 0.2, 1.9, 3.8 μM and 12.5 μM, respectively but fell short of exceeding the parent antibiotic’s potency. Using a medicinal inorganic chemistry approach, we sought to further potentiate the activity of the construct.

Here, we introduce the second generation Trojan horse compound, Ga-D5 (Galbofl oxacin) as a ciprofloxacin-bearing mimic of albomycin natural product with a serine linker and explore its potential for a concerted diagnosis and therapy approach (Fig. 2). We incorporated a protease-sensitive serine linker in close analogy to the parent natural product albomycin.\textsuperscript{31–34} We demonstrate that this structural modification is critical to further enhance the in vitro and in vivo antibiotic activity of Galbofl oxacin against S. aureus beyond the potency of the parent antibiotic ciprofloxacin.

**Experimental section**

**Materials and methods**

All starting materials were purchased from Acros Organics, Alfa Aesar, Sigma Aldrich, or TCI America and used without further purification. NMR spectra (\(\text{\textsuperscript{1}H}\) and \(\text{\textsuperscript{13}C}\)) were collected on a 700 MHz Advance III Bruker, 500 MHz or 400 MHz Bruker instrument at 25 °C and processed using TopSpin 3.5pl7. \(\text{\textsuperscript{19}F}\) NMR spectra were collected on a 400 MHz Bruker instrument at 25 °C using TFA as an internal standard (\(\delta = 76\) ppm). Chemical shifts are reported as parts per million (ppm). Mass spectrometry: low-resolution electrospray ionization (ESI) mass spectrometry and high-resolution (ESI) mass spectrometry was carried out at the Stony Brook University Institute for Chemical Biology and Drug Discovery (ICB&DD) Mass Spectrometry Facility with an Agilent LC/MSD and Agilent LC-UV-TOF spectrometers, respectively. UV-VIS spectra were collected with the NanoDrop spectrophotometer for UV-VIS spectra were collected with the NanoDrop Agilent LC/MSD and Agilent LC-UV-TOF spectrometers, respectively. Drug Discovery (ICB&DD) Mass Spectrometry Facility with an Agilent 5110 ICP-OES. A 10-point standard with respect to the Stony Brook University Institute for Chemical Biology and Chemical Science Edge Article

**Synthesis of coordination complexes of D5**

Ga and Fe complexes of D5 were synthesized using the following protocol. Galbofl oxacin. D5 (0.010 g, 0.009 mmol, 1 eq.) was dissolved in DMF (1 mL) and Ga(NO\(_3\))\(_3\) (0.007 g, 0.029 mmol, 3 eq.) dissolved in 10 mM CH\(_3\)COONa (\(\text{pH} = 5\)) was added. The pH of the solution was adjusted to 6 by adding 0.1 M NaOH. The reaction mixture was stirred for 1 h at 60 °C and overnight at room temperature. Solvent was removed in vacuo and product was purified by preparative HPLC (Method A, product elutes at 46% B) to afford Ga-D5/Galbofl oxacin (0.006 g, 0.005 mmol, 62%) as a white solid. Calculated mass for Ga-D5/Galbofl oxacin (C\(_{44}\)H\(_{45}\)F\(_2\)GaN\(_4\)O\(_{13}\)): 1071.3; found 1072.4 [M + H\(^+\)]. Retention time (method D): 7.13 min (purity/peak area: >99%). Fe-D5. D5 (0.005 g, 0.004 mmol, 1 eq.) was dissolved in DMF (1 mL) and FeCl\(_3\) (0.002 g, 0.012 mmol, 3 eq) was added. The reaction mixture was stirred for 1 h at room temperature. Solvent was removed in vacuo and product was purified by preparative HPLC (method A, product elutes at 48% B) to afford Fe-D5 (0.006 g, 0.003 mmol, 87%) as a red solid. Calculated mass for Fe-D5 (C\(_{44}\)H\(_{45}\)F\(_2\)FeN\(_4\)O\(_{13}\)): 1058.3; found 1059.3 [M + H\(^+\)]. Retention time (method D): 7.10 min (purity/peak area: >99%).

**Antimicrobial activity assay**

Antibacterial activity of apo-D5, its corresponding Fe and Ga complex and fragment A was determined by measuring their minimum inhibitory concentrations (MIC) using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. All aqueous solutions and media were prepared using deionized water. All liquids and media were sterilized by autoclaving (220 °C, 1 h) before use. Iron deficient Mueller–Hinton broth (MHB) (cation adjusted) was used for these assays. 5.25 g of MHB was added to 250 mL of deionized water. Aqueous solutions of 1 M Ca\(^{2+}\) (0.418 mL) and 1 M Mg\(^{2+}\) (0.155 mL) to 250 mL of MHB was added to the broth.
The broth was autoclaved for 1 h at 220 °C. To make the broth iron-deficient, 4.06 mL of 1 mg mL⁻¹ sterile (autoclaved) aq. solution of 2,2'-bipyridine (DP) was added to 250 mL of cation adjusted MHB. MIC experiments were conducted in triplicate and carried out at 3 different instances, resulting in n = 9 for each reported MIC₉₀, to ascertain a maximum error margin of <5% for each reported MIC value.

In general, 0.3 mM stock solutions of testing compounds were prepared. 10 μL solution of testing compound (0.3 mM) was added to the first well of the 96-well plate and serial dilutions were made down each row of the plate. 40 μL of growth media and 50 μL of diluted bacterial inoculum was also added to each well, resulting in a total volume of 100 μL and a concentration gradient of 0.3 × 10⁻⁷ M to 0.92 × 10⁻¹² M. The dilution rate was adjusted keeping in mind the MIC of ciprofloxacin (positive control). The plates were incubated at 37 °C for 18 h and each plate was examined for bacterial growth using a plate reader (EPOCH2NS, Biotek microplate spectrometer). The MIC was recorded as the lowest compound concentration (μM) required to inhibit >90% of bacterial growth as judged by the absorbance of the culture media relative to the negative control.

### Cytotoxicity experiments

HEK-293 cells were grown in MEM with 1% sodium pyruvate, 1% l-glutamine, 100 units per mL Pen Strep and 10% Fetal Bovine Serum. The cells were maintained at 37 °C in a humidified incubator under an atmosphere containing 5% CO₂. Modified Eagle’s Medium (MEM), containing 10% fetal calf serum, 1% penicillin and streptomycin, was used as growth medium. HEK-293 cells were detached from the wells with trypsin and EDTA, harvested by centrifugation and re-suspended again in cell culture medium. The assays were carried out on 96 well plates with 6000 cells per well for normal human embryonic kidney cells (HEK-293). After 24 h of incubation at 37 °C and 5% CO₂, the cells were treated with the compounds (with final DMSO concentrations of 0.5%) with a final volume of 200 μL per well. For a negative control, one series of cells was left untreated. The cells were incubated for 48 h followed by adding 50 μL MTT (2.5 mg mL⁻¹). After an incubation time of 2 h, the medium was removed and 200 μL of DMSO were added. The formazan crystals were dissolved, and the absorption was measured at 550 nm, using a reference wavelength of 620 nm. Each test was repeated in quadruplicates in three independent experiments. Results were plotted as % cell viability at different concentrations of samples tested.

\[
\text{% Cell viability} = \frac{OD_{370} \text{ of test sample}}{OD_{370} \text{ of negative control}} \times 100
\]

### In vitro cleavability assessment

**In vitro** cleavage of D5 and Galbofloxacin was performed using a proteinase K enzyme assay. Conjugates (10 μM) were incubated with proteinase K (20 ×, 200 μM, dissolved in Tris buffer, pH = 7) at 37 °C. Aliquots were removed at select time points, and were monitored for cleavage on analytical HPLC (D5 – method C, Galbofloxacin – method D). Cleavage was confirmed by appearance of a second peak corresponding to the cleaved fragment A and was expressed as % cleavage over time.

### Radiolabeling with ⁶⁷Ga and bacterial uptake of ⁶⁷Ga in wt bacterial strains

⁶⁷Ga-citrate was received from Jubilant Radiopharma at an average specific activity of 140.9 MBq mL⁻¹. The ⁶⁷Ga-citrate solution was converted to ⁶⁷Ga-chloride using a previously described protocol.²⁸ The average specific activity of the resultant ⁶⁷Ga-chloride solution used for radiolabeling was 131.3 MBq mL⁻¹. For radiolabeling of D5, an aliquot of (⁶⁷GaCl₃ 3.7 MBq, 30 μL) was mixed with a solution of D5 (30 μL, 0.01 mM) in chelex resin treated water. The pH of the solution was adjusted with HEPES (100 mM, 100 μL) to 7.4. Complexation was monitored by radio-HPLC, method D. Radiolabeling was found to proceed after 5 minutes at room temperature. ⁶⁷Ga-D5: tₚ = 7.13 min (purity/peak area: 99%).

Wt E. coli (Mg 1655), wt S. aureus (RN4220) and wt P. aeruginosa (PAO1) were grown overnight in 5 mL LB (Fe-deficient) at 37 °C. The overnight cultures were inoculated in 10 mL iron deficient LB and incubated at 37 °C until the OD₆₀₀ reached 0.4. Uptake was initiated by adding ⁶⁷Ga complexes (10 μL, 0.18 MBq) to culture tubes containing 10 mL bacterial inoculum and incubating at 37 °C. Aliquots (1 mL) were removed after 10 min, 20 min, 30 min, 1 h and 2 h and centrifuged for 3 min (34 000 rpm). The supernatant was removed, and the bacterial pellet was washed with DPBS (1 mL, 3×). The assay was performed in five replicates and in parallel with a ⁶⁷Ga-citrate control. All tubes were counted using an automated gamma counter to quantify retained radioactivity in pellets in comparison to the 1 mL parent inoculum.

To challenge active transporter-mediated uptake, the bacterial cultures were preincubated with 200× Fe-LDFC. ⁶⁷Galbofloxacin (10 μL, 0.18 MBq) was added after 30 min. Aliquots (1 mL) were removed after 10 min, 20 min, 30 min, 1 h and 2 h and centrifuged for 3 min. Pellet were washed with DPBS (3×) and retained activity was quantified as described above.

### Biodistribution of naïve and infected Balb/c mice

All animal experiments were performed using protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University accredited through Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International, Federal assurance #A3011-01). 0.3–0.6 MBq of ⁶⁷GaGalbofloxacin and ⁶⁷Ga-citrate (control) were intravenously injected via tail vein catheter in naïve mice. Mice were sacrificed 1 h p.i. and select organs were harvested. Radioactivity was counted by using a gamma counter, and the radioactivity associated with each organ was expressed as % ID g⁻¹. For infected mice biodistribution, mice were given a S. aureus (10⁵ CFU, 50 μL) in left triceps. After 1 h p.i. mice were sacrificed and select organs were harvested. Radioactivity was counted by using a gamma counter, and the
radioactivity associated with each organ was expressed as % ID g⁻¹.

Metabolite analysis

100 μL of urine was directly injected on the radio HPLC. Eluate was collected in 30 s increments from 0–15 minutes. Activity in each tube was quantified using a gamma counter. The counts were used to reconstruct the metabolite trace which was then compared to the HPLC traces of the original ⁶⁷Ga
dofloxacin complex.

Galb
doxyxacin efficacy study

All animal experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Stony Brook Medicine. Bacterial stocks were freshly prepared before inoculation. In general, bioluminescent S. aureus Xen 29 strain was cultured in MHB at 37 °C to mid log phase (OD₆₀₀ = 0.4, 2 × 10⁸ cells per mL). Bacterial cells were harvested by centrifugation (34 000 rpm, 3 min) and washed with sterilized MHB. Final inoculums were generated by diluting bacteria to the desired concentrations in MHB.

To induce localized infection model, mice were injected with 2 × 10⁷ bacterial cells [2 × 10⁸ cells per mL inoculum, 50 μL] into the left forelimb muscle of mice. Test compounds were administered intravenously 1 h and 25 hours post bacterial inoculation. The study included five groups of mice: cipro
doxacin as positive control: 284.4 μmol kg⁻¹ (4 mice) and 9.3 μmol kg⁻¹ (4 mice); vehicle (saline) as a negative control (4 mice); and Galb
doxyxacin: 9.3 μmol kg⁻¹ (4 mice) and 2.3 μmol kg⁻¹ (4 mice). Muscle size and survival was monitored for 7 days post-infection. The mice were also imaged on a small animal imager every 12 hours for 7 days to monitor the infection burden and treatment efficacy. After 7 days, mice were euthanized, injected and control muscle from each cohort was extracted and plated for CFU determination.

Results and discussion

Synthesis of siderophore antibiotic conjugate D5 and its metal complexes: galb
doxyxacin and Fe-D5

The structure of D5 draws direct structural inspiration from the natural product albomycin; the presence of the serine linker is essential for the release of the t-seryl transferase inhibitor portion of the natural product inside the bacterial cytoplasm.³⁴–³⁷ Thus, we proposed that the introduction of serine to our cipro
doxacin-conjugates could result in enhanced efficacy by way of drug cargo release, a concept validated by Nolan and coworkers on enterobactin derived constructs.³⁴,³⁸ We implemented a conjugation strategy mirroring the connectivity of the natural product albomycin and our first-generation lead D2. The acetylated, amino protected linear desferrichrome precursor P5 was obtained by employing the synthetic strategy pioneered by Miller and coworkers.³⁵,³⁹–⁴² The serine linker was introduced to the building block P5 by functionalizing cipro
doxacin first with Fmoc-β-alanine,³⁸ followed by N-terminal deprotection and subsequent reaction of the free amine of fragment A³ with Fmoc-Ser(O’Bu)-OH with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, Hexa
dfluorophosphate Benzotriazole Tetramethyl Uronium (HBTU) mediated amidation using a solution phase coupling approach. A second amidation step with the protected, linear desferrichrome-derived building block P5 following Fmoc
deprotection of the serine residue (Scheme S1) was employed to link the proto-siderophore P5 with the drug cargo precursor (2). The final product D5 was attained in a <2% overall yield after 16 steps following final purification using reverse phase chromatography (Fig. S1–S17†).

Complexation of D5 with Ga(III) and Fe(III) salts formed Galb
doxyxacin and Fe-D5 in aqueous, buffered media at pH 6 and pH 7, respectively. The corresponding complexes were further purified using reverse phase chromatography and isolated as white and orange-red powders, respectively. Characterization via high performance liquid chromatography (HPLC), high resolution mass spectrometry (HRMS) and inductive coupled plasma-optical emission spectrometry (ICP-OES) was employed to affirm purity and produce consistent complex concentrations for subsequent in vitro and in vivo studies based on the quantitation of metal content (Fig. S5, S6, S16 and S17†).

Growth inhibition in vitro bacterial strains

With apo-
metal-free), Ga and Fe complexes of D5 in hand, we next probed their ability to inhibit bacterial growth in a conventional minimum inhibitory concentration for 98% inhibited growth (MIC₉₈) assay. We selected E. coli K12 and P. aeruginosa PA01 as Gram-negative model organisms, and S. aureus RN4220 as the Gram-positive model organism. Fe
deficient media was prepared by addition of 2,2’-bipyridine (DP) to mimic the tightly iron controlled in vitro environment.³⁵,⁴²–⁴³ The parent drug cipro
doxacin was utilized as a control giving an MIC₉₈ of 0.93 μM, which is consistent with literature values. apo-D5 and Galb
doxyxacin were determined to have MIC₉₈ of 0.93 μM in wt E. coli, retaining the growth inhibitory action of cipro
doxacin and comparable to the antibiotic activity of our first-generation conjugate Ga-D2. In accordance with our previous findings, Fe(III) complexes remain significantly less active: Fe-D5 showed a 2-fold decrease in activity in E. coli in comparison to apo-D5 and Galb
doxyxacin. We hypothesize that the concerted delivery of Fe at elevated concentrations is growth promoting and efficiently offsets the inhibitory action of the siderophore-mediated delivery of the

Table 1 Summary of MIC₉₈ (μM) determined in E. coli, S. aureus and P. aeruginosa. GP: growth promoting

| Compound     | E. coli  | S. aureus | PA01   |
|--------------|----------|-----------|--------|
| Cipro
doxacin  | 0.93 ± 0.00 | 0.93 ± 0.00 | 0.93 ± 0.00 |
| apo-D5      | 0.93 ± 0.02 | 0.93 ± 0.00 | 30 ± 0.04 |
| Galb
doxyxacin | 0.93 ± 0.00 | 0.093 ± 0.00 | 15 ± 0.03 |
| Fe-D5       | 1.9 ± 0.02  | 7.5 ± 0.00  | GP     |
However, S. aureus compared to cipro produced growth promoting effects (Fig. S18, Table 1). This qualifies Galboflexin as one of the most active synthetic Trojan horse compounds against S. aureus measured to date. The apo-D5 compound showed and MIC$_{90}$ of 0.93 μM, whereas Fe-D5 showed decreased activity (MIC$_{90}$ = 7.5 μM) (Fig. 3A, Table 1), indicating that complexation with Ga(III) is essential to produce the synergistic, growth inhibitory effect.

D5 and its metal complexes were also assessed for its antimicrobial activity in the FhuA transporter deficient mutant E. coli AN193. Fig. S19 summarizes the corresponding results: the previously observed growth inhibitory activity in E. coli of apo-D5, Galboflexin and Fe-D5 is completely muted, while ciproflexin retains its growth inhibitory activity as found with the wt E. coli strain. This demonstrates that internalization and activity of Galboflexin remains FhuA-dependent, affirming that siderophore-mediated active transport of D5-constructs remains essential to produce growth inhibition.

In vitro cleavage of galboflexin and apo-D5
As growth inhibition experiments confirmed that Galboflexin exhibited increased potency, we assessed apo-D5 and Galboflexin as substrates for proteinase mediated cleavage adjacent to the serine residue. In vivo, the antibacterial active moiety is released by peptidase N. The same reaction can be mimicked in vitro by proteinase K, a broad-spectrum serine protease (Fig. 3B). We incubated 10 μM of apo-D5 ($t_R = 7.28$ min), Fe-D5 ($t_R = 6.96$ min) or Galboflexin ($t_R = 7.13$ min) with proteinase K (20×, 200 μM, in tris buffer, pH = 7) at 37 °C and monitored cleavage by analytical HPLC, which reveals release of fragment A ($t_R = 7.03$ min, Fig. 3B and D). Under these conditions, proteinase K cleaved apo-D5 completely in 15 min (Fig. S21, Table S1†), whereas the cleavage for Galboflexin is slower, with a $t_{1/2}$ of 4 h, with complete cleavage to fragment A after 8 h (Fig. 3D, Table S1†). The cleavage of Fe-D5 was slowest with 34% conjugate still intact after 24 h incubation with proteinase K (Fig. S21, Table S1†). The results for apo-D5, Fe-D5 and Galboflexin are consistent with prior enzymatic cleavage studies on albomycin and desferri-albomycin. The results also indicate higher dissociation constant for Fe-D5 as compared with Galboflexin.

While apo-D5, Fe-D5 and Galboflexin are proteinase substrates, it is conceivable that dissociation of the metal ion in the bacterial cytoplasm results in accelerated cleavage of the conjugate to release the antibacterial payload. Importantly, cleavage of the construct does not occur outside the bacterial envelope, as fragment A is completely inactive in all tested wt bacterial strains (E. coli, S. aureus and P. aeruginosa, Fig. 3C and S20†). These results affirm the importance of siderophore mediated active uptake of Galboflexin prior to release of the antibiotic payload.

Cytotoxicity analysis
As we had identified Galboflexin as a potent antibiotic against S. aureus, and with potential for prospective in vivo applications, we explored its toxicity to mammalian cells next. Specifically, we determined the anti-proliferative activity of Galboflexin and apo-D5 against normal human embryonic kidney cells (HEK-293) using cisplatin, gallium(III) citrate and ciproflexin as controls. As indicated in Fig. 4, the Galboflexin conjugate displays no in vitro toxicity up to 80 μM, with a slightly reduced...
cell viability (85%), comparable to ciprofloxacin, at 100 μM (Fig. S22†). In comparison, the benchmark cytotoxic cisplatin produces reduced cell viability of 60% at 2 μM compound concentration (Fig. S22†). apo-D5 and Ga(III) citrate are nontoxic to HEK-293 human cells at all tested concentrations (Fig. 4).

Radiochemical labeling with ⁶⁷Ga and radiochemical uptake experiments in wt bacterial strains

We next employed a radiochemical tracer approach to further elucidate and quantitate in vitro and in vivo properties of Galbofloxacin. To this end, we employed ⁶⁷Ga (t₁/₂ = 79 hours, Eγ (avg) = 93.3 keV), an isotope with multi-day half-life that enables bacterial uptake and pharmacokinetic studies. The formation of the ⁶⁷Galbofloxacin complex is feasible under mild radiolabeling conditions (pH 7, 100 mM HEPES, 5 minutes) (Fig. 5A) quantitatively with ligand concentrations of 10⁻⁶ M. The complexation was monitored using a radio-HPLC (Fig. 5B, method D).

The radiolabeled ⁶⁷Galbofloxacin complex provides a convenient tool to probe time-dependent compound uptake in bacteria relative to weakly complexed ⁶⁷Ga-citrate. ⁶⁷Galbofloxacin was prepared as outlined previously, with an average specific activity of 1.9 MBq μmol⁻¹. E. coli, S. aureus and P. aeruginosa (3.2 × 10⁸ CFU) were incubated in Fe-deficient LB with 0.18 MBq of ⁶⁷Galbofloxacin at 37 °C. Aliquots were removed at 10, 20, 30, 60 and 120 minutes followed by separation of pellets from supernatant and counting of retained radioactivity in each fraction. For all the tested strains, ⁶⁷Galbofloxacin shows time dependent increase in uptake with a peak uptake at 120 minutes and statistically significant, enhanced uptake in comparison to ⁶⁷Ga-citrate, for E. coli and P. aeruginosa (Fig. S23, S24, Tables S2 and S3†). Notably, for S. aureus, ⁶⁷Galbofloxacin uptake was comparable to ⁶⁷Ga-citrate uptake (Fig. 5C, Table S4†). We hypothesize that ⁶⁷Ga-citrate is efficiently taken up by S. aureus due to its similarity to staphyloferrin, a citric acid based siderophore naturally produced by S. aureus.⁶⁶ To probe siderophere specific active transport to the bacterial cytoplasm, we also challenged ⁶⁷Galbofloxacin uptake with the Fe complex of parent siderophore (Fe-LDFC). All three bacterial cultures were incubated for 30 min with 200× excess Fe-LDFC before addition of ⁶⁷Galbofloxacin. We observed statistically significant depression of ⁶⁷Galbofloxacin uptake within the bacterial pellet at all time points (Fig. 3D, S25, S26, Tables S5, S6 and S7†).

In vivo biodistribution and pharmacokinetics in naïve and infected mice models

With ⁶⁷Galbofloxacin uptake validated in vitro using bacterial uptake assays, we investigated biodistribution ⁶⁷Galbofloxacin in vivo next. Balb/c mice (female, 8 weeks) were injected with

Fig. 4  MTT assay of Galbofloxacin, apo-D5 and Ga-citrate in HEK-293 cell line shows low cytotoxicity for all tested conjugates (n = 3 × 5).

Fig. 5  (A) Schematic description of the radiochemical complexation of apo-D5 with ⁶⁷Ga. ⁶⁷Ga-complexation proceeds in 5 min, 25 °C, pH 7. (B) A representative radiolabeling HPLC trace for the characterization of ⁶⁷Galbofloxacin (tR = 7.13 min, left axis, ⁶⁷Ga counts per second), in comparison with HPLC characterization of the apo-D5 ligand (tR = 7.28 min, right axis, absorbance at 280 nm) is shown. Free ⁶⁷Ga elutes at 0.7 minutes. (C) Time-dependent, radiochemical bacterial uptake studies in S. aureus of ⁶⁷Galbofloxacin in iron deficient, DP-treated media show that uptake is comparable with ⁶⁷Ga-citrate, which acts as a staphyloferrin (natural siderophore) mimic for S. aureus (n = 5). (D) On pre-incubation with 200× Fe-LDFC, the uptake of ⁶⁷Galbofloxacin in S. aureus is attenuated significantly after 10 min (n = 5).
Galbofloxacin and $^{67}$Ga-citrate intravenously, followed by biodistribution and analysis of metabolites in the urine using radio-HPLC at 1 hour post injection. Naive biodistribution revealed rapid, renal clearance of all both $^{67}$Galbofloxacin and $^{67}$Ga-citrate (Fig. 6, Table S8†). Metabolite study revealed 5% intact $^{67}$Galbofloxacin radiochemical complex in the urine (Fig. S27†). To quantitate the bacterial uptake of $^{67}$Galbofloxacin in vivo, we induced a localized, soft tissue infection in mice using $10^5$ CFU S. aureus infection in the left triceps. $^{67}$Galbofloxacin showed a significant, enhanced uptake ($P \leq 0.05$) in the infected muscle as compared to the control muscle (Fig. 6, Table S8†), in contrast to $^{67}$Ga-citrate, which did not distinguish infected and control muscle tissues. Overall, favorable pharmacokinetics, as indicated by rapid renal excretion, no significant off-target organ uptake, and increased uptake in infected muscle qualifies $^{67}$Galbofloxacin for further evaluation for therapy in an animal model of soft tissue infection.

Growth inhibition in bioluminescent S. aureus strain Xen 29 and in vivo therapy studies

The bioluminescent S. aureus strain Xen 29 has previously been used to monitor in vivo antibiotic efficacy using preclinical bioluminescence imaging non-invasively. Therefore, before using S. aureus Xen 29 for in vivo therapy studies, the in vitro antibacterial activity of Galbofloxacin was assessed in S. aureus Xen 29. MIC assays were performed in Fe deficient media as described for the wt strains. The parent drug ciprofloxacin was employed as a control with MIC$_{99}$ of 3.8 $\mu$M. Galbofloxacin exhibits MIC$_{99}$ of 0.93 $\mu$M, 4× more potent than ciprofloxacin. The 96 well plates for both ciprofloxacin and Galbofloxacin were imaged on small animal optical imager (Fig. 7A and C) to quantify the radiance value due to the bioluminescent bacteria for each well. The MIC results based on OD$_{600}$ and ROI analysis coincided for both test compounds as shown in Fig. 7B and D. The 4-fold higher activity of Galbofloxacin as compared to ciprofloxacin in S. aureus Xen 29, confirmed this strain as an ideal model system for in vivo therapy studies.

The in vivo therapeutic activity of Galbofloxacin was evaluated in S. aureus Xen 29 triceps infection model. 8 week-old female Balb/c mice were infected intramuscularly with $2 \times 10^7$ bacterial cells in the left forelimb and treatments were administered intravenously at 1 h and 25 h post infection. The
treatments included 4 mice in each cohort of ciprofloxacin high dose (284.4 μmol kg⁻¹), ciprofloxacin medium dose (9.3 μmol kg⁻¹), Galbofloxacin medium dose (9.3 μmol kg⁻¹), Galbofloxacin low dose (2.3 μmol kg⁻¹) and vehicle (saline) (Table S9†). Mice were imaged on a small animal optical imager every 12 hours and monitored for change in muscle size and survival (Fig. 8A) for 7 days. The muscle size for the vehicle and ciprofloxacin (9.3 μmol kg⁻¹) increased rapidly, for Galbofloxacin (2.3 μmol kg⁻¹) it increased gradually, whereas for ciprofloxacin (284.4 μmol kg⁻¹) and Galbofloxacin (9.3 μmol kg⁻¹) cohort the muscle size decreased over the course of 7 days (Fig. S28 and S29†). Bioluminescence imaging and ROI analysis revealed increased and eventually fatal infection for ciprofloxacin (9.3 μmol kg⁻¹) on day 1 (Fig. 8C, S31, S40, Table S10†), whereas the infection burden decreased for Galbofloxacin (9.3 μmol kg⁻¹) over the course of 7 days (Fig. 8C, S47, S44, Table S10†) and efficiently reduced morbidity. Untreated mice had a survival of 0% after 12 h (Fig. 8B–D, S40† Tables 2 and S10†). Infected muscle from each cohort was plated after euthanizing and infection burden was determined by counting the CFUs (Fig. S39,† Table 2) and imaging the agar plates on IVIS scanner (Fig. S30, S32, S34, S36 and S38†). Mice treated with medium dose ciprofloxacin (9.3 μmol kg⁻¹) had a survival of 0% after day 1 (Fig. 8C, Table 2), whereas the survival was 100% for same dose Galbofloxacin mice at day 7 (Fig. 8D, Table 2).

Mice receiving Galbofloxacin at 2.3 μmol kg⁻¹ dose exhibited increased survival, albeit infection persisted. By day 4.5 the low dose Galbofloxacin cohort showed slowed progression of infection when compared to the vehicle treated cohort (Fig. S35†). The survival rate was 25% on day 7 for mice dosed with 2.3 μmol kg⁻¹ Galbofloxacin (Fig. 8D, Table 2). Mice dosed with ciprofloxacin (284.4 μmol kg⁻¹) survived for 7 days and showed efficient reduction of infection, indicating expected dose dependency (Fig. 8C, S33,† Table 2).

Conclusions
Here, we show for the first time that a Ga-siderophore antibiotic conjugate, Galbofloxacin exhibits both superior in vitro potency and in vivo efficacy when compared to its parent antibiotic ciprofloxacin. In our study, Galbofloxacin shows an order of magnitude higher potency in comparison to the parent drug ciprofloxacin against S. aureus with MIC₉₈ of 93 nM. Moreover, Galbofloxacin shows higher potency than apo-D5 (MIC₉₈ = 0.93...
µM) indicating the synergistic effect of Ga complexation, siderophore-mediated transmembrane transport and cytoplasmic release of the antibiotic. MIC assay with FhuA deficient E. coli results in no growth inhibition, demonstrating siderophore mediated active uptake remains essential for Galbobroxacin. MTT assay in HEK-293 cells demonstrates no toxicity for Galbobroxacin up to 80 µM, comparable to ciprofloxacin at 100 µM. Proteinase K assay was carried out to demonstrate the time dependent, proteolytic cleavage of Galbobroxacin. The results indicate 50% cleavage of Galbobroxacin after 4 h in the presence of 20× proteinase K. We also quantified bacterial uptake for the conjugate and measured in vivo pharmacokinetics using 67Galbobroxacin. 67Galbobroxacin can be synthesized radiochemically under mild conditions and with high radiochemical yields. The complex shows time dependent siderophore mediated active uptake in wt bacterial strains. Biodistribution of 67Galbobroxacin in naive mice and in a mouse model of intramuscular infection reveals renal clearance and significantly enhanced uptake in infected muscle when compared to 67Ga-citrate, which showed no difference. Finally, an in vivo therapy study with Galbobroxacin in a S. aureus Xen 29 murine infection model demonstrates that the high in vitro potency of Galbobroxacin is directly reflective of its in vivo efficacy. Mice dosed twice with 9.3 µmol kg⁻¹ of Galbobroxacin survived for 7 days with the infection burden decreasing and eventually resolving completely over the course of study, whereas the infection burden was fatal on day 1 for mice dosed with same molar dose of ciprofloxacin.

Galbobroxacin constitutes the first, rationally designed gallium coordination complex with superior potency to combat Gram-positive soft tissue infection when compared to its parent antibiotic. Galbobroxacin also demonstrates that Trojan horse antibiotics can successfully reprise their in vitro potency in vivo. The expansion of the scope of treatable infections by modified metal, antibiotic payload as well as mechanistic studies to further elucidate the potentiating, growth inhibitory effect of Ga sideromycins on bacterial cells are underway.

Data availability

All experimental and image data is shown in the ESI† and additional raw data files can be obtained from the authors upon request.

Author contributions

Experiments were designed by AP and EB, experiments were conducted by contributions from authors. The manuscript was written and edited by all authors.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The National Institutes for General Medicine (NIGMS) are acknowledged for funding (R35GM142770). Yong Li is acknowledged for his valuable suggestions during the therapy study.

References

1 CDC, Biggest Threats and Data | Antibiotic/抗microbial Resistance, CDC, 2019.
2 Y. Guo, G. Song, M. Sun, J. Wang and Y. Wang, Front. Cell. Infect. Microbiol., 2020, 10, 107.
3 S. Y. C. Tong, J. S. Davis, E. Eichenberger, T. L. Holland and V. G. Fowler, Clin. Microbiol. Rev., 2015, 28, 603–661.
4 S. Y. C. Tong, J. S. Davis, E. Eichenberger, T. L. Holland and V. G. Fowler, Clin. Microbiol. Rev., 2015, 28, 603–661.
5 R. J. Turner, Microbiol. Biotechnol., 2017, 10, 1062–1065.
6 A. Pandey and E. Boros, Chem.–Eur. J., 2021, 27, 7340–7350.
7 U. E. Schaible and S. H. E. Kaufmann, Nat. Rev. Microbiol., 2004, 2, 946–953.
8 T. A. Wencziewicz and M. J. Miller, Top. Med. Chem., 2018, 26, 151–183.
9 R. C. Hider and X. Kong, Nat. Prod. Rep., 2010, 27, 637–657.
10 G. L. A. Mislin and I. J. Schalk, Metallomics, 2014, 6, 408–420.
11 C. Ji, R. E. Juárez-Hernández and M. J. Miller, Future Med. Chem., 2012, 4, 297–313.
12 S. C. Andrews, A. K. Robinson and F. Rodriguez-Quíñones, FEMS Microbiol. Rev., 2003, 27, 215–237.
13 M. J. Miller, H. Zhu, Y. Xu, C. Wu, A. J. Walz, A. Vergne, J. M. Roosenberg, G. Moraski, A. A. Minnick, J. McKee-Dolence, J. Hu, K. Fennell, E. Kurt Dolence, L. Dong, S. Franzblau, F. Malouin and U. Möllmann, BioMetals, 2009, 22, 61–75.
14 T. A. Wencziewicz and M. J. Miller, J. Med. Chem., 2013, 56, 4044–4052.
15 T. Zheng and E. M. Nolan, J. Am. Chem. Soc., 2014, 136, 9677–9691.
16 H. K. Zane, H. Naka, F. Rosconi, M. Sandy, M. G. Haygood and A. Butler, J. Am. Chem. Soc., 2014, 136, 5615–5618.
17 J. A. Karlowsky, M. A. Hackel, M. Tsuji, Y. Yamano, R. Echols and D. F. Sahm, Int. J. Antimicrob. Agents, 2019, 53, 456–466.
18 T. Aoki, H. Yoshizawa, K. Yamawaki, K. Yokoo, J. Sato, S. Hisakawa, Y. Hasegawa, H. Kusano, M. Sano, H. Sugimoto, Y. Nishitani, T. Sato, M. Tsuji, R. Nakamura, T. Nishikawa and Y. Yamano, Eur. J. Med. Chem., 2018, 155, 847–868.
19 G. G. Zhanel, A. R. Golden, S. Zelenitsky, K. Wiebe, C. K. Lawrence, H. J. Adam, T. Idowu, R. Domalaon, F. Schweizer, M. A. Zhanel, P. R. S. Lagacé-Wiens, A. J. Walkty, A. Noreddin, J. P. Lynch III and J. A. Karlowsky, Drugs, 2019, 79, 271–289.
20 J. Y. Wu, P. Srinivas and J. M. Pogue, Infect. Dis. Ther., 2020, 9, 17–40.
21 W. Neumann, M. Sassone-Corsi, M. Raffatellu and E. M. Nolan, J. Am. Chem. Soc., 2018, 140, 5193–5201.
22 T. J. Sanderson, C. M. Black, J. W. Southwell, E. J. Wilde, A. Pandey, R. Herman, G. H. Thomas, E. Boros, A.-K. Duhme-Klair and A. Routledge, ACS Infect. Dis., 2020, 6, 2532–2541.
23 M. Ghosh, Y. M. Lin, P. A. Miller, U. Möllmann, W. C. Boggess and M. J. Miller, ACS Infect. Dis., 2018, 4, 1529–1535.
24 M. Ghosh, P. A. Miller, U. Möllmann, W. D. Claypool, V. A. Schroeder, W. R. Wolter, M. Kuckow, H. Yu, S. Li, W. Huang, J. Zajicek and M. J. Miller, J. Med. Chem., 2017, 60, 4577–4583.
25 A. Pandey, C. Savino, S. H. Ahn, Z. Yang, S. G. Van Lanen and E. Boros, J. Med. Chem., 2019, 62, 9947–9960.
26 C. E. Arnold, A. Bordin, S. D. Lawhon, M. C. Libal, L. R. Bernstein and N. D. Cohen, Vet. Microbiol., 2012, 155, 389–394.
27 C. R. Chitambar, Biochim. Biophys. Acta, Mol. Cell Res., 2016, 1863, 2044–2053.
28 C. H. Goss, Y. Kaneko, L. Khuu, G. D. Anderson, S. Ravishankar, M. L. Aitken, N. Lechtzin, G. Zhou, D. M. Czyz, K. McLean, O. Olakanmi, H. A. Shuman, M. Teresi, E. Wilhelm, E. Caldwell, S. J. Salipante, D. B. Hornick, R. J. Siehnel, L. Becker, B. E. Britigan and P. K. Singh, Sci. Transl. Med., 2018, 10, eaat7520.
29 C. Goss, D. Hornick and M. Aitken, Pediatr. Pulmonol., 2012, 47, 303.
30 Y. Wang, B. Han, Y. Xie, H. Wang, R. Wang, W. Xia, H. Li and H. Sun, Chem. Sci., 2019, 10, 6099–6106.
31 Z. Lin, X. Xu, S. Zhao, X. Yang, J. Guo, Q. Zhang, C. Jing, S. Chen and Y. He, Nat. Commun., 2018, 9, 3445.
32 G. F. Gause, Br. Med. J., 1955, 2, 1177–1179.
33 T. E. Clarke, V. Braun, G. Winkelmann, L. W. Tari and H. J. Vogel, J. Biol. Chem., 2002, 277, 13966–13972.
34 Z. L. Reitz, M. Sandy and A. Butler, Metallomics, 2017, 9, 824–839.
35 V. Braun, K. Gunther, K. Hantke and L. Zimmermann, J. Bacteriol., 1983, 156, 308–315.
36 A. Pramanik, U. Stroher, J. Krejci, A. Standish, E. Bohn, J. Paton, I. Autenrieth and V. Braun, Int. J. Med. Microbiol., 2007, 297, 459–469.
37 A. Hartmann, H. P. Fiedler and V. Braun, Eur. J. Biochem., 1979, 99, 517–524.
38 T. Zheng, J. L. Bullock and E. M. Nolan, J. Am. Chem. Soc., 2012, 134, 18388–18400.
39 E. K. Dolence, A. A. Minnick, C. E. Lin, M. J. Miller and S. M. Payne, J. Med. Chem., 1991, 34, 968–978.
40 C. J. Adams, J. J. Wilson and E. Boros, Mol. Pharm., 2017, 14, 2831–2842.
41 C. J. Adams, J. J. Wilson and E. Boros, Mol. Pharm., 2017, 14, 2831–2842.
42 R. E. Juárez-Hernández, P. A. Miller and M. J. Miller, ACS Med. Chem. Lett., 2012, 3, 799–803.
43 E. W. Hunsaker and K. J. Franz, Inorg. Chem., 2019, 58, 13528–13545.
44 A. Pramanik, U. Stroher, J. Krejci, A. Standish, E. Bohn, J. Paton, I. Autenrieth and V. Braun, Int. J. Med. Microbiol., 2007, 297, 459–469.
45 W. Saenger, in Handbook of Proteolytic Enzymes, 2013, vol. 3, pp. 3240–3242.
46 S. Konetschny-Rapp, G. Jung, J. Meiwees and H. Zahner, Eur. J. Biochem., 1990, 191, 65–74.
47 M. van Oosten, T. Schäfer, J. A. C. Gazendam, K. Ohlsen, E. Tsompanidou, M. C. de Goffau, H. J. M. Harmsen, L. M. A. Crane, E. Lim, K. P. Francis, L. Cheung, M. Olive, V. Ntziachristos, J. M. van Dijl and G. M. van Dam, Nat. Commun., 2013, 4, 2584.
48 T. Hertlein, V. Sturm, U. Lorenz, K. Sumathy, P. Jakob and K. Ohlsen, Antimicrob. Agents Chemother., 2014, 58, 1630–1638.
49 T. Hertlein, V. Sturm, U. Lorenz, K. Sumathy, P. Jakob and K. Ohlsen, Antimicrob. Agents Chemother., 2014, 58, 1630–1638.
50 Y. Li, F. Daryaee, G. E. Yoon, D. Noh, P. M. Smith-Jones, Y. Si, S. G. Walker, N. Turkman, L. Meimetis and P. J. Tonge, ACS Infect. Dis., 2020, 6, 2249–2259.