Siderophores as drug delivery agents: application of the "Trojan Horse" strategy. BioMetals

ARTICLE *in* BIOLOGY OF METALS · MARCH 2009

Impact Factor: 2.5 · DOI: 10.1007/s10534-009-9219-2 · Source: PubMed

Biometals (2009) 22:615–624
DOI 10.1007/s10534-009-9219-2

Siderophores as drug delivery agents: application of the "Trojan Horse" strategy

Ute Möllmann · Lothar Heinisch · Adolf Bauernfeind · Thilo Köhler · Dorothe Ankel-Fuchs

Received: 5 November 2008 / Accepted: 27 January 2009 / Published online: 12 February 2009

Abstract The outer membrane permeability barrier is an important resistance factor of bacterial pathogens. In combination with drug inactivating enzymes, target alteration and efflux, it can increase resistance dramatically. A strategy to overcome this membrane-mediated resistance is the misuse of bacterial transport systems. Most promising are those for iron transport. They are vital for virulence and survival of bacteria in the infected host, where iron depletion is a defense mechanism against invading pathogens. We synthesized biomimetic siderophores as shuttle vectors for active transport of antibiotics through the bacterial membrane. Structure activity relationship studies resulted in siderophore aminopenicillin conjugates that were highly active against Gram-negative pathogens which play a crucial role in destructive lung infections in cystic fibrosis patients and in severe nosocomial infections. The mechanism of action and the uptake of the compounds via specific iron siderophore transport routes were demonstrated. The novel conjugates were active against systemic *Pseudomonas aeruginosa* infections in mice with ED_{50} values comparable to the quinolone ofloxacin and show low toxicity.

Keywords Siderophores · Aminopenicillin conjugates · Permeability barrier · Efflux · *Pseudomonas aeruginosa*

U. Möllmann (✉) · L. Heinisch
Leibniz Institute for Natural Product Research and Infection Biology - Hans Knoell Institute, Beutenbergstrasse 11a, 07745 Jena, Germany
e-mail: ute.moellmann@hki-jena.de

A. Bauernfeind
Micoer Institute, Hesseloherstr 4, 80802 Munich, Germany

T. Köhler
Department of Microbiology and Molecular Medicine, Centre Médical Universitaire, Geneva, Switzerland

D. Ankel-Fuchs
Gruenenthal GmbH, 52099 Aachen, Germany

Introduction and background

The continuous increase in resistance of bacterial pathogens against the available anti-infective drugs urgently demands new efficient therapeutics (Shlaes et al. 2004). The outer membrane permeability barrier of Gram-negative pathogens is a crucial resistance factor. Reduced permeability of the cell envelope to antibiotics potentiates other resistance mechanisms like inactivating enzymes, efflux pumps, and target modification, and it reduces the final drug concentration at the target site. The outer membrane permeability barrier is particularly strong in increasingly problematic Gram-negative bacterial pathogens like *Pseudomonas aeruginosa, Burkholderia cepacia*,

and *Stenotrophomonas maltophilia*. These species are the major pathogens associated with pneumonia and bacteremia in immunocompromized hosts, with destructive lung infections in cystic fibrosis (CF) patients, and with severe nosocomial infections in ventilated patients, the latter of which is accompanied with a high mortality rate. *P. aeruginosa* is well known for its intrinsic resistance to a wide range of antimicrobial agents and its ability to develop multidrug resistance following antibiotic therapy. For an effective treatment of these human pathogens which are of great concern, new classes of antimicrobials with novel mechanisms of action and new approaches to increase the efficacy of traditional anti-bacterials are urgently needed (Talbot 2008; Talbot et al. 2006).

There are several approaches to circumvent permeability mediated resistance; one of them is the combination of drugs with elements destabilizing the bacterial outer membrane. Another opportunity is to use facilitated transport by bacteria itself, i.e., to misuse essential bacterial nutrient uptake systems by a Trojan Horse strategy. Several natural antibiotics mimic substrates for peptide, for sugar phosphate, for nucleoside, for polyamine and for iron siderophore uptake routes (Zähner et al. 1977).

However, the bacterial iron transport system is the most appropriate for the Trojan Horse approach due to the delicate balance of iron homeostasis in all organisms. Iron is essential for nearly all living organisms and plays a central role in diverse enzymes and biological functions. However, it is rarely available in the environment as well as in the infected host. Under aerobic conditions and at physiological pH, iron exists as hydroxide polymer. The solubility product is 10^{-38} M. The concentration in serum and tissues is 10^{-24} M. But the concentration required for bacterial growth by passive diffusion of the iron is at least 10^{-6} M (Braun and Killmann 1999). In the infected host, iron withholding is an important defense mechanism (Weinberg 1995). Constitutive elements are the iron binding, trafficking and recycling proteins transferrin (in blood) and lactoferrin (in body fluids), and ferritin as the intracellular storage protein. Additionally, a lot of processes are induced directly by the invading pathogens to reduce the free available iron. It starts with reduced assimilation of dietary iron, includes increased synthesis of the iron binding proteins, trafficking and scavenging by neutrophiles, and even immunoglobulins to iron repressible cell surface proteins are synthesized (Weinberg 1995).

In response to the restricted access to soluble iron, bacteria evolved high-affinity iron uptake pathways comprized of ferric iron specific carriers, termed siderophores, and the cognate membrane receptors. The pathway is activated under iron depletion by derepression of the fur repressor, which needs ferrous iron as a corepressor. Siderophores are excreted and sequester ferric ions. The complex is recognized by the relevant membrane receptors and is translocated into the cell. Iron is released by esterase activity, reduced to ferrous iron, then included in the different proteins and the fur repressor (Neilands 1982).

The main structural types of siderophores are catecholates, hydroxamates, and citrate based polycarboxylates (Fig. 1). Enterobactin (Fig. 2; O'Brien et al. 1970, 1971; Pollack and Neilands 1970) is one of the best described and strongest representatives of the catecholate siderophores (Raymond et al. 2003). It is produced by enterobacteria like *Escherichia coli* and *Salmonella*. Enterobactin is a triscatechol derivative of a cyclic triserine lactone. It consists of three 2,3-dihydroxy benzoic acid metal binding units attached via amide linkage to a trilactone backbone. Breakdown product is 2,3-dihydroxy benzoyl serine (2,3-DHBS) as linear monomer, dimer, and trimer (Raymond et al. 2003).

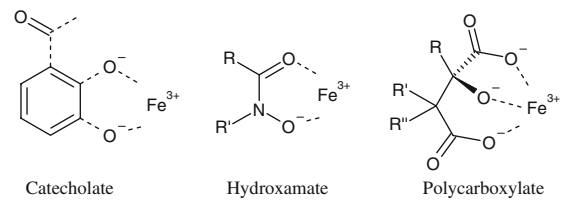


Fig. 1 Main structural types of siderophores as iron complexes

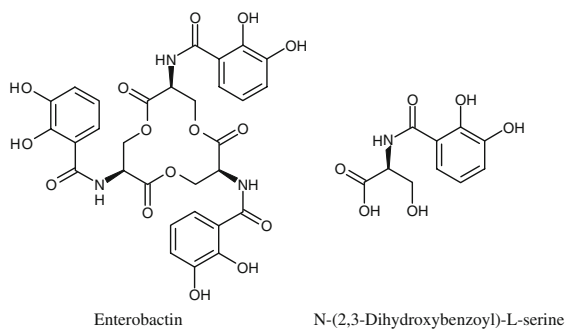


Fig. 2 Enterobactin and its breakdown product 2,3 DHBS

Bacteria express multiple siderophore uptake systems to assure access to the essential rare iron. *E. coli* has uptake routes for hydroxamates, citrate, catecholates, for endogenous and exogenous siderophores (Guerinot 1994). Out of the three catecholate receptors one, FepA, recognizes the cyclic trimer enterobactin, the two others, Cir and Fiu, the linear breakdown product 2,3-DHBS. While the receptors in the outer membrane are specific for individual siderophores and the transfer proteins in the cytoplasmic membrane are specific for the siderophore class, the TonB protein complex is universal and essential for all Fe^{3+} siderophore uptake routes by providing the energy for the transport process from the cytoplasm via ATPase activity.

In the infected host the siderophores have to compete for the rare iron with the host protein transferrin and lactoferrin. Bacteria can survive in the host only if the siderophores have a stronger affinity to ferric iron than the host proteins. For a lot of bacterial pathogens it was shown, that they can employ siderophores to obtain iron from transferrin or lactoferrin (Weinberg 1995) and that loss of siderophore biosynthesis is associated with loss of virulence (Wright et al. 1981).

In summary, bacterial iron uptake mechanisms are essential, multiple, very efficient and important virulence factors which are expressed in the infected host. Therefore, the iron uptake mechanisms are ideal targets to circumvent membrane associated drug resistance by the Trojan Horse approach. This means that a drug, which is unable to cross the bacterial membrane barrier, is linked to a siderophore. The Fe^{3+}-siderophore complex is recognized by the cognate receptor as a substrate providing the essential iron, and is actively transported across the outer membrane with the drug attached.

Examples of natural siderophore drug conjugates (SDC) are the sideromycins albomycin, produced by *Actinomyces subtropicus* (Gause 1955; Benz et al. 1982; Fiedler et al. 1985), ferrimycins, produced by *Streptomyces griseoflavus* (Bickel et al. 1960, 1965; Sackmann et al. 1962) and salmycin, produced by *Streptomyces violaceus* (Vertesy et al. 1995; Pramanik and Braun 2006). Albomycins are composed of ferrichrome, a tris(N^5-acetyl, N^5-hydroxyornithine) peptide, and a nucleoside-analogous thioribosyl pyrimidine moiety linked by a serine spacer (Benz et al. 1982; Bickel et al. 1965). Ferrimycins are hybrids of ferrioxamine B and an antibiotically active group. Salmycin consists of an amino disaccharide linked by a dicarboxylic spacer to the ferrioxamine B-type siderophore danoxamine (Pramanik and Braun 2006). The first artificial SDCs were synthesized in the group of Zähner in 1977. Ferricrocin and ferrioxamin B were attached to sulfonamides. However, antibacterial activity was lost, particularly when the sulfonamide was linked without a spacer directly to the siderophore. With increasing knowledge about siderophore transport mechanisms, the concept developed towards synthetic siderophores and β-lactams as drug moiety. In the following years several SDC were published. These were mainly cephalosporins, carbacephalosporins, carbapenems or monobactams with one or two catecholate or mixed catecholate hydroxamate moieties. (Arisawa et al. 1991; Ghosh et al. 1996; Chang et al. 1997; Roosenberg et al. 2000). Antibacterial activity of the SDCs was increased when compared to those of the non-modified drugs under conditions of iron starvation, preferentially against *P. aeruginosa* strains.

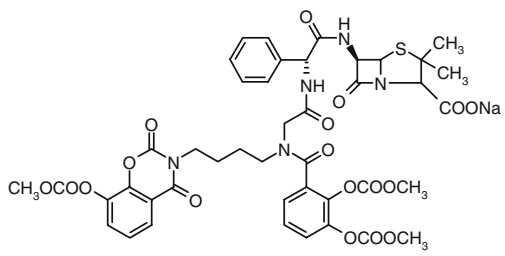
There are clear benefits of β-lactam antibiotics as drug moiety in SDCs. First, the targeted penicillin binding proteins (PBPs) are located in the periplasm. Only the outer membrane has to be crossed by the SDC to get access. Second, in contrast to most other types of antibiotics, the attachment site of the β-lactam drug to the target is different to the linkage site for the siderophore moiety. The conjugate can become active as a whole, without splitting off the siderophore moiety. Here we review the results of in vitro and in vivo studies that have examined the antimicrobial characteristics of new SDCs with aminopenicillins as drug moieties (rather than cephalosporins, carbapenems, and monobactams).

Synthesis and analysis of siderophore analogs and their drug conjugates

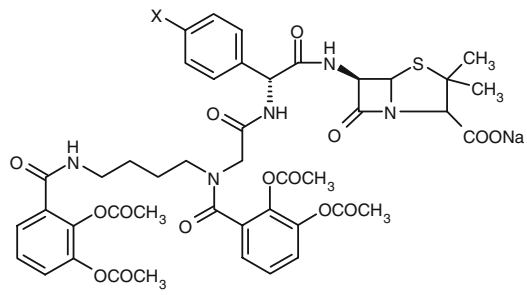
As a first step, siderophore moieties were synthesized as described (Schnabelrauch et al. 2000; Heinisch et al. 2002a). To identify and to optimize their siderophore-like properties, the siderophore analogues were analyzed for their iron-complexing capacity using the chrome azurol S (CAS) assay (Schwynn and Neilands 1987). Growth promotion assays under iron depletion, using a panel of

siderophore indicator strains, were applied to demonstrate the recognition of the iron complexes by outer membrane siderophore receptors with a subsequent translocation across the membrane and a release of the iron. (Schumann and Möllmann 2001; Mies et al. 2008) The most active siderophore analogues were selected as vectors for the synthesis of drug conjugates.

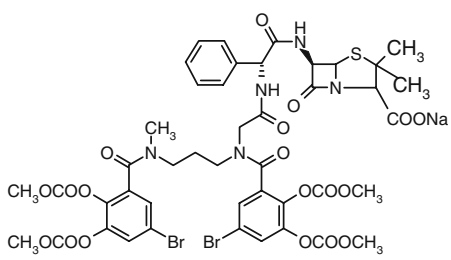
A substantial number of different siderophore aminopenicillin conjugates have been synthesized (Heinisch et al. 2002b, 2003; Wittmann et al. 2002). A correlation between siderophore structure and in vitro antibacterial efficacy was found, and lead structures were optimized with respect to optimum spacer length and number of catechol moieties. The continuous structure activity relationship (SAR) studies led to siderophore moieties with biscatecholate, triscatecholate, and substituted hydroxamate groups based on secondary di- or tri-aminoacids and on the diaminoacids L-ornithine and L-lysine. Drug moieties were the aminopenicillins ampicillin in compounds **9924067, 9924109, 9924129, 9924154**, and **10024013**, and amoxycillin in compound **9924155** (Fig. 3). A special feature of the conjugates is the acylation of the catecholate groups forming acetoxy groups (**9924154, 9924155**, **10024013**), acetoxy- and



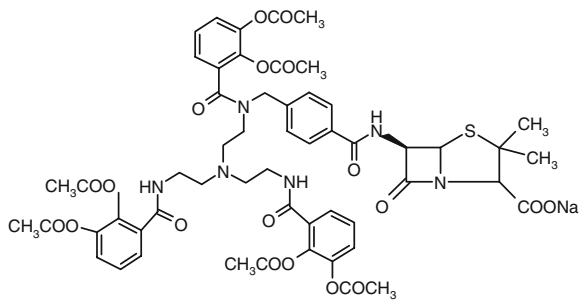
9924067



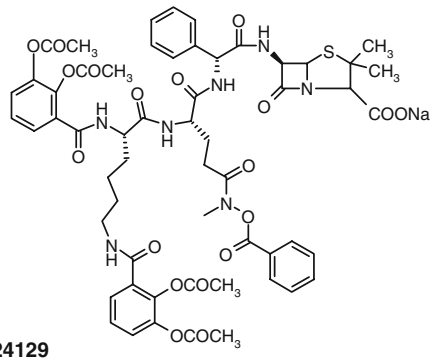
9924154: X = H, **9924155**: X = OH



9924109



10024013



9924129

Fig. 3 Structures of siderophore aminopenicllin conjugates

substituted hydroxamate groups (**9924129**), methoxycarbonyloxy groups (**9924109**), methoxycarbonyloxy and cyclic dioxobenzoxazinyl groups (**9924067**) (Wittmann et al. 2000). Thus they can operate like a prodrug preventing negative side effects of the catechol groups. The conjugates were studied for their mode of action, for their antimicrobial profile and in vitro activity, for their in vivo activity in a mouse infection model, and for toxicological side effects.

Mode of action

The mode of action was studied related to the influence of iron, of permeability, of porin usage, of TonB, of siderophore receptor selectivity, and of efflux sensitivity on antibacterial activity. Influence of iron, demonstrated by assays in iron-repleted and iron-depleted media confirmed the concept. Under iron-depleted conditions, e.g., when the siderophore pathways are activated, and which reflects the situation in vivo in the infected host, antibacterial activity of the conjugates is significantly increased (Fig. 4).

Permeability of the compounds was determined using a wild-type strain and a mutant with alterations in the outer membrane barrier. The minimal inhibition concentration (MIC) of the β-lactam moiety ampicillin for the permeability mutant *P. aeruginosa* K799/61 differs drastically from the MIC for the respective wild type strain *P. aeruginosa* K799/WT (Zimmermann 1980), in strong contrast to the conjugates, indicating that the conjugates enter the bacterial cell independently from a functional outer membrane permeability barrier. The permeation of the β-lactam drugs and the SDC was also expressed by the MIC ratio of *P. aeruginosa* wild type strain K799/WT and permeability mutant K799/61. It was 500 for ampicillin and below ten for most conjugates, while the MIC of the conjugates were generally much lower than those of the β-lactam antibiotics (Table 1).

Usually β-lactams like azlocillin and ampicillin diffuse through the water-filled porin channels OmpF and OmpC in *E. coli*. Using mutants with deletions in these porins and an OmpC-overexpressing mutant, it could be demonstrated that the activity of the β-lactam antibiotic depends on the presence of these porins, according to the difference in the diameters of the inhibition zones. This was almost not visible for the conjugates. The moderate increase in activity against the OmpF-overexpressing mutant showed that still some diffusion of the SDCs was possible. In contrast to the β-lactams, inhibition zone diameters of the conjugates for the porin mutants did not differ crucially if porins were deleted (−) or overexpressed (+++), but decreased essentially if TonB was deleted, which provides the energy and is essential for all active siderophore iron uptake routes (Table 2). These results confirmed, that the activity of the SDCs depends on active Fe^{3+}-siderophore uptake routes.

Defined siderophore deletion mutants were used in an agar diffusion assay to investigate the specific iron transport pathway misused by the conjugates (Table 3). There was no influence of the enterobactin receptor FepA on activity, neither of the β-lactams nor of the conjugates, demonstrated by the deletion mutant H873. Activity of the conjugates against the siderophore receptor mutants, where either *cir*

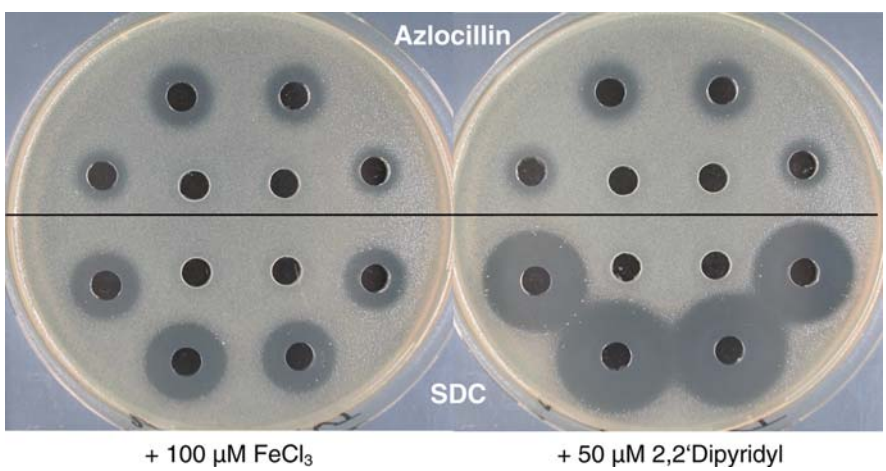


Fig. 4 Influence of Fe^{3+} on antibacterial activity. Inhibition zones of azlocillin and siderophore ampicillin conjugate **9925129** (SDC) on Mueller-Hinton agar plates inoculated with *P. aeruginosa* SG 137; left plate iron repleted, right plate iron depleted

Table 1 Activity against *P. aeruginosa* wild-type strain and permeability mutant

Compounds	*Pseudomonas aeruginosa*		MIC ratio K799/WT:K799/61
	K799/WT wild type representing permeability	K799/61 impaired outer membrane representing target activity	
Benzylpenicillin	100	0.4	250
Ampicillin	100	0.2	500
Carbenicillin	25	0.2	125
Mezlocillin	6.25	0.1	62.5
Azlocillin	3.12	0.1	32
9924067	0.32	0.08	4
9924109	0.16	0.02	8
9924129	0.64	0.04	16
9924154	0.08	0.02	4
9924155	0.16	0.08	2

MIC in mg/l

Table 2 Influence of outer membrane porins OmpF and OmpC and of TonB on antibacterial activity against relevant *E. coli* wild type and mutant strains in the agar diffusion assay

Mutant	*E. coli*				
	KB4	KB5	PLB268	AB2847	BR158
ompF	−	+	+	+	+
ompC	+	−	+++	+	+
tonB	+	+	+	+	−
Compound					
9924067	29.5	31	35	30.5	15.5
9924109	31	32	35	31.5	15.5
9924129	26	23	27	24	12
9924154	31.5	31	34	29.5	12.5
9924155	31.5	29.5	33	30	11.5
10024013	24	21	27.5	23.5	13.5
Azlocillin	16	15	32	20	20
Ampicillin	10.5	17	25	24	24

Concentration of SDC: 5 μg per agar well of 9 mm in diameter. Inhibition zones in mm

(H1875) or *fiu* (H1877) were deleted, slightly decreased. However, inhibition zone diameters of the conjugates, but not of the β-lactams, decreased drastically in the siderophore receptor mutant H1876 where both genes, *cir* and *fiu*, were deleted. These are the receptors also used by the linear enterobactin breakdown products of 2,3-DHBS (Hantke 1990). These results finally confirmed, that the catecholate-SDC can misuse bacterial iron uptake routes, particularly those via the catecholate receptors for 2,3-DHBS, Cir and Fiu, but not for enterobactin.

Table 3 Influence of siderophore receptors on antibacterial activity against relevant *E. coli* wild type and mutant strains in the agar diffusion assay

Mutant/receptor	H1443	H1876	H873	H1877	H1875
FepA	+	−	−	−	−
Cir	+	−	+	+	−
Fiu	+	−	+	−	+
Compound					
9924067	30	12.5	30	27.5	26
9924109	31	15.5	30	29.5	28
9924129	27	12	26	22	25
9924154	34	12	33	30.5	27
9924155	32	11	32	29	26
10024013	24.5	13	25	22	22.5
Ampicillin	20.5	20	19.5	20	20.5
Azlocillin	16	16	17	16	16

Concentration of SDC: 5 μg per agar well of 9 mm in diameter. Inhibition zones in mm

Thus reduced permeability can be circumvented efficiently by misuse of the essential iron uptake pathways and siderophore receptors as specific transporters for the SDCs.

In vitro proof of principle

The in vitro antibacterial activity profile of the conjugates was studied in comparison to the individual drug moiety ampicillin as well as to the antibiotics currently accepted as "gold standard"

for the therapy of infections with problematic Gram-negative enteric bacteria and especially Gram-negative glucose non-fermenting rods like *P. aeruginosa* and *S. maltophilia*.

The compounds showed an excellent activity against reference laboratory strains applied for basic evaluation (Table 4). Compared to ampicillin there was a more than 1,000-fold increase in activity against difficult-to-treat strains like *P. aeruginosa* and *S. maltophilia*. There was at least a 100-fold increase in activity against enterobacteria. Activity against enterobacteria is comparable to meropenem. Against *P. aeruginosa* and *S. maltophilia* activity was clearly better than meropenem. As previously reported (Chang et al. 1997), SDCs showed reduced activity against Gram-positive bacteria, like *Staphylococcus aureus*, which exhibit a different cell wall structure. An exception and a first example of a different structural type of SDCs is compound **10024013** (Fig. 3), demonstrating reasonable activity against both, Gram-negative and Gram-positive bacteria.

The results against the laboratory reference strains could be confirmed against extended numbers of clinically isolated strains by determination of MIC_{50} (Table 5). Due to the crucial role of Gram-negative non-fermenting bacteria, for a broad collection of clinical isolates of *P. aeruginosa*, *S. maltophilia* and *B. cepacia* the distribution of MICs was investigated (Table 6). A considerable number of aminopenicillin non-susceptible strains was highly susceptible to the respective SDC compounds. The conjugates were active even against carbapenem-resistant clinical strains of *P. aeruginosa* and *S. maltophilia*. The majority of strains were inhibited at MICs below

Table 4 In vitro antibacterial activity against a set of laboratory reference strains

Compound	MIC (mg/l)						
	Pseudomonas aeruginosa		*S. maltophilia*	*Klebsiella pneumoniae*	*E. coli*	*Serratia marcescens*	*Stapylococcus aureus*
	SG 137	ATCC 27853	GN 12873	ATCC 10031	ATCC 25922	SG 621	SG 511
9924067	0.04	0.31	0.08	0.005	0.02	0.02	5
9924109	<0.005	0.05	0.02	<0.005	<0.005	0.02	6.25
9924129	0.01	0.2	0.04	0.01	0.1	0.05	12.5
9924154	<0.005	0.05	0.005	<0.005	<0.005	<0.005	3.12
9924155	0.01	0.1	0.005	<0.005	0.01	<0.005	3.12
10024013	<0.05	0.2	<0.05	0.1	0.78	0.4	0.78
Meropenem	0.2	0.4	>100	0.06	0.04	0.06	0.1
Azlocillin	6.25	6.25	12.5	3.125	6.25	50	0.4
Ampicillin	>100	>100	>100	6.25	6.25	25	0.4

Table 5 In vitro activity against Gram-negative clinical isolates

Compound	MIC_{50} (mg/ml)				
	S. maltophilia $n = 52$	*Burkholderia* spp. $n = 38$	*Acinetobacter* spp. $n = 49$	*E. coli* $n = 27$	*Enterobacter cloacae* *Serratia marcescens* *Citrobacter freundii* $n = 8$
9924067	0.06	0.25	1	8	2
9924109	0.25	0.5	0.5	0.25	2
9924129	≤0.03	1	>4	16	16
9924154	0.06	0.13	0.5	8	2
9924155	0.06	0.5	0.25	8	4
Ampicillin	≥128	128	128	32	>128
Meropenem	≥128	1	0.25	2	16

Table 6 In vitro antibacterial activity against clinical isolates of *P. aeruginosa* (including imipenem resistant strains), *Burkholderia*, and *Stenotrophomonas maltophilia* (including meropenem resistant strains)

Compound	Strain	No. of isolates	MIC_{50} (mg/l)	MIC_{90} (mg/l)
9924154/155	*P. aeruginosa*	41	≤0.06/≤0.06	8/8
Ciprofloxacin	*P. aeruginosa*	41	0.25	16
9924154/155	*P. aeruginosa* (*Imi R*)	81	0.125/≤0.06	1/2
Imipenem	*P. aeruginosa* (*Imi R*)	81	>128	>128
9924154/155	*S. maltophilia*	52	0.06/0.06	4/4
Meropenem	*S. maltophilia*	52	>128	≥128
9924154/155	*Burkholderia* spp.	38	0.125/0.5	≥4/≥4
Meropenem	*Burkholderia* spp.	38	1.0	4

0.25 mg/ml (Schneider et al. 1999; Ankel-Fuchs et al. 2000; Möllmann et al. 2001a).

Resistance against *P. aeruginosa* is increasingly influenced by overexpressed efflux pumps (Guinea et al. 2000; Schweizer 2003). In contrast to the β-lactams and quinolones, the siderophore aminopenicillin conjugates were no substrates for efflux pumps in *P. aeruginosa*, since MICs were similar for wild type (WT) and the efflux pump overproducing strains MexAB-OprM, MexCD-OprJ or MexEF-OprN (Köhler et al. 1997; Möllmann et al. 2001b; Table 7).

In vivo proof of principle

In vivo efficacy of selected SDCs was tested at MDS Panlabs, Taiwan, in the mouse protection test with *P. aeruginosa* ATCC9027. Ofloxacin served as control substance. Immunocompetent mice (ten animals per group) were challenged i.p. with a lethal dose ($LD_{98–100}$) of *P. aeruginosa* in 5% mucin. Siderophore aminopenicillin conjugates were administered i.v. 30 min and 4 h post infection. The effective doses for 50% survival (ED_{50}) were 20.4 mg/kg for compound **9924067**, 5.6 mg/kg for **9924109**, 10.4 mg/kg for **9924129**, 7.3 mg/kg for **9924154**, 5.0 mg/kg for **9924155**, and 4.5 mg/kg for ofloxacin, respectively, indicating a promising therapeutic potential of the conjugates.

Table 7 Influence of *P. aeruginosa* efflux systems on antibacterial activity

Compound	*P. aeruginosa*			
	PAO1 WT	PAO M1 MexAB-OprM++	PAO J1 MexCD-OprJ++	PAO N1 MexEF-OprN++
Azlocillin	3.12	12.5	3.12	3.12
Meropenem	0.62	2.5	0.62	1.25
Ciprofloxacin	≤0.05	0.2	0.4	0.78
9924067	0.31	0.31	0.31	0.16
9924109	0.16	0.16	0.16	0.08
9924129	0.31	0.31	0.31	0.16
9924154	0.08	0.08	0.08	0.04
9924155	0.08	0.08	0.08	0.08

MIC in mg/l

Toxicological findings

Preliminary mutagenicity and toxicology data of **9924154** and **9924155** were obtained in non-GLP studies. In vitro assays (Ames test, SOS/umu test and micronucleus test) were negative. Single doses of 250 mg/kg and 500 mg/kg applied to mice and rats i.v. were well tolerated. Multiple i.v. dosing of both compounds to rats (2 weeks, 50–250 mg/kg) was well tolerated. The NOEL (no effect levels) were 200 mg/kg for **9924154** and 250 mg/kg for **9924155**.

Conclusion

The efficacy of traditional antibacterials, in particular the reduced permeation of aminopenicillins through the outer membrane of Gram-negative pathogens, can be increased by conjugation of the antibiotic to a siderophore moiety allowing facilitated transport via essential, species specific Fe^{3+} siderophore transport pathways. In addition to the active uptake by the bacteria itself, the siderophore aminopenicillin

conjugates are not substrates for efflux pumps in *P. aeruginosa*. The preclinical data on the described conjugates indicate good in vitro activity against Gram-negative pathogens and good in vivo activity against *P. aeruginosa*, no mutagenic potential, and good tolerance in mice and rats. The activity profile and the specific active uptake in bacteria under iron depletion could be beneficial for the treatment of lung infections in CF patients.

Acknowledgments We thank Klaus Hantke, University of Tübingen, Germany, for the generous support with the porin mutants as well as with the iron transport mutants, Franz-Josef Schmitz, University of Düsseldorf, Germany, for testing in vitro activity against clinical *P. aeruginosa* isolates, Monika Golembiewski and Irmgard Heinemann for their excellent technical assistance. Financial support by German Ministry of Education and Research, BMBF grant #0311232 is gratefully acknowledged.

References

Ankel-Fuchs D, Matthiesen T, Bauernfeind A, Möllmann U, Heinisch L, Härtl A (2000) In vitro and in vivo characteristics of new siderophore-β-lactam conjugates with enhanced activity against glucose-non-fermenting Gram-negative bacteria. 10th ECCMID, Stockholm, Sweden, May 2000. Abstract WeP171

Arisawa M, Sekine Y, Shimizu S, Takano H, Angehrn P (1991) In vitro and in vivo evaluation of Ro 09–1428, a new parenteral cephalosporin with high antipseudomonal activity. Antimicrob Agents Chemother 35:653–659

Benz G, Schröder T, Kurz J, Wünsche C, Karl W, Steffen GJ, Pfitzner J, Schmidt D (1982) Konstitution der Deferriform der Albomycine δ_1, δ_2 und ε. Angew Chem 94:552–553

Bickel H, Gäumann E, Nussberg G, Reusser P, Vischer E, Voser W, Wettstein A, Zähner H (1960) Stoffwechselprodukte von Actinomyceten. 25 Mitteilung: Über die isolierung und charakterisierung der ferrimycine A_1 und A_2, neuer antibiotika der sideromycin-gruppe. Helv Chim Acta 43:2105–2118. doi:10.1002/hlca.19600430730

Bickel H, Mertens P, Prelog V, Seibl J, Walser A (1965) Constitution of ferrimycin A1. Antimicrob Agents Chemother 5:951–957

Braun V, Killmann H (1999) Bacterial solutions to the iron-supply problem. Trends Biochem Sci 24:104–109. doi:10.1016/S0968-0004(99)01359-6

Chang MH, Koh HY, Choh YS, Choi KI (1997) Novel anti-Pseudomonas Cephalosporins. Curr Pharm Des 3:209–226

Fiedler H-P, Walz F, Zähner H (1985) Albomycin: studies on fermentation, isolation, and quantitative determination. Appl Microbiol Biotechnol 21:341–347. doi:10.1007/BF00249977

Gause GF (1955) Recent studies on albomycin, a new antibiotic. BMJ 12:1177–1179

Ghosh A, Ghosh M, Niu C, Malouin F, Moellmann U, Miller MJ (1996) Iron transport-mediated drug delivery using mixed-ligand siderophore-beta-lactam conjugates. Chem Biol 3:1011–1019. doi:10.1016/S1074-5521(96)90167-2

Guerinot ML (1994) Microbial iron transport. Annu Rev Microbiol 48:743–772. doi:10.1146/annurev.mi.48.100194.003523

Guinea J, Cercenado E, Garcia-Garrote F, Cuevas O, Martin-Pedroviejo J, Bouza E (2000) Prevalence of efflux pumps among clinical isolates of multiple antibiotic resistant *Pseudomonas aeruginosa*. Abstr Intersci Conf Antimicrob Agents Chemother 40:87

Hantke K (1990) Dihydroxybenzoylserine, a siderophore for *E.coli*. FEMS Microbiol Lett 67:5–8

Heinisch L, Gebhardt P, Heidersbach R, Reissbrodt R, Möllmann U (2002a) New synthetic catecholate-type siderophores with triamine backbone. Biometals 15:133–144. doi:10.1023/A:1015293900133

Heinisch L, Wittmann S, Stoiber T, Berg A, Ankel-Fuchs D, Möllmann U (2002b) Highly antibacterial active aminoacyl penicillin conjugates with bis-catecholate siderophores based on secondary diamino acids and related compound. J Med Chem 45:3032–3040. doi:10.1021/jm010546b

Heinisch L, Wittmann S, Stoiber T, Scherlitz-Hoffmann I, Ankel-Fuchs D, Möllmann U (2003) New tris- and tetrakis-catecholate siderophores based on polyazaalkanoic acids and their β-lactam conjugates. Arzneim-Forschung. Drug Res 53:188–195

Köhler T, Michea-Hamzehpour M, Henze U, Gotoh N, Curty LK, Pechère JC (1997) Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol Microbiol 23:345–354. doi:10.1046/j.1365-2958.1997.2281594.x

Mies KA, Gebhardt P, Möllmann U, Crumbliss AL (2008) Synthesis, siderophore activity and iron(III) chelation chemistry of a novel mono-hydroxamate, bis-catecholate siderophore mimic: *N*(alpha),-*N*(epsilon)-bis[2,3-dihydroxybenzoyl]-l-lysyl-(gamma-*N*-methyl-*N*-hydroxyamido)-l-glutamic acid. J Inorg Biochem 102:850–861. doi:10.1016/j.jinorgbio.2007.11.021

Möllmann U, Heinisch L, Ankel-Fuchs D, Köhler T (2001a) Mechanism of action of new Siderophore Acylaminopenicillins. Abstr Intersci Conf Antimicrob Agents Chemother 16–19 Dec 2001, 41 abstract no. F-377

Möllmann U, Heinisch L, Bauernfeind A, Schneider I, Schmitz FJ, Ankel-Fuchs D (2001b) Antibacterial activity of new Siderophore Acylaminopenicillins. Abstr Intersci Conf Antimicrob Agents Chemother 16–19 Dec 2001, 41 abstract no. F-378

Neilands JB (1982) Microbial envelope proteins related to iron. Annu Rev Microbiol 36:285–309. doi:10.1146/annurev.mi.36.100182.001441

O'Brien IG, Cox GB, Gibson F (1970) Biologically active compounds containing 2,3-dihydroxybenzoic acid and serine formed by *Escherichia coli*. Biochim Biophys Acta 201:453–460

O'Brien IG, Cox GB, Gibson F (1971) Enterochelin hydrolysis and iron metabolism in *Escherichia coli*. Biochim Biophys Acta 237:537–549

Pollack JR, Neilands JB (1970) Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem Biophys Res Commun 38:989–992. doi:10.1016/0006-291X(70)90819-3

Pramanik A, Braun V (2006) Albomycin uptake via a ferric hydroxamate transport system of *Streptococcus pneumoniae* R6. J Bacteriol 188:3878–3886

Raymond KN, Dertz EA, Sanggoo SK (2003) Bioinorganic chemistry special feature: Enterobactin: an archetype for microbial iron transport. Proc Natl Acad Sci USA 100: 3584–3588. doi:10.1073/pnas.0630018100

Roosenberg JMII, Lin YM, Lu Y, Miller MJ (2000) Studies and syntheses of siderophores, microbial iron chelators, and analogs as potential drug delivery agents. Curr Med Chem 7:159–197

Sackmann W, Preusser P, Neipp L, Kradolfer F, Gross F (1962) Ferrimycin A, a new iron containing antibiotic. Antibiot Chemother 12:34–45

Schnabelrauch M, Wittmann S, Rahn K, Möllmann U, Reissbrodt R, Heinisch L (2000) New synthetic catecholate-type siderophores based on amino acids and dipeptides. Biometals 13:333–348. doi:10.1023/A:1009297610755

Schneider I, Bauernfeind A, Miehle S, Ankel-Fuchs D (1999) Activity of aminopenicillin-siderophore-conjugates against *Stenotrophomonas maltophilia*. 39th Interscience Conference on antimicrobial agents and chemotherapy, San Francisco, California, September 1999, Abstract 400, p 298

Schumann G, Möllmann U (2001) A screening system for siderophores as potential drug delivery agents in mycobacteria. Antimicrob Agents Chemother 45:1317–1322. doi:10.1128/AAC.45.5.1317-1322.2001

Schweizer HP (2003) Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet Mol Res 2:48–62

Schwynn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56. doi:10.1016/0003-2697(87)90612-9

Shlaes DM, Projan SJ, Edwards JE Jr (2004) Antibiotic discovery: state of the state. ASM News 70:275–281

Talbot GH (2008) What is the pipeline for Gram-negative pathogens? Expert Rev Anti Infect Ther 6:39–49. doi: 10.1586/14787210.6.1.39

Talbot GH, Bradley J, Edwards JE, Gilbert D, Scheld ME, Bartlett JG (2006) Bad bugs need drugs: an update on the development pipeline from the antimicrobial availability task force of the infections diseases society of America. Clin Infect Dis 42:657–668. doi:10.1086/499819

Vertesy L, Aretz W, Fehlhaber HW, Kogler H (1995) Salmycin A–D, antibiotika aus *Streptomyces violaceus*, DSM 8286, mit siderophor-aminoglycosid-struktur (salmycin A–D, antibiotics from *Streptomyces violaceus*, DSM 8286, having a siderophor-aminoglycoside structure). Helv Chim Acta 78:46–60. doi:10.1002/hlca.19950780105

Weinberg ED (1995) Acquisition of iron and other nutrients in vivo. In: Roth JA et al (eds) Virulence mechanisms of bacterial pathogens. ASM Press, Washington, DC, pp 79–93

Wittmann S, Scherlitz-Hofmann I, Möllmann U, Ankel-Fuchs D, Heinisch L (2000) 8-Acyloxy-1,3-benzoxazine-2,4-diones as siderophore components of antibiotics. Arzneim-Forsch Drug Res 50:752–757

Wittmann S, Schnabelrauch M, Scherlitz-Hoffmann I, Möllmann U, Ankel-Fuchs D, Heinisch L (2002) New synthetic siderophores and their β-lactam conjugates based on amino acids and dipeptides. Bioorg Med Chem 10:1659–1670. doi: 10.1016/S0968-0896(02)00044-5

Wright AC, Simpson LM, Oliver JD (1981) Role of iron in the pathogenesis of *Vibrio vulnificus* infections. Infect Immun 34:503–507

Zähner H, Diddens H, Keller-Schierlein W, Nägeli H-U (1977) Some experiments with semisynthetic sideromycins. Jpn J Antibiot 30:S201–S206

Zimmermann W (1980) Penetration of β-lactam antibiotics into their target enzymes in *Pseudomonas aeruginosa*: comparison of a highly sensitive mutant with its parent strain. Antimicrob Agents Chemother 18:94–100