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Iron trafficking as an antimicrobial target

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Abstract Iron is essential for the survival of most organisms. Microbial iron acquisition depends on multiple, sometimes complex steps, many of which are not shared by higher eukaryotes. Depriving pathogenic microbes of iron is therefore a potential antimicrobial strategy. The following minireview briefly describes general elements in microbial iron uptake pathways and summarizes some of the current work aiming at their medicinal inhibition.

Keywords Iron · Antibiotic · Siderophore

Introduction

The bottom of the food chain is a marvelously inventive place. The diverse and changing environments that microbes inhabit, coupled with the perils of unicellular existence, demand a certain resourcefulness. Readers of *Biometals* are aware of the special challenges posed by the need to acquire iron: an absolutely essential but highly insoluble metal in most biota, and a jealously protected one inside the human body. Microbial systems for Fe uptake and trafficking are consequently highly developed and fundamentally interesting. Limiting Fe under laboratory conditions can be detrimental or lethal, offering a means for limiting microbial growth. The potential for medicinally impeding Fe metabolism is a commonly, if sometimes uncritically, cited justification for in-depth biological studies of Fe acquisition. In fact, derailing the Fe supply train, while plausible as an antimicrobial strategy, is still largely untested in practice. The following mini-review summarizes several components of Fe trafficking pathways that are currently being pursued as or have the potential to serve as promising antimicrobial targets.

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Existing antibiotics and current needs

New antimicrobial targets are needed as infectious diseases spread globally and as resistance to available antibiotics grows (Gold and Moellering 1996; CDC 2006; Walsh 2000). The World Health Organization cites infectious diseases as the current number one killer of children and young adults around the globe. In the hour that one might spend reading this issue of *Biometals*, over 1,500 people will die of infectious diseases, over half of them children under five. This amounts to more than 13 million deaths a year: one in two deaths in developing countries. Disabling illnesses are expected to add substantially to the human and economic toll of infection (Brundtland 1999). As recent news stories have highlighted, dangerous

diseases are increasingly transported across borders. In 2000, ~46% of newly identified US TB cases originated in other countries (WHO 2005). The spread of TB has been hastened by the lack of public health surveillance for this disease and by the concurrent HIV/AIDS epidemic. Current first-line treatments for many infectious diseases are often many decades old, and resistance to many of them is widespread. Even newer antibiotics such as itraconazole (2001) and voriconazole (2002) have already been compromised by resistance. *Klebsiella pneumonia*, for example, is a ubiquitous, clinically important, opportunistic Gram negative pathogen that is often associated with infections acquired in hospital settings (Ko et al. 2002; Yu et al. 2007). In 2003, 20.6% of >1,000 hospital isolates of *K. pneumoniae* were resistant to 3rd generation cephalosporins, representing a nearly 50% increase in nonsusceptible *K. pneumoniae* between 2002 and 2003 alone (Cardo et al. 2004). Not only new antibiotics, but likely antibiotics that work in new ways (vide infra), are needed to stem the tide of infectious agents, resistant or otherwise.

Successful antibiotics from penicillin to quinine have traditionally targeted facets of microbial biochemistry that are essential for and, as much as possible, unique to the pathogen. Most existing antimicrobial strategies fall into one of four categories (Walsh 2000). The first three target enzymes involved in (1) cell-wall biosynthesis; (2) protein synthesis; and (3) nucleic acid metabolism and repair. Category (4) antimicrobials interfere with membrane integrity via, for example, the use of membrane-permeable ion transporters (ionophores) that upset the cellular osmotic balance. Each of these strategies clearly aims at interrupting an essential cellular process. Of all of these, (1) has perhaps the greatest potential for pathogen specificity, at least toward bacterial pathogens, as the peptidoglycan component of the cell wall is unique to them. Each of the others has the potential for cross-reacting with human or animal host cells that depend on similar biochemical pathways, with consequent toxicity. In fact, many antibiotics in classes (2)–(3) are especially lethal to rapidly proliferating cells and consequently have anticancer activity. Interestingly, rapidly proliferating cells also have amplified iron requirements. Angiogenesis inhibition, a powerful anti-cancer strategy in principle if not in current therapeutic application, starves growing cells of critical supplies, not the least of which is iron.

In contrast to these established antimicrobial approaches, interruption of the Fe trafficking is a plausible but still largely unproven means of clinically controlling pathogens. However, two classic anti-malarials provide important proof-of-concept examples, in which successful drugs target the Fe trafficking and storage mechanisms of *Plasmodium falciparum* (Bray et al. 2005). *Quinine* is a natural product quinoline found in the bark of the cinchona tree. It and its derivatives have been used as malaria remedies since the 17th century. *Artemisinin* is a sesquiterpene lactone with an activated peroxide, and likewise a plant product (from *Artemisia annua*). It has been in use as an antimalarial in China since at least the fourth century. Though their precise mechanisms of action are still occasionally debated, both indisputably interfere with *P. falciparum's* unusual Fe trafficking pathway, in which hemoglobin-derived heme is taken up and stored as crystalline hemin. Direct use/storage of heme makes sense in light of the malaria parasite's preferred habitat inside the hemoglobin-packed human erythrocyte. Quinine and related compounds are believed to disrupt the stacking and consequently prevent crystallization of heme molecules. The Fe(II) in ferroheme can reductively cleave the artemisinin peroxide bond, generating a radical species that cross-links to and interferes with metabolism and storage of the ferriheme.

Fe trafficking: major whistle-stops

Artemisinin and quinine likely interfere with a metabolic endpoint (iron storage). A typical iron-acquisition pathway, however, has several steps, any of which might in principle serve as a potential target (Bullen et al. 2005; Byers and Arceneaux 1998; Miethke and Marahiel 2007; Ratledge and Dover 2000; Weinberg 1998). A tremendous amount of work, particularly focusing on Gram negative bacteria, has been done in this area; several relevant steps are summarized only in the most schematic form, below:

(1) *Ligand exchange* A pathogen will generally encounter iron in a chelated form in which the ligand is either a protein (e.g., the transport proteins

transferrin or lactoferrin in plasma or milk, respectively) or the porphyrin ring. As a first step toward acquisition, many bacterial and fungal pathogens secrete a siderophore or hemophore which binds iron(III) or heme with high affinity. A hemophore may act in conjunction with proteolytic enzymes, acting to release heme from a protein (e.g., hemoglobin). Exchange of the iron into the pathogen-derived ligand allows for its specific recognition and uptake. The human immune system already assaults this step of the trafficking pathway when infection is sensed, by mounting an "iron blockade": the removal of transferrin-bound Fe(III) from circulation and into intracellular storage within ferritin, where it is presumed to be unavailable to extracellular pathogens (Weinberg and Weinberg 1995). Direct usage of ferritin iron by intracellular pathogens is undescribed in vivo (Gobin and Horwitz 1996), but possible.

(2) *Diffusion* Once repackaged in a siderophore or hemophore, the newly speciated iron must diffuse back to the microbe. Mammalian biochemistry has again devised a countermeasure against the diffusion step, at least for catecholate-containing ferrisiderophores. As an example, the mammalian immunoprotein siderocalin, first identified as neutrophil gelatinase-associated lipocalin, picks off catecholate-dependent ferrisiderophores before their safe return to the pathogen (Goetz et al. 2002; Fischbach et al. 2006; Holmes et al. 2005; Nelson et al. 2005). The ferrisiderophores are entirely encompassed by this protein, which eventually is excreted by the host (Goetz et al. 2002; Yang et al. 2002). Siderophore-binding proteins aside, it is unclear how pathogens contend with the apparent inefficiency of Fe-acquisition systems that depend on diffusion of secreted molecules back to themselves. Under conditions of Fe-stress, which are expected to be common in most pathogenic milieu, microbes can secrete extremely large amounts of siderophore, even exceeding their own mass within a relatively short period of time (Hersman et al. 2000). The importance of iron apparently justifies the metabolic investment.

(3) *Specific recognition and membrane transport* Bacterial ferrisiderophores are recognized and internalized by specific, Ton-B-dependent outer membrane receptors that couple to and are driven by the electrochemical gradient of the inner membrane of Gram negative bacteria (Pawelek et al. 2006). It is generally understood that siderophores and receptors have a 1:1 relationship, at least in Gram negative bacteria, though exceptions to this rule have been identified (Vasil and Ochsner 1999). Heme or hemophores are likewise recognized and internalized by specific outer membrane receptors (Mazmanian et al. 2003). Some organisms, notably some ancient eukaryotic protozoans, have outer membrane receptors for the direct endocytosis of human transferrin or hemoglobin (Britigan et al. 1998; Fast et al. 1999; Krishnamurthy et al. 2005; van Luenen et al. 2005). In each case, specific, high-affinity receptors at the outer membrane allow for the efficient uptake of the iron source and the rejection of other molecules.

The importance of specific recognition has been demonstrated in several ways. In one dramatic example, it was shown that the siderophore mycobactin T is an avid growth promoter for *Mycobacterium tuberculosis*, its native producer. By contrast, mycobactin S, a siderophore from the congener species *M. smegmatis*, cannot be incorporated by and is moreover highly toxic to *M. tuberculosis*. Structural analyses showed that these large natural products were completely identical except for the handedness of one stereocenter (Hu and Miller 1997). It has been suggested that bacteria use small structural differences in their siderophores as a means of outcompeting rivals for limited Fe. Indeed, laboratory growth media are often supplemented with siderophores or unnatural chelators that a particular species is incapable of metabolizing in order to create a simulated Fe challenge. EDTA is a well-known biocontrol additive in foods, due in part to the chelator's avid association with Fe, which it sequesters in a form that is unavailable to non-siderophore producing bacteria. In each case, Fe is made less bioavailable by binding it to a chelator that a particular organism cannot use.

(4) *Intracellular trafficking* Many events in the care and handling of iron after it traverses the outer membrane are less well-defined. The site and agents of iron reduction, the identities of intracellular Fe(II) transporting ligands, and the timing of Fe reduction with release from the siderophore are not well understood. Iron encounters ferritin as Fe(II), becoming oxidized as part of the mineralization process. By the same token, iron is reduced as it is mobilized from the ferritin stores back into the cytoplasm (Liu and Theil 2005). Hence, newly incorporated Fe(III) must eventually be reduced, and internal trafficking at least at some places must involve Fe(II). In the case of Gram negative bacteria, reduction could occur in

either the periplasm or cytoplasm. If reduction occurs in the cytoplasm, either the ferrisiderophore (more likely) or Fe(III) alone may cross from the periplasm through the inner membrane via an ATP-powered ABC transporter. Once in the cytoplasm, release of Fe(III) from the siderophore could occur via enzymatic cleavage particularly for very tightly bound Fe(III)/siderophore complexes. The iron from ferrienterobactin and fusarinines, for example, is released via hydrolysis of their ester backbones in the cytoplasm (Brickman and Mcintosh 1992; Kragl et al. 2007). How the Fe(III) is subsequently reduced and transported is unknown. Similarly, in organisms that directly utilize heme, opening of the porphyrin macrocycle is catalyzed in the cytoplasm by heme oxygenase (Skaar et al. 2004; Wegele et al. 2004). In that case, the released iron would have to be reduced or, if already reduced, maintained stably as Fe(II).

Alternatively, reduction and consequent release of Fe(II) from a siderophore for Gram negative species could occur in the periplasm, followed by movement through an inner membrane, GTP-dependent transporter, FeoB. Transport could occur for Fe(II) alone or in conjunction with an Fe(II) binding ligand (Kammler et al. 1993; Dhungana and Crumbliss 2005). Whether in the periplasm or cytoplasm, Fe(II) chelators could have multiple roles. First, they could protect Fe(II) from rapid reoxidation. Second, the Fe(II)/chelator binding energy could help to "pay" the energy toll for reducing the ferrisiderophore iron. Ferrisiderophores can have fairly negative reduction potentials, requiring a significant driving force to reduce the bound iron. Finally, such chelators could be important for transport into or proper trafficking within the cytoplasm (Dhungana and Crumbliss 2005).

(5) *Storage* A large number of organisms, including bacteria, plants, and animals, store iron in ferritins as crystalline iron oxide (ferrihydrite). Fungi are a major exception, storing iron instead inside intracellular siderophores (Miethke and Marahiel 2007). These may structurally resemble the secreted siderophores but with modifications that prevent their cellular export. Other exceptions include several eukaryotic protozoa, including *P. falciparum*, *Trypanosoma*, and *Leishmania sp.* (Sutak et al. 2008). These and other blood-feeding pathogens may take up heme directly, storing iron inside vacuoles as crystalline hemozoin. The details of iron storage in many non-ferritin-producers are not well understood.

Possible targets

Fe trafficking could in principle be chemically interrupted at any of the steps outlined above. A large fraction of known antibiotics and drugs in general act as enzyme inhibitors. Enzyme inhibition is amenable to established methods of rational drug design, including the development of structure-activity relationships via systematic variation of the structures of inhibitors. We will consequently consider enzymatic targets first.

Siderophore biosynthesis

Siderophore biosynthesis stands out as *the* central enzyme-mediated event of Fe trafficking by many species. Eliminating siderophore production would block both the release of iron from host molecules (step (1) above) and the pathogen's mechanism for transporting Fe(III) through the outer membrane (step (3)). Many organisms depend primarily or entirely on siderophore-mediated mechanisms for both processes. As a consequence, genetic knockouts of siderophore biosynthesis genes in several organisms severely restrict growth and/or virulence (Cendrowski et al. 2004; Chen et al. 2004; Dale et al. 2004; Hissen et al. 2005; Kang and Armstrong 1998; Schrettl et al. 2004). Other organisms appear to be less impaired by similar gene knockouts, reflecting their ability to compensate for the lost siderophore in other ways: e.g., production of a second siderophore, use of other molecules in the host environment for mediating Fe entry as "xenosiderophores", uptake of heme (if available), etc.

Organisms that rely on a single siderophore for one or more essential functions are likely to be especially vulnerable to interference with siderophore biosynthesis. For example, fungi often use variants of the same siderophore to recruit iron, transport it through the cell wall, and store it intracellularly (step (5)) (Haas et al. 2008; Johnson 2008; Philpott 2006). In *Mycobacteria*, water-soluble mycobactins scavenge iron from the environment; more lipophilic mycobactin variants remain mounted in the cell wall. These may similarly serve as an iron storage reservoir, or they may have other yet unknown biological functions. As a consequence, certain mycobacterial and fungal species may be particularly susceptible to chemical interruption of siderophore biosynthesis.

Pioneering work has focused on inhibition of the biosynthesis of aryl-capped siderophores, including mycobactin T in *M. tuberculosis*. The mycobactin scaffold is assembled by a mixture of non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs). Multi-modular NRPS/PKS enzymes catalyze the biosynthesis of a large number of siderophores, antibiotics, and other natural products in bacteria, fungi, and plants. "Modular" refers to the organization of the enzymes into a series of catalytic units, each of which activates a particular monomer and carries out a single initiation, chain elongation, or termination step (Donadio et al. 1991, Marahiel et al. 1997, von Dohren et al. 1997). NRPSs use amino acids to build peptidic products without an RNA template, while PKSs synthesize polyketides in a series of carbon condensations similar to those in fatty acid biosynthesis (Crosa and Walsh 2002; Kleinkauf and Döhren 1996). Monomers are activated for chain-incorporation by ATP-mediated adenylation (formation of monomer-adenosine monophosphate (AMP) conjugates). Both NRPSs and PKSs use the nucleophilic sulfhydryl at the end of a Coenzyme-A-derived pantetheinate arm as the "handle" that grasps the monomer or growing product (Schlumbohm et al. 1991; Lambalot et al. 1996; Pfeifer et al. 1995).

Successful inhibition strategies to date have targeted aryl acid activating enzymes including MbtA, a NRPS that initiates assembly of the mycobactin backbone at its aryl acid (salicylate) head group (Fig. 1; Quadri 2000; Finking et al. 2003). Solution of the crystal structure of DhbE, a so-called "stand-alone" adenylation domain that recognizes and activates 2,3-dihydroxybenzoate (DHB) for incorporation into bacillibactin, set the stage for this work (May et al. 2002). As an isolated enzyme and not a subdomain of a much larger NRPS like MbtA, detailed structural characterizations of the enzyme and its DHB-AMP complex were feasible. The DhbE structures were used to build homology models for MbtA, which shares 76% identity with DhbE at the active site. An added, critical observation came from work with mechanistically related adenylating enzymes, including amino acid tRNA synthetases. These were shown to bind their acyl-AMP intermediates 2–3 times more tightly

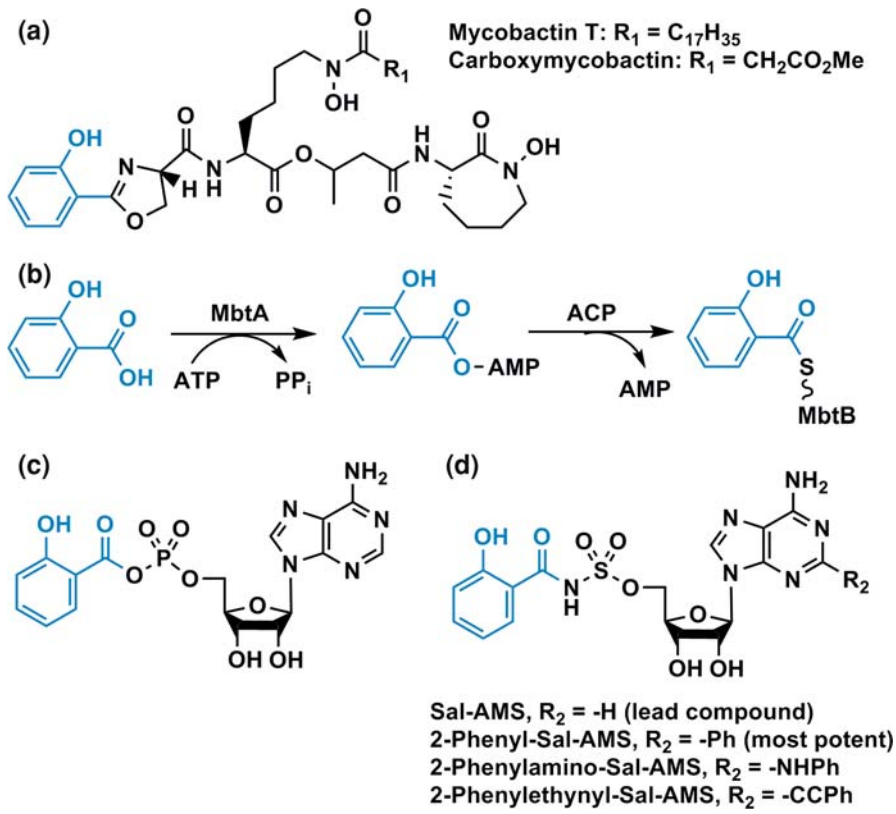

Fig. 1 **a** Mycobacterial siderophores. The salicylate-derived aryl headgroup activated by MbtA is shown in light blue throughout. **b** Reaction catalyzed by MbtA. ArCP is the aryl carrier protein to which MbtA transfers its adenylated substrate. **c** Sal-AMP is the native product of MbtA. **d** Various analogs to Sal-AMP were designed and tested as enzyme inhibitors and antituberculor agents

than their carboxylic acid and ATP substrates (Forrest et al. 2000).

By analogy, the MbtA inhibitor 5′-*O*-[*N*-salicyl-sulfamoyl]adenosine (Sal-AMS) was designed as a catalytically inactive analog of the enzyme product salicyl-AMP, with a sulfamoyl group in place of the phosphate linker (Fig. 1c, d) (Ferreras et al. 2005). The compound was evaluated for its activity against MbtA as well as the enzymes YbtE and PchD, NRPSs which adenylate salicylic acid as part of the biosynthesis of yersiniabactin (by *Yersina pestis*) and pyochelin (*Pseudomonas aeruginosa*), respectively (Miethke et al. 2006). Sal-AMS was shown to be a tight-binding inhibitor, with half maximal enzyme inhibitory concentration (IC_{50}) values ranging from 10.7 to 14.7 nM. Inhibition of MbtA and YbtE was competitive with respect to ATP and noncompetitive with salicylate, indicating that Sal-AMS and ATP bind the same enzyme form and that inhibition occurs at the initial adenylation step. Sal-AMS was further shown to inhibit production of mycobactin by *M. tuberculosis* and yersiniabactin by *Y. pestis*, respectively. Finally, the compound inhibited the growth of both organisms under iron-limiting conditions, with inhibitory concentrations inhibiting bacterial proliferation to 1% (MIC_{99}) equal to 2.2 μM (*M. tuberculosis*) and 51.2 μM (*Y. pestis*).

Sal-AMS itself is a modestly effective lead compound that provides an important proof-of-concept. More potent inhibitors based on Sal-AMS, with better chemical and pharmacokinetic properties, were subsequently developed in a series of structure-activity relationship studies carried out by Aldrich and coworkers (Neres et al. 2008; Qiao et al. 2007b; Somu et al. 2006a, b; Vannada et al. 2006). Initial work focused on the critical phosphate "linker" portion of Sal-AMP, which was replaced with a series of groups of varying charge and susceptibility to hydrolysis. Stability under biological conditions is particularly important, as the 5′-*O*-(sulfamoyl)adenosine hydrolysis product is broadly cytotoxic (i.e., via mechanisms independent of MbtA). *β*-ketosulfonamide linked compounds were found to be equally effective at inhibiting MbtA while more resistant to hydrolysis. Using Sal-AMS as a template, further variations in its glycosyl, aryl, and nucleobase domains were examined systematically (Neres et al. 2008). Only modifications at the nucleobase led to improved inhibition (Qiao et al. 2007a, b; Somu et al. 2006b). The DhbE/DHB-AMP crystal structure revealed hydrophobic protein-purine interactions with poorly aligned hydrogen bonding, suggesting that modifications at the nucleobase would be well-tolerated. Indeed, Sal-AMS analogs with substitutions at the purine C-2 (2-phenylamino-, phenylethyl-, and 2-phenyl-Sal-AMS, Fig. 1) were more potent than the parent compound, with apparent inhibition constants of 0.94, 0.40, and 0.27 nM, respectively. The best compound, 2-phenyl-Sal-AMS, had 24 times the potency of the lead compound (Sal-AMS) and a high binding affinity ($K_D = 0.27$ pM). This compound also had substantial in vitro antitubercular activity, with MIC_{99} equal to 39 nM (Neres et al. 2008).

We are taking a somewhat broader approach by focusing on the enzymes that produce hydroxamate, the chelating portion of several siderophores. This is a strategy with many practical advantages. First, while siderophore scaffolds are structurally extremely diverse, most rely on one of three bidentate chelating moieties: hydroxamic acid, catecholate, or α-hydroxy-carboxylic acid. Second, the chelating portions are clearly necessary for siderophore function. Third, the hydroxamic acids are not the strongest known chelators but they are specifically associated with the virulent forms of several organisms. This is ascribed to the hydroxamate's ability to evade siderophore-binding siderocalin, since this immunoprotein specifically binds catecholate-dependent siderophores through cation-pi interactions (vide supra). Finally, the monooxygenase and acyl transferase enzymes that produce hydroxamate are so-called "tailoring enzymes" that act independently of large NRPS or PKS complexes. They are consequently much simpler to prepare, study, and structurally characterize in atomic detail.

Biosynthesis of hydroxamate-dependent siderophores typically begins with hydroxylation of L-lysine or L-ornithine at the side chain amine (see Fig. 2b). The hydroxylamine may be subsequently formylated or acylated by a GNAT-family acyltransferase, yielding the bidentate chelator (Vetting et al. 2005). Acylation can also modify the siderophore's solubility, depending on the nature of the acyl -*R* group (see Fig. 1a). The doubly modified amino acid building block is then woven into the siderophore backbone by NRPS-dependent or NRPS-independent pathways. The latter couple activated (phosphorylated) carboxylic acids to the α-amine of the modified lysine or ornithine, using conventional non-modular enzymes.

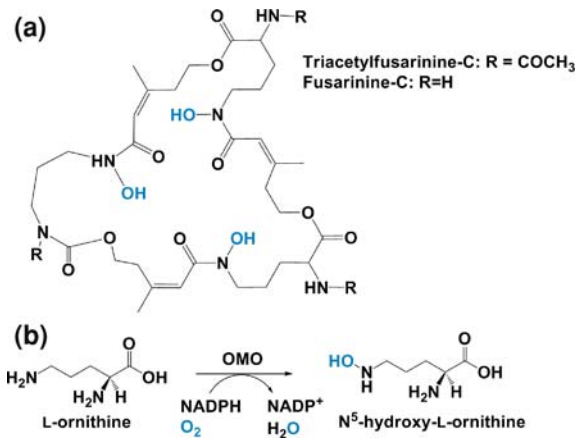

Fig. 2 **a** Fungal siderophores (*Aspergillus fumigatus*). The hydroxyl groups added by N^5-ornithine monooxygenase (OMO) are shown in light blue. **b** Reaction catalyzed by N^5-ornithine monooxygenase, a flavin adenine dinucleotide (FAD) and NADPH dependent enzyme

Such pathways were recently shown to be much more widespread than ever expected, having been identified in greater than 80 diverse bacteria (Challis 2005; Vetting et al. 2005). Alternatively, hydroxamate is generated by formation of an amide bond between the hydroxylated amine and a carboxylic acid via NRPS-dependent or –independent pathways, as in the biosynthesis of fusarinine-C (Fig. 2). Finally, hydroxamate can be formed by direct hydroxylation of an amide. Though chemically more difficult due to the high pK_a/weak nucleophilicity of the amide -NH, such a route has nonetheless been proposed for mycobactins (Fig. 1), where hydroxylation and acylation are believed to be final biosynthetic steps (Moody et al. 2004). Each of these biosynthetic routes involves an amine or amide hydroxylation step catalyzed by a flavin-dependent monooxygenase. Flavin monooxygenases consist of at least four families, each with distinct chemical mechanisms and primary structural features. The siderophore-associated monooxygenases (SMOs) constitute their own family. In higher eukaryotes, monooxygenation is a nonessential step in amine catabolism. Hence, SMOs may be a particularly clean antimicrobial target.

Other enzymatic targets

Once retrieved by the pathogen, the iron from siderophores must be released, either by reduction to Fe(II) or by cleavage of the siderophore backbone (e.g., by a specific esterase, vide supra). These enzymatic steps are so far unexplored targets. In the case of the presumed siderophore reductase, this is likely due to the still nebulous understanding of siderophore reductases even in otherwise well-characterized organisms. Many flavin reductases are known to reduce iron and iron-chelates somewhat non-specifically in vitro. These enzymes have been classified as so-called two-component systems, in which one enzyme reduces and releases flavin and a second retrieves it for reduction chemistry (Mazoch et al. 2004; Meyer and Halle 1992; Schroder et al. 2003). In the case of siderophores, the flavin reductase is believed to reduce and release flavin, which can react directly with chelated Fe(III). More recently, the [2Fe-2S]-cluster-containing *fhuF* gene product was isolated from *E. coli* and shown to reduce Fe(III) bound to ferrioxamine B. This enzyme is flavin-independent, and consequently represents an alternative to the two-component systems. FhuF production is upregulated via a Fur-binding promoter under conditions of iron stress (Matzanke et al. 2004). Under the same conditions, however, a *fhuF*-knockout was not growth restricted, suggesting that *E. coli* at least has additional means of mobilizing iron from the several siderophores it is known to use.

Non-enzymatic and non-siderophore targets

Iron trafficking systems have many essential, non-enzymatic components. Perhaps most obvious among these are the highly specific cell-surface siderophore and hemophore receptors. The siderophore/receptor interaction has already been exploited for "Trojan Horse" drug delivery (Roosenberg et al. 2000), in which an antibiotic or cytotoxic agent is chemically tethered to a siderophore and the complex is actively internalized by a specific pathogen. Targeting Fe uptake itself, a small molecule that specifically binds to and thereby blocks a siderophore or heme uptake receptor could be used to more simply block iron entry into cells. Such an approach might be expected to suffer from built-in redundancies in uptake pathways, such as the ability of an organism to use multiple siderophores, or from the ability of the organism to rapidly evolve improved receptors.

Finally, siderophore/hemophore-independent steps of the Fe-trafficking process could conceivably make

good antimicrobial targets, provided they are well chosen and as pathogen-specific as possible. For example, recent work has shown that metal trafficking may limit growth of pathogens which live inside mammalian macrophages (Wyllie et al. 2002). The natural resistance-associated macrophage protein (NRAMP) family consists of the transmembrane proteins Nramp1 and Nramp2. Nramp1 is expressed exclusively in cells of the immune system. It is recruited to the membrane of the phagosome as an invading pathogen is engulfed. Mutations in Nramp1 have long been known to genetically predispose an individual to certain diseases, including leprosy and tuberculosis. It was more recently shown that the Nramp proteins are divalent cation transporters, and that Nramp1 may be involved in moving phagosomal cations back to the cytoplasm for general use (Canonne-Hergaux et al. 1999; Huynh and Andrews 2008; Searle et al. 1998). By controlling divalent cation concentrations (Fe(II), Mn(II), and possibly Zn(II)), Nramp1 may also regulate the inter-phagosomal replication of bacteria. Interestingly, some phagosomal pathogens, including the protozoa (e.g., *Leishmania* sp.), depend on phagosomally available Fe sources but do not produce siderophores. These organisms may be particularly amenable to highly selective, chemical interference with their Fe metabolic pathways, provided that the phagosomal compartment can be successfully reached (Wilson et al. 2002).

Outlook

Though microbial iron metabolism has been much studied and even co-opted in order to effect drug uptake, direct assaults on iron trafficking are few. The multiple, sometimes redundant pathways used by microbes to ensure the success of iron acquisition make this a potentially challenging area for the development of therapeutic antimicrobials. A particular cause for concern is the likelihood that, in the event that one supply train is blocked, another will be activated, or that existing genes will evolve to somehow compensate. Such concerns are common to all antimicrobial efforts, however, and should not preclude further investigations. Initial studies targeting siderophore biosynthesis suggest this could be a promising future direction.

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