Bacterial iron uptake: a promising solution against multidrug resistant bacteria

S. Fardeau¹, C. Mullié¹, A. Dassonville-Klimpt¹, N. Audic², A. Sasaki¹,², P. Sonnet¹

1 - Laboratoire des Glucides, CNRS-UMR 6219, UFR de Pharmacie, Université de Picardie Jules Verne, 1 rue des Louvels, 80037 Amiens cedex 1, France
2 - Pharmamens, UFR de Pharmacie, 1 rue des Louvels, 80037 Amiens France

The emergence of nosocomial diseases driven by multidrug resistant bacteria is a major issue for our healthcare system. Mechanisms underlying multidrug resistance in these bacteria can be linked to the decreased permeability of bacterial membranes towards antibiotics, either by structural modifications inducing reduced passive diffusion, or by decreasing active uptake systems. Micro-organisms need iron which is present in low quantity in biological media for their development and absorb it via specific receptors or uptake mechanisms. Bacteria synthesize low-weight molecules called siderophores that strongly bind extracellular ferric iron and are recognized by membrane receptors and transported into the bacterial cytoplasm. In this paper, we propose to review the various siderophores and uptake complexes existing in Gram-negative bacteria, the naturally occurring molecules using a Trojan horse strategy via bacterial iron uptake systems to enter and kill bacteria and to make a state-of-the-art of the synthetic molecules so far developed using siderophore-like (iron-chelating) agents coupled with antibiotic molecules.

Keywords: siderophore; iron uptake; iron chelators;“Trojan Horse” strategy; antibiotic.

1. Introduction

Iron is abundant on Earth; it is the fourth most common element in the crust of the planet. Organisms have exploited this transition metal and its properties: its ability to loose or gain an electron allows iron to play a key role in the reduction-oxidation reactions. As a consequence, its presence can be found in various enzymes. Indeed, iron is involved in many key metabolic processes like Krebs cycle, oxidative phosphorylation or electron transport [1]. Soluble Fe(II) is oxidized to Fe(III) which forms ferric hydrate complexes resulting in low free Fe(III) concentration between 10⁻¹⁸-10⁻²⁴ mol/L [2]. Consequently, this free Fe(III) low concentration is below the required level to sustain bacterial life, which has been evaluated as at least 10⁻⁶ mol/L [3]. To survive in iron-deficient medium, bacteria have developed several solutions: (i) scavenge heme as an iron source, (ii) absorb and metabolize iron proteins (lactoferrin, ferritin) or (iii) develop specific receptors to assimilate ferrous ions [4]. Alternatively, they can synthesize low molecular weight molecules, called siderophores, possessing a high affinity for ferric iron and able to scavenge bound iron from their environment due to their superior binding strength. The ferri-siderophore complex must then cross the membrane before delivering iron within the cytoplasm. Throughout this review, we will give an overview of the chemical structure of siderophores, their recognition and transport, their roles in the intermicrobial fight and their future as therapeutic agents by coupling antibiotics to iron chelator siderophores analogues.

2. Chemical structure of siderophores

Siderophores form tight and stable complexes with ferric ion and most of them could be divided into three main classes, depending on the oxygen ligands for Fe(III) coordination, which are catecholates, hydroxamates or hydroxycarboxylates (Fig.1a). These ligands can be found, respectively, in aerobactin, ferrichrom and enterobactin siderophores which are synthetized by the corresponding bacterial strains (Fig.1b). In some of these compounds, the iron-chelating parts are linked together via a carbon backbone, allowing the siderophore to distort itself to reach an optimal configuration to chelate the metallic ion with a ferric iron-siderophore ratio of 1:1. Different iron-siderophore ratios can be found. For example, cepabactin, produced by Burkholderia cepacia, has a ferric iron-siderophore ratio of 1:3 whereas it is of 1:2 for pyochelin due to the assistance of the nitrogen atom of the dihydrothiazole ring (Fig. 1b).
3. Siderophore recognition and transport

Herein, we will focus on the Gram-negative siderophore recognition and transport. Gram-positive bacteria only possess a single membrane whereas Gram-negative bacteria possess an outer and an inner membrane to protect themselves from hazardous elements, like toxins or degrading enzymes. The presence of porins on the outer membrane allows low molecular weight molecules (below 500 Da) to bypass this barrier [5]. Siderophores, having higher molecular masses, have to be absorbed via a specific uptake system. This absorption system consists in an outer membrane receptor, a periplasmic binding protein and a cytoplasmic membrane protein belonging to the ATP-binding cassette transporter (ABC-transporter) superfamily. Once siderophores reach the cytoplasm, the ferric iron they carry is reduced and the iron-free siderophore degraded or recycled by excretion through an efflux pump system.

3.1 The Outer Membrane Receptor

More than 500 siderophores have been described, related to different kind of microorganisms, and the number is still growing [6]. This large diversity implies in each case a specific recognition by bacterial receptors on the outer bacterial membrane. The crystal structures of the receptors from enterobactin (FepA) [7], ferrichrom (FhuA) [8], citrate chelator (FecA) [9] and pyoverdin (FptA) [10] have been described.

The structures of outer membrane receptors (OMR) described in the literature display similarities: a transmembrane β-barrel domain and a N-terminal domain forming a plug that obstructs the barrel to prevent exogenous elements from entering the periplasm [11, 12]. The crystal structures of various outer membrane receptors also show similarities in their architectures: 10 periplasmic loops (with lengths ranging from 2 to 10 amino acid residues), a 22-β-strand barrel, orientated with an axis of 45° from the axis of the β-barrel, and 11 extracellular loops. Even if the β-barrels from different bacterial strains present different dimension (for instance, the dimension of FepA are 44 Å length, 30 Å width and 70 Å height while FhuA are 46 Å, 39 Å and 69 Å), they share common characteristics: the β-barrels extend above the lipid bilayer of the outer membrane and their structure is kept with hydrogen bonds and salt bridges between the different strands. The extracellular loops of the β-barrels, composed of 11 loops, represent nearly 50 % of the total structure and their length is about 30 to 40 Å above the outer membrane.

Two interaction models have been described for the recognition between the ferric-siderophore complex and the extracellular loops of the OMR. In the first, the OMR is directly loaded with an iron-bound chelator. Then, an extracellular loop closes the outer binding pocket formed by the OMR, preventing the release of the bound complex. Structures of FhuA with various siderophores have been described in the literature (ferrichrome [13], ferricrocin [8] phenylferricrocin and albomycin [14]). Interestingly, these various molecules share a similar hydroxamate-chelating part which interacts on the third and eleventh loops. The deletion of these loops negates the binding and transport of the chelators. Once the interactions are sufficient, the absorption of iron-bound chelators can proceed, even if their
structures are different. However, the closure mechanism of FhuA is still unknown. The second model follows the same pattern except for one point: an iron-free siderophore is already bound with the extracellular recognition site of the receptor. An iron-loaded siderophore substitutes its iron-free counterpart and, once the binding is achieved, a conformational change closes the extracellular part of the receptor upon the binding pocket. This mechanism has been described for FecA/citrate siderophore [15] and FpvA/pyoverdin [16]. Structural studies on FecA and citrate siderophore demonstrate the importance of the seventh and eighth loops in the binding and absorption of the ferric-citrate complex. Indeed, their deletions disable the ability of FecA to absorb citrate siderophores [17]. The ligand-free citrate also binds to the extracellular site but without inducing its closure [15]. The hypothetical mechanism of action implies the substitution of the ligand-free citrate by an iron-citrate complex, then the conformational changes leading to the absorption of the complex occur. The crystal-structure of FepA-enterobactin has not yet been elucidated [18]; some reports, via specific mutant bacteria, have underlined the role of specific loops [19] but none has done a breakthrough on this subject. Nevertheless, interesting experiments with synthetic tricatecholate-chelators designed to mimic enterobactin have been performed with *Escherichia coli* expressing FepA [19,20]. Iron-loaded chelators MECAM and TRENCA (Fig. 2), after their recognition by FepA as catechol siderophores, promoted the bacterial growth.

![Fig. 2. Synthetic enterobactin analogues.](image_url)

The *N*-terminal domain of the OMR is referred to as the plug domain (also termed as the cork or hatch domain, Fig. 3). It obstructs the lumen of the OMR and consists of four-stranded β-sheet with loops and helices around it. Its position is kept into the receptor by 40 to 70 hydrogen bonds and two salt bridges between a glutamic acid residue from the β-barrel and an arginine residue from the cork. Two characteristic segments of the plug deserve further description. First, the apices on its top interact with the iron-siderophore complex, as seen with FhuA and ferrichrome [8] and with FecA and ferric-citrate [15]. However, experiments with the inversion between the plug domain of FepA and FhuA have shown no difference in the absorption of ferric-enterobactin and ferrichrome, respectively [21]. These results suggest a lesser importance of the substrate-plug domain recognition in the absorption process, as compared with the extracellular recognition. The plug domain also presents a short amino acid sequence that allows the iron-siderophore complex to reach the periplasm. Indeed, this linear portion of the plug domain, called TonB-box, interacts with TonB. The complex TonB-TonB-box has been described in the literature for various bacterial species [22-24].

In bacteria, TonB proteins interact with OMR proteins that carry out high-affinity binding and energy-dependent uptake of specific substrates into the periplasmic space (Fig. 3). In the absence of TonB, these receptors bind their substrates but substrates do not reach the periplasmic space. Hence, in the case of iron-siderophore complexes, TonB, along with two other proteins, ExbB and ExbD, implements the transport of the chelator from the OMR to the periplasmic space [25,26].
However, its exact role is still unknown, only hypotheses have been formulated [27,28]. The mechanism of action of TonB therefore remains a matter of discussion. At present, four different models have been proposed:

- (i) the shuttle model implies a difference of potential energy. First TonB, in an unenergized state, is charged and put in a different conformational state by the ExB/ExD system with the proton motive force of the membrane [29-35]. Then, TonB binds the OMR and releases its stored potential energy. A conformational change subsequently happens in the plug domain of the receptor, leading to the transfer of the substrate into the periplasm. This model was proposed when first studies underlined that TonB interacted with both the outer membrane and the cytoplasmic membrane [36]. However, the structural conformation of TonB in the two states of the process has not yet been described.

- (ii) the pulling model was proposed in likeness of proteins such as protein G and ubiquitin [37, 38]. Recently, molecular dynamics simulations have shown in silico that the pulling of the plug domain by TonB may happen [39] and thus would allow the substrate to move in the periplasm. However, this model has no in vivo experimental substantiation.

- (iii) the assisted model follows recent data obtained with the ferrichrom uptake system. Indeed, several experiments (phage display, fluorescence spectroscopy) hinted that the periplasmic binding protein FhuD and TonB could form a 1:1 complex [40]. TonB would subsequently present FhuD to the FhuA OMR and also interact with the receptor to induce a conformational change in the plug domain. FhuD would then bind to the iron-siderophore complex. The last step would see the complex siderophore-FhuD being brought to its inner membrane transporter FhuBC by TonB.

- (iv) the propeller model was proposed on two bases: the first crystal structure of the C-terminal domain of TonB showed a dimeric structure which was seen as a propeller [41] and the similarity between ExbB/ExbD with the flagellar motor proteins MotA/MotB [42]. The TonB and the TonB-box interact together and a rotation is generated by ExbB/ExbD using the proton motive force of the cytoplasmic membrane. This induces the passage of the siderophore into the periplasmic space, either by a conformational change or by the removal of the plug domain. This model faces two problems: first, an anchor has to exist to fix ExbB/ExbD during the movement but is still unknown and second, recent structural works on TonB present the protein as a monomer and not a dimer, which is a requirement for this model [43-46].
3.2 Periplasmic Binding Proteins (PBP) and ATP-binding cassette transporter

PBPs are a large class of bacterial proteins bringing different molecules from the OMR to their cytoplasmic membrane transporters. Their number is still increasing with time. A structure-based classification resulted in 8 different clusters [47]. Ferric-siderophore complexes binding proteins are included in cluster 8, along with vitamin B₁₂ binding proteins. Little work has been published concerning the exact structures of the siderophore-binding proteins; most publications report on the structure of *E. coli* FhuD, the ferric-hydroxamate binding protein, and its interactions with various hydroxamate chelators [48]. The complex iron-siderophore-PBP is transported across the inner membrane by an ATP-binding cassette transporter (ABC transporter) to deliver the loaded iron chelator to the bacterial cytoplasm. Up to now, five ABC transporter subtypes are known to be involved in the energy transport of the Fe-siderophore uptake in Gram-negative bacteria [49]. In the cytoplasm, two mechanisms are implied in the iron release from siderophores: (i) ferrisiderophore hydrolysis or (ii) Fe(III)-siderophore reduction to Fe(II)-siderophore by an iron reductase. In the case of enterobactin, bacteria have to hydrolyze its cyclic backbone with a specific esterase called Fes [50]. The iron reduction follows the hydrolysis of the ester function; enterobactin can thus chelate a ferric iron and transport it only once. Iron-siderophore reduction is the main process since Fe(II) is probably the preferred form of iron used by bacteria due to its better solubility as compared to Fe(III). The ferri-siderophore reductases have been identified in various bacterial species (for instance in *E. coli* [50], *Pseudomonas aeruginosa* [51] and *Legionella pneumophila* [52]). The reduction reaction, using NADH or NADPH, proceeds as follows:

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\text{Fe(II)-Siderophore} + \text{NAD(P)}^+ \rightarrow \text{Fe(II)-Siderophore} + \text{NAD(P)}^+ + \text{H}^+
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The ion Fe(II) is then removed from the siderophore and immediately assimilated by apoproteins. Interestingly, *E. coli* reductase shows an ubiquitous ability, reducing a wide array of ferri-siderophores, even those synthesized by other bacterial strains [50]. The substrates tested were aerobactin, ferrichrome arthrobactin, various hydroxamate siderophores and ferric dicitrates. More surprisingly, the synthetic analogues of enterobactin, MECAM and MECAMS (Fig 2.), were also reduced. After the enzymatic reductions of Fe(III)-siderophores, the iron-free siderophores are excreted from the bacteria, across the membranes, to start a new cycle. The export mechanism is still poorly understood but the intervention of an efflux pump (EP) system is believed to be crucial. Such transport proteins are involved in the extrusion of metabolites or toxic substrates (such as antibiotics) from the bacteria to the external environment. EPs may be specific for one substrate or may transport a range of structurally dissimilar compounds. To the best of our knowledge, only two siderophore excretion systems have been reported in the literature. The first characterized is the enterobactin export system in *E. coli*. (a membrane protein called EntS) that transports the free siderophore to the periplasm [53] and then to the outer membrane via the outer membrane channel tunnel protein TolC [54]. The second exports pyoverdin, a specific siderophore from *P. aeruginosa*, and implies PvdRT-OpmQ EP [55]. Mutant bacteria with dysfunctional PvdRT-OpmQ EP were shown to accumulate pyoverdin in their periplasm.

4. The role of siderophores in the intermicrobial fight

In optimal conditions (temperature, abundant presence of nutrients, no competition with other bacterial strains), bacteria can grow and divide extremely rapidly. But in biologic media, due to the presence of few nutrients, the competition for life is hard and bacteria have evolved various strategies to survive. In biologic media, iron is strongly bound to proteins like lactoferrin. To scavenge the iron from these proteins, bacteria can use: (i) their own specific siderophores such as pyoverdin and pyochelin for *P. aeruginosa*, or (ii) other siderophores that have been synthesized by other bacterial strains. In the latter case, bacteria have developed new receptors on their outer membrane to absorb the siderophore. For example, enterobactin is synthesized by *E. coli* [56], *Salmonella typhimurium* [57], *Klebsiella pneumonia* [58] and various enterobacteriaceae. *P. aeruginosa*, in the presence of iron-enterobactin complexes, is able to generate new siderophore uptake systems in order to absorb these complexes [59]. This opportunistic strategy enables, for a given bacterial strain, the use of different kinds of siderophores from various other bacterial strains without the necessity to synthesize them. Therefore, in the intermicrobial fight, bacteria bring two major answers to enhance their chances of survival: (i) synthesize specific siderophores to avoid the use of their own siderophores by other bacterial strains and (ii) excrete toxic molecules coupled to iron-chelating agents to kill other bacterial strains able to absorb these agents; this solution is called the “Trojan Horse” strategy.

The first solution implies specific siderophore recognition by OMRs of the producing bacteria. As previously shown, siderophores have specific receptors and uptake systems according to the chelating function used. However, if a more specific chelating fragments or a different iron-chelator ratio is implied, the absorption has to be done by a specific receptor. *P. aeruginosa* is able to produce pyoverdin and pyochelin, two specific siderophores that cannot be synthesized or used by other bacterial strains. Another possibility is the addition of a structural modification on the siderophore, subsequently followed by a change in the uptake system, to obtain a specific siderophore. For example, since enterobactin can be used by several other species, *Salmonella enterica* and *Escherichia coli* synthesize salmochelin, an enterobactin analogue, by addition of a β-glucosyl moiety catalyzed by the IroB enzyme [60, 61].
Salmochelin is no longer a ligand for FepA but is specifically recognized by the specific OMR IroN [62]. Moreover, like FepA, IroN also recognizes other catecholate chelators.

The second solution is the “Trojan Horse” strategy (Fig. 4). Indeed, the uptake system of iron-siderophore complexes is efficient to absorb large molecules which would not pass the bacterial membranes otherwise. Bacteria can excrete different kind of molecules, called bacteriocins, which are toxic to other bacterial strains. Some bacteriocins, such as sideromycins or microcins, possess a siderophore covalently linked to an antibiotic moiety. This association enhances the antibiotic penetration via the siderophore OMR. [63]

Among sideromycins produced by Streptomyces spp. or Actinomyces spp., albomycin is the more thoroughly described (Fig. 5). Albomycin shows, like ferrichrome in Fig.1, three hydroxamate functions linked on a peptidic backbone. The interactions with OMR FhuA and the crystal structure of bounded FhuA-albomycin have been reported [15]. Albomycin is transported by FhuD to the ABC transporter FhuB-FhuC [64]. Inside the bacteria, the peptidase N (pepN) cleaves the linker and the chelator moiety is excreted. The seryl thioribosyl pyrimidin antibiotic fragment stays inside the cell and inhibits the transcription of RNA by acting on the seryl-t-RNA synthetase [65]. Without the action of pepN, albomycin presents no inhibitory activity toward seryl-t-RNA synthetase [66, 67].

Microcins are toxic peptides, with a molecular weight below 10 kDa and a high pH stability, secreted by enterobacteria (mostly Escherichia coli) [68]. Produced under nutrient depletion conditions, they show potent antibacterial activity against bacteria closely related to E. coli, with minimum inhibitory concentrations (MICs) in the nanomolar range. They are recognized by OMR as siderophores. For example, MccJ25, a 21-residue peptide, is absorbed by bacteria through the FhuA receptor [69]. MccE492 is a linear trimer of N-(2,3-dihydroxybenzoyl)-L-serine (Fig. 5) linked to a 84-residue peptide through a D-glucose moiety [70]. Its siderophore moiety, structurally close to enterobactin, seems to be recognized by the OMR FepA.

Fig. 4. Schematic representation of the “Trojan Horse” strategy.

Fig. 5. Structures of albomycin and microcin E492 (the iron-chelator part is pale-gray, the linker is black and the bacteriostatic agent is dark gray).
Since Gram-negative bacteria have developed strategies to export antibiotics through their outer membrane, the coupling of antibiotics with small synthetic iron chelators as siderophore analogues seems to be a very interesting way to target infections caused by bacteria that are resistant through a decrease in antibiotic accumulation. Such bacteria reduce the drug accumulation in their cytoplasm by modifying their membrane (and so their permeability) [71], and/or by overexpressing their efflux systems, expelling antibiotic molecules outside the bacteria [72].

5. The use of bacterial iron uptake as a therapeutic approach

To obtain an efficient antibiotic, the siderophore receptors must recognize and transport the entire molecule (siderophore-linker-antibiotic) into the cell. The choice of the iron-chelator part is crucial. It should ideally be recognized by the greatest number of bacteria and the more important pathogens such as P. aeruginosa. In a first attempt, natural siderophores coupled to antibiotics have been used in proof of concept for this strategy such as the pyochelin-norfloxacin conjugates that presented antimicrobial activities against P. aeruginosa [73]. The use of siderophores with inversed chirality centers, by use of D amino acids instead of L, has shown interesting results [74]. For example, the enantio-enterobactin (Fig. 6) keeps the same constant of association for iron and is well recognized and absorbed by the OMR. But, after reaching the cytoplasm, the ester-backbone is not recognized by hydrolyzing enzymes and the iron not freed in the cytoplasm. However, siderophores are natural products that are isolated from bacteria with low yields. This is a serious limitation for further therapeutic developments. That is why, in following attempts, the use of synthetic iron chelators as siderophore analogues was proposed to overcome the problems of siderophore production and specific recognition.

Various iron chelators have been developed by pharmaceutical firms: they are mainly used in treatment of haemochromatosis (or iron overload). Three molecules are currently used (Fig. 6): the bidentate chelator deferiprone, the tridentate chelator deferasirox and the hexadentate chelator deferoxamine. These molecules are currently used and well-supported by the human body. Therefore, they should not be harmful to our organism. It must be noticed that OMRs only recognize specific chelating functions as shown in Fig. 1. Experiments have demonstrated that, in the presence of these synthetic iron-chelators, a bacterial growth inhibition has been shown, supposedly driven by the iron sequestration from the biological medium [75, 76]. Synthetic iron chelators MECAM and TRENCA are well absorbed by different strains of bacteria [19, 20]. Nevertheless, the resulting bacterial growth is weaker as compared to the action of the enterobactin. The synthetic catechol-chelators are interesting siderophore analogues, well absorbed by bacteria through the Fep uptake system. The CacCAM [77] can be covalently linked to antibiotics via its carboxylic acid function (Fig. 6). Currently, several research teams like ours are still at work to identify new iron chelators that could act as siderophore analogues. These chelators must ideally be accessible by an efficient synthesis which would authorize a large scale production.

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Fig. 6. Synthetic iron chelators.
The second aspect of the “Trojan Horse” strategy concerns the choice of the antibiotic moiety, covalently fixed to the iron chelator by a linker. The choice of an antibiotic with a broad-spectrum of action is also important to have biologic activity toward a maximum of bacterial strains. The examples available in the literature mainly deal with two therapeutic families: quinolones and β-lactams. The next step in a rational drug design is to add the iron-chelating fragment without blocking the antibiotic activity. Indeed, if, because of the iron chelator presence, the interaction between the antibiotic and its target is limited as compared to that of the free antibiotic, the antibacterial activity will also be reduced or lost. So, it is crucial to link the iron chelator to a biologically inactive part of the antibiotic. β-lactams act through impeding the bacterial cell-wall building. Therefore, they only need to be transported as far as the periplasmic space of bacteria to be effective. β-lactams, linked to iron chelators such as catechols or hydroxamic acids, have been evaluated on various bacterial strains [78-80]. It has been shown that adding other chemical elements to the β-lactam core do not affect the antibiotic action. For example, the β-lactam-iron chelator compound A (Fig. 7) presents a better antibacterial activity on Gram negative bacteria than the antibiotic alone.

Because of their broad spectrum of action toward bacteria, quinolones are another interesting antibiotic family. They interfere with DNA replication by inhibiting key enzymes gyrases and topoisomerases. These enzymes are located in the bacterial cytoplasm. Therefore, to be effective, the antibiotic-linker-iron chelator complex needs to reach the cytoplasm and release its antibiotic moiety in it. With this objective, different quinolone-chelator conjugates have been synthetized such as the chelator (hydroxyl carboxylic acid)-fluoroquinolone B which possesses interesting antibacterial activities on Gram-negative bacteria [81]. The coupling of β-lactam or fluoroquinolone antibiotics to the desferridanoxamine (C) has shown better antibiotic activities in comparison with the corresponding drugs alone [82]. With regard to the biological activities, the linker is also important as it has been demonstrated with the pyochelin-ciprofloxacin conjugates D1 and D2. When the linker is a peptide-bond (D1), no antibacterial activity is observed. When a labile linker like an ester (cleavable by cytoplasmic esterases) in compound D2 is used, an antibacterial action is shown [83]. Consequently, a strong linker, like a peptide bond or a carbon chain, can inhibit the therapeutic activity.
6. Conclusion

The use of bacterial iron uptake as therapeutic approach is promising but much remains to be learned due to the few available examples in the literature of antibiotic-siderophore conjugates. The nature of the iron chelator is crucial for the Gram-negative bacteria siderophore recognition and transport of the conjugate. In this “Trojan Horse” strategy, the nature of the antibiotic (cytoplasmic target or not) and the linker are also important. A better understanding of OMR structures and of their mechanism of action is still an interesting challenge.

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References


