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REVIEW

IRON METABOLISM IN THE CELL AS A TARGET IN THE DEVELOPMENT OF POTENTIAL ANTIMICROBIAL AND ANTIVIRAL AGENTS

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The search and creation of innovative antimicrobial drugs, acting against resistant and multiresistant strains of bacteria and fungi, are one of the most important tasks of modern bioorganic chemistry and pharmaceuticals. Since iron is essential for the vital activity of almost all organisms, including mammals and bacteria, the proteins involved in its metabolism can serve as potential targets in the development of new promising antimicrobial agents. Such targets include endogenous mammalian biomolecules, heme oxygenases, siderophores, protein 24p3, as well as bacterial heme oxygenases and siderophores. Other proteins that are responsible for the delivery of iron to cells and its balance between bacteria and the host organism also attract certain particular interest. The review summarizes data on the development of inhibitors and inducers (activators) of heme oxygenases, selective for mammals and bacteria, and considers the characteristic features of their mechanisms of action and structure. Based on the reviewed literature data, it was concluded that the use of hemin, the most powerful hemooxygenase inducer, and its derivatives as potential antimicrobial and antiviral agents, in particular against COVID-19 and other dangerous infections, would be a promising approach. In this case, an important role is attributed to the products of hemin degradation formed by heme oxygenases *in vitro* and *in vivo*. Certain attention has been paid to the data on the antimicrobial action of iron-free protoporphyrinates, namely complexes with Co, Ga, Zn, Mn, their advantages and disadvantages compared to hemin. Modification of the well-known antibiotic ceftazidime with a siderophore molecule increased its effectiveness against resistant bacteria.

Key words: iron; heme oxygenase; siderophores; antibacterial and antiviral activity; hemin; protein 24p3

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INTRODUCTION

The wide distribution of resistant and multi-resistant bacterial and fungal strains stimulates a search and creation of innovative antimicrobial drugs with original mechanisms of action.

Iron plays an important role in the life of almost all living organisms, including mammals and bacteria [1–3]. This suggests that iron metabolism in the cells may be considered as an attractive target for the development of new antimicrobial agents with new mechanisms of action.

Iron metabolism in mammals involves such agents as heme oxygenases, siderophores, protein 24p3, while bacteria have their own specific heme oxygenases and siderophores. The creation of inhibitors and inducers of heme oxygenases, selective for mammals and bacteria, may become one of the promising directions for the design of new antimicrobial agents.

In this regard, hemin attracts special attention. It is the most powerful inducer of heme oxygenases; hemin derivatives and products of its degradation can be used to achieve this goal [4]. In this regard, metal complexes of protoporphyrinates that do not contain iron are of particular interest. Another promising direction developed by many researchers in this field is the modification of molecules of known antibiotics by siderophores (the "Trojan horse" strategy). Thus, such a covalent modification of the antibiotic ceftazidime made it possible to increase its effectiveness against resistant bacteria [5].

This review summarizes literature data on the development of inhibitors and inducers (activators) that are selective for mammalian and bacterial heme oxygenases.

1. PROTEIN 24p3

The 24p3 protein is a member of the lipocalin family of proteins that bind various hydrophobic molecules (steroids, etc.). The 24p3 protein is a small (≈60 kDa) proteinase resistant glycoprotein [6]. It is present in the intercellular space of neutrophils, hepatocytes, lung cells, bone marrow, adipose tissue, macrophages, thymus, ducts of healthy mammary glands, prostate, and kidneys [7]. The 24p3 protein does not interact directly with iron ions, but binds it as a part of a low molecular weight compound, iron chelate, with the so-called siderophore 2,5-dihydroxybenzoic acid (Fig. 1) [8].

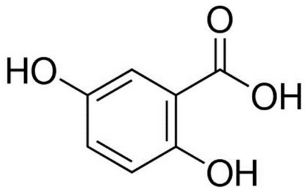


Figure 1. Structural formula of 2,5-dihydroxybenzoic acid.

1.1. Mammalian Siderophores

Siderophores are low molecular weight, high affinity iron chelating compounds secreted by the cells of living organisms to deliver iron ions Fe^{3+} to them.

In mammalian cells enzymatic synthesis of 2,5-dihydroxybenzoic acid involves 3-hydroxybutyrate dehydrogenase (BDH2). Enzymes with a function similar to BDH2 have been also found in yeast, zebrafish, and bacteria [9].

In cultured mammalian cells, as well as in yeast cells and zebrafish embryos, a deficiency of 2,5-dihydroxybenzoic acid leads to an abnormal accumulation of cytoplasmic iron and a deficiency of mitochondrial iron; this is associated with a decrease in iron transport from the soluble part of the cell to mitochondria [9]. In *in vitro* experiments, siderophore-deficient cells showed elevated levels of reactive oxygen species (ROS). Such cells were more sensitive to oxidative stress than normal cells. In *in vivo* experiments, mice unable to synthesize siderophores had abnormal accumulation of iron in the spleen and, to a lesser extent, in the liver [10]. Feeding such mice with food containing large amounts of iron led to their death. On the other hand, feeding such mice with iron-deficient food resulted in severe anemia [10].

The regulation of the iron content in the studied mammalian cells (rat, mouse, dog kidney cells, as well as human kidney tumor cells) occurs as follows [11]. The 24p3 protein, containing a complex of iron ions with a siderophore, binds to the 24p3R receptor on the surface of mammalian cells, penetrates into the cell, and releases iron ions bound to the 24p3 protein. This leads to an increase in the concentration of cytoplasmic iron necessary for mammalian cells. The 24p3 protein, which does not contain iron, interacts with the surface receptor 24p3R on the membrane, penetrates into the cell and, after binding to the intracellular siderophore, forms a complex with iron and transfers it to the extracellular environment, thereby reducing the concentration of intracellular iron in mammalian cells [11] (Fig. 2).

In addition, "the fight against bacterial infections in mammals", is accompanied by a decrease in the amount of circulating 2,5-dihydroxybenzoic acid, as well as its complexes with iron; this reduces the supply of iron ions, entering bacterial cells [12].

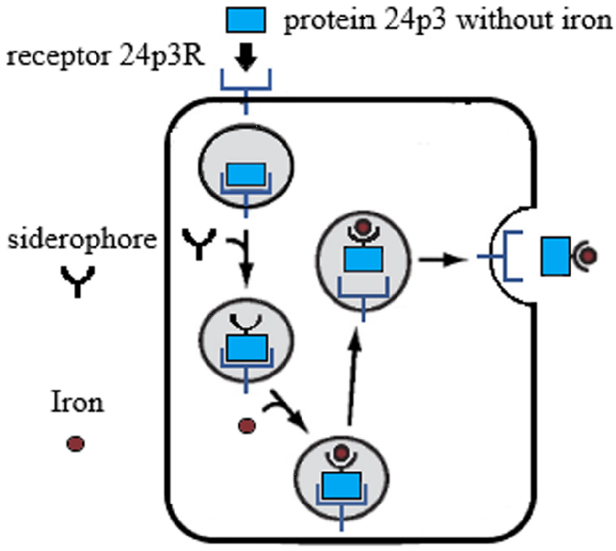


Figure 2. Schematic representation of the release of iron from mammalian cells by the 24p3 protein. Explanations are given in the text (adapted from [11]).

2. BACTERIAL SIDEROPHORES

The main function of siderophores in bacteria, yeasts, and fungi (more than 500 species in total) is to capture iron bound to host proteins and deliver iron to the bacterial cell. Due to the high ability of bacterial siderophores to bind to iron (binding constant 10^{22}–10^{50} mol/l), they remove iron from proteins such as transferrin, lactoferrin, but do not remove iron from heme-containing proteins [2]. In addition, bacterial siderophores are capable of forming complexes with water-insoluble ferric salts [2]. Most microorganisms synthesize at least one siderophore [13]. Depending on the chemical structure, bacterial siderophores can be divided into five classes: catecholates and phenolates ("aryl caps"), hydroxamates (α-hydroxycarboxylic acids), carboxylates (dicarboxylic and tricarboxylic acids), and mixed-type siderophores. For example, *Yersinia* strains (a causative agent of bubonic plague) secrete a siderophore, yersiniabactin (Fig. 3). Under conditions of iron deficiency the bacterium *Pseudomonas aeruginosa* secretes the siderophores pyoverdin, ornibactin, cepabactin (Fig. 3), and pyochelin [2]. The main siderophore is pyoverdin. Under conditions of iron deficiency, *P. aeruginosa* is also able to secrete salicylic acid. However, the authors of [14] do not consider it as a siderophore, because salicylic acid has a low binding constant to Fe^{3+} at physiological pH.

The mechanisms responsible for absorption of complexes of bacterial siderophores with iron differ in Gram-positive and Gram-negative bacteria. The main difference is that Gram-positive bacteria do not have an outer membrane through which complexes of siderophores with iron must move. Gram-negative bacteria have receptors on their outer membrane that can recognize the siderophore-iron

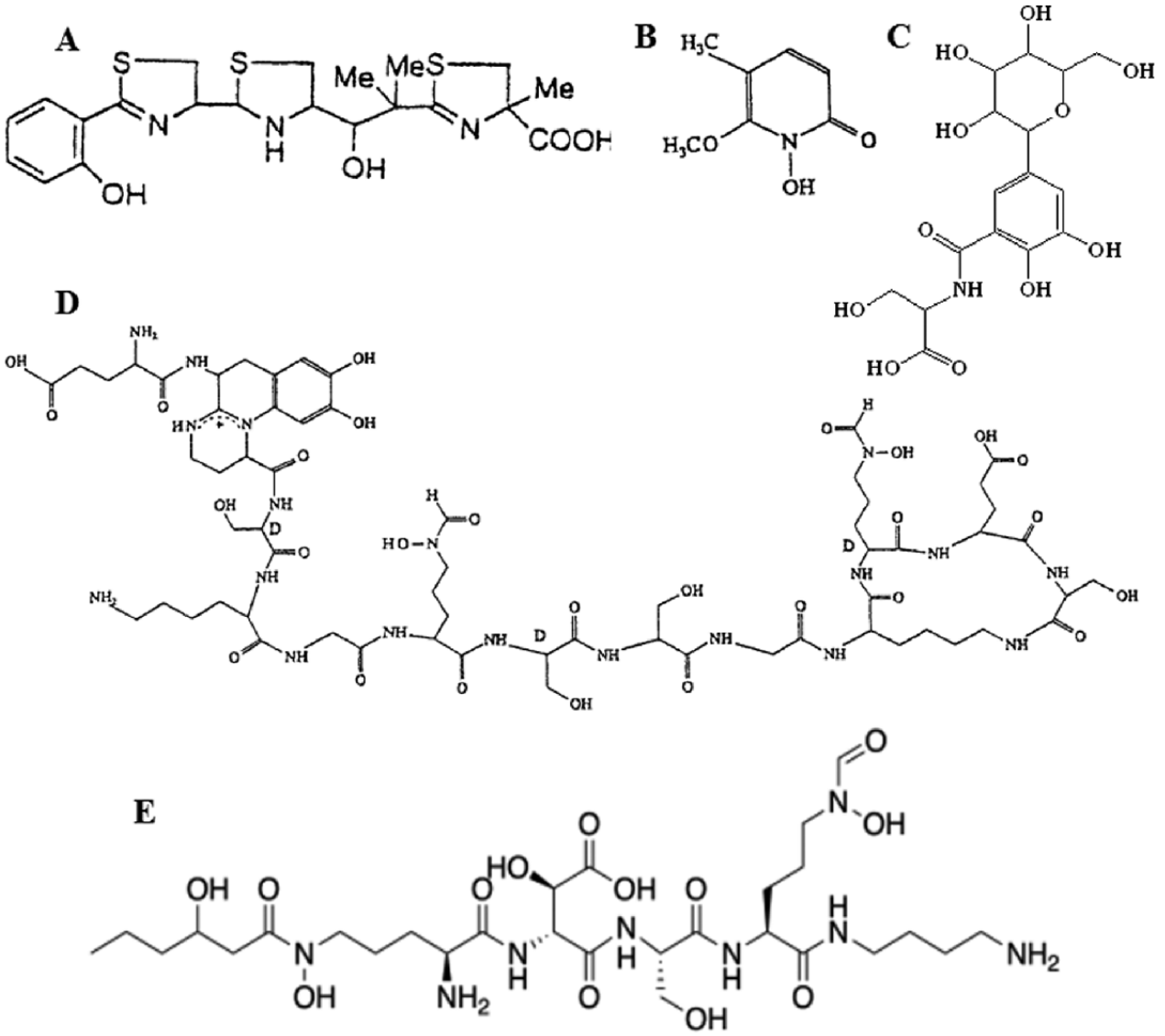


Figure 3. Structural formulas of siderophore yersiniabactin (A), cepabactin (B), salmochelin (C), pyoverdin (D), ornibactin (E).

complex. After binding of the complex to the receptor, the latter undergoes a conformational change, and the siderophore-iron complex penetrates into the periplasmic space and then into the cytosol. In the cytosol, iron is reduced and then binds to ATP-binding transport proteins of the ABC family on the cytoplasmic membrane (Fig. 4).

Since Gram-positive bacteria do not have an outer membrane, siderophores are imported directly into the cell using a membrane-located binding protein and ATP-binding transport proteins of the ABC family. The further fate of siderophores after the release of iron is not well understood. In some cases, siderophores can be reused or hydrolyzed to release iron [16, 17]. For example, in *P. aeruginosa*, the release of iron from the complex with pyoverdin occurs without chemical degradation of the siderophore. After the release of iron, the latter moves into the extracellular environment and is able to capture new iron ions. On the contrary, the separation of the enterobactin iron complex in *Salmonella* and *Escherichia coli* bacteria is carried out via hydrolysis [17].

3. THE ANTIBACTERIAL ACTIVITY OF PROTEIN 24p3

The iron-binding part of the bacterial siderophore enterobactin contains 2,3-dihydroxybenzoic acid (Fig. 5), which is structurally related to the mammalian siderophore 2,5-dihydroxybenzoic acid [9]. This structural similarity obviously explains that the 24p3 protein is able to capture not only the mammalian siderophore, but also the siderophores of bacterial cells.

Binding to bacterial siderophores in the intercellular space, the mammalian protein 24p3 prevents the entry of iron ions into bacterial cells, thereby exhibiting bacteriostatic activity. At the same time,

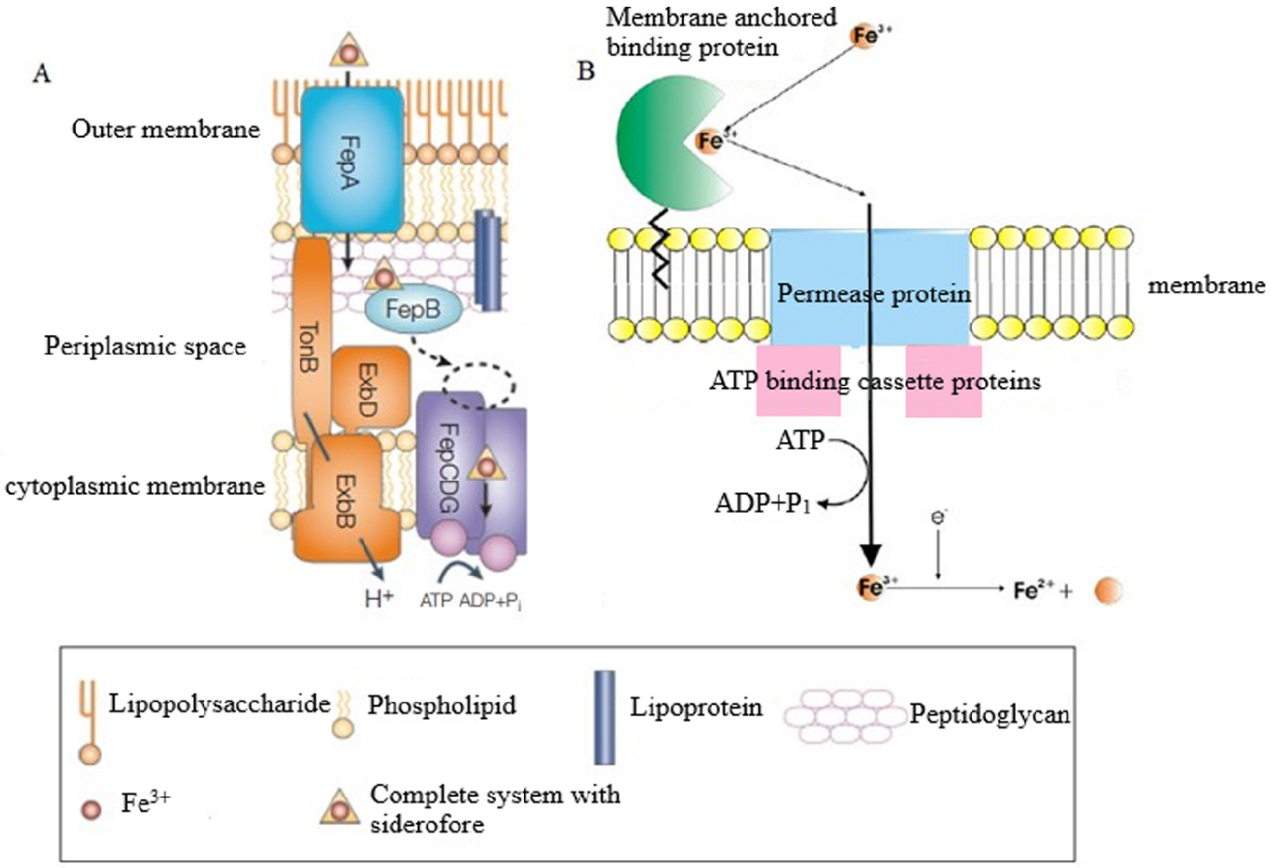


Figure 4. The uptake system of the iron-siderophore complex in Gram-negative (**A**) and Gram-positive (**B**) bacteria. Surface receptor proteins (FepA) are in blue, periplasmic proteins (FepB) are shown in cyan, ATP-gated transporters (FepCDG) are in purple, TonB, ExbD are membrane-anchored periplasmic proteins, ExbB is an integral membrane protein (adapted from [15, 16]). The color version of this figure is available in the electronic version of this article.

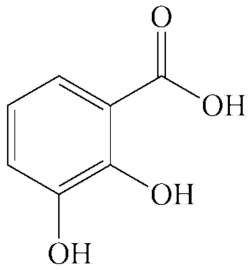


Figure 5. Structural formula of 2,3-dihydroxybenzoic acid.

there is no information in the literature about the possibility of penetration of the 24p3 protein into bacterial cells [18].

24p3 binds to the bacterial siderophore enterobactin, which exists in both iron-bound and free forms. The resultant complex of iron-bound enterobactin with 24p3 is then transported to mammalian cells, in which iron is stored. In contrast to the first scenario, the complex of iron-free enterobactin with the 24p3 causes intensive release of the pro-inflammatory cytokine interleukin-8 (IL-8) by epithelial cells [19], which attracts neutrophils. *In vivo* neutrophils play an important role in protecting mice from the development of bacteremia and sepsis caused by *Klebsiella pneumoniae* [20].

Interestingly, during mutagenesis, Gram-negative bacterial strains, including strains of *E. coli*, *K. pneumoniae*, etc., as well as Gram-positive *Staphylococcus aureus*, released modified siderophores (for example, yersiniabactin and salmochelin (Fig. 3)), which were not captured by the 24p3 protein [19].

Addition of 5 μM 24p3 to iron-deficient *E. coli* cells caused a 20-fold inhibition of cell growth. Administration of additional amounts of Fe^{3+} iron ions to the medium resulted in the loss of the ability to bind additional amounts of iron ions by 24p3 and this contributed to restoration of cell growth [8]. According to the authors of [8], 24p3 lacks its own antibacterial activity, and the decrease in the bacterial cell growth in its presence is associated with a decrease in the amount of iron available for bacteria in the culture.

The minimum inhibitory concentration (MIC) of the 24p3 exposed to *E. coli*, *S. aureus*, *Staphylococcus epidermidis*, *P. aeruginosa*, *K. pneumoniae*, *Enterococcus faecalis*, *Proteus mirabilis* cells varied in the range of 6.5–12.5 ng/ml [21]. The antibacterial activity of this protein was confirmed during incubation with platelets, which could be used to prevent infection during storage of the platelet concentrate [21].

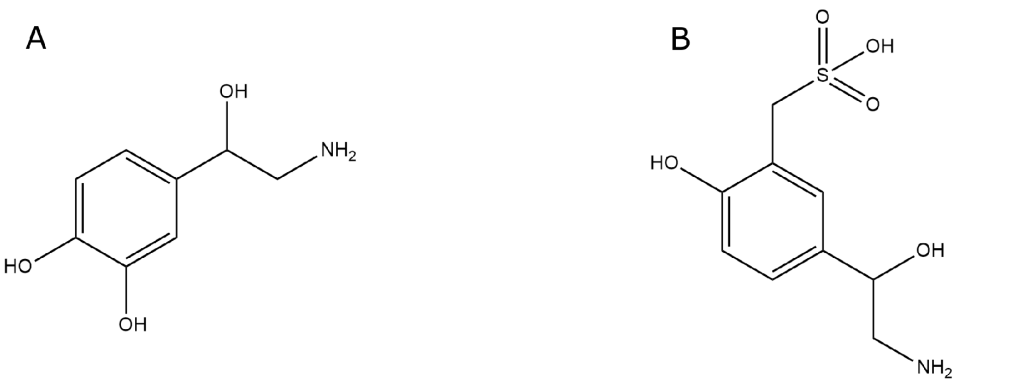


Figure 6. Structures of norepinephrine (**A**) and norepinephrine-3-O-sulfate (**B**). Although norepinephrine and norepinephrine-3-O-sulfate are not yet considered as bacterial siderophores, their iron uptake has been identified as another property of these molecules.

Interestingly, ε-polylysine inhibits the growth of *S. aureus*, *Bacillus cereus*, and *Klebsiella oxytoca* in platelet concentrates at significantly higher concentrations as compared to the 24p3 protein: from 50 μg/ml to 200 μg/ml [22].

In the model of infection of a monolayer culture of IPEC-1 epithelial cells with *E. coli K88* cells, the endogenous 24p3 protein secreted by IPEC-1 epithelial cells was insufficient to suppress the bacterial cell growth [23]. Administration of additional amounts of 24p3 to cells (0.1 μg/ml, 0.5 μg/ml, and 1 μg/ml) caused a dose-dependent inhibition of *E. coli K88* cell growth, was observed.

It is important to note that additional daily administration of 24p3 to mice at a dose of 150 ng/g i.p. for 16 weeks did not cause appearance of side effects [24].

4. ALTERNATIVE SOURCES OF IRON UPTAKE BY BACTERIA

Bacterial cells can capture not only their own iron-containing siderophores, but also mammalian siderophores, 2,5-dihydroxybenzoic acid [12, 25], as well as neuroendocrine catecholamines, such as norepinephrine, adrenaline, and dopamine (Fig. 6). The structures of these compounds are similar to the structures of bacterial siderophores [26].

In vitro experiments have shown [27] that addition of 50 μM norepinephrine and 30% bovine serum (BSA) to the nutrient medium with bacteria promoted bacterial growth. The addition of BSA alone to the nutrient medium contributed to a decrease in the bacterial growth to 10^5 cells/ml, while addition of norepinephrine alone had no noticeable effect on the bacterial growth (Fig. 7). Analysis of the BSA fraction revealed that, in addition to albumin, it also contained transferrin, which, apparently, bound iron ions, thus preventing iron uptake by bacterial cells and exerting a bacteriostatic effect. Norepinephrine, in turn, is able to bind iron from the complex with transferrin and transfer it to bacterial cells.

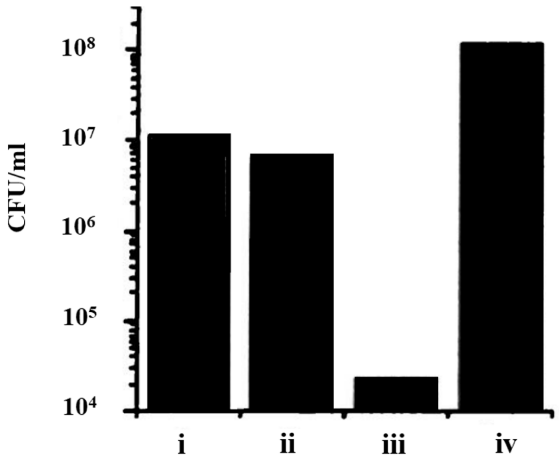


Figure 7. Effect of bovine serum on the growth of *E. coli E2348/69* bacteria after 18 h. **i** – control group without additives; **ii** – addition of 50 μM norepinephrine to the nutrient medium; **iii** – addition of 30% bovine serum to the nutrient medium; **iv** – addition of 50 μM norepinephrine and 30% bovine serum to the nutrient medium (adapted from [27]).

The ability of bacterial cells to capture complexes of norepinephrine with iron, may be attributed to the similarity of its structure with the structures of bacterial siderophores. It should be noted that, in contrast to norepinephrine, the pharmacologically inactive metabolite norepinephrine-3-O-sulfate (Fig. 6) is unable to bind iron from the complex with transferrin and thus support the growth of bacterial cells [27].

Since norepinephrine and other neuroendocrine catecholamines are present in the blood of all mammals, an increase in their content during stressful and disease states can increase bacterial growth. For example, severe tissue damage in patients with trauma or burns causes a massive systemic release of norepinephrine [28]. Administration of 4 mg or 40 mg of noradrenaline to mice a day before infection with the bacterium *Salmonella enterica Typhimurium ATCC14028*, increased the number of CFU of bacteria in the liver by 2.6 times and 3.5 times and in the caecum by more than 10 times and 20 times, respectively [29].

Other systems for iron uptake by bacteria also exist. For example, some *Salmonella* strains can use citrate ferric as a source of iron ions [2]. Iron ions can also be delivered to bacterial cells by endogenous α-keto/α-hydroxy acids [30]. Addition of pyoverdinic, α-ketoglutaric, α-ketoisocaproic, α-hydroxyisovaleric and other acids to the iron-deficient medium led to a slight increase in the growth of *Salmonella* strains, which were unable to secrete their own siderophores due to mutations. Moderate bacterial growth may indicate a lower efficiency of iron uptake in the presence of these acids as compared to bacteria capable of releasing siderophores. Taking into consideration the ubiquity of α-keto/α-hydroxy acids in cells of all types, they may play a role as agents that stimulate bacterial growth under conditions where conventional endogenous siderophores are ineffective [30]. In the future, additional studies are needed for better understanding of the role of the above-described endogenous mammalian biomolecules in host physiology and their influence on the "tug of war" process between bacteria and the host for iron [31].

5. HEME OXYGENASES IN MAMMALS AND BACTERIA

Heme is known to play an important regulatory role in cell biology. Heme is synthesized in both mammalian and bacterial cells [1]. Since an excess of intracellular heme has a toxic effect, there is a system of its regulation in cells. An excess of heme in blood plasma and in cells can oxidize lipids, denature proteins, and disrupt the integrity of the cell cytoskeleton. Heme can also reduce the activity of cytosolic enzymes, including some glycolytic enzymes, glucose-6-phosphate dehydrogenase, and glutathione reductase [32]. In addition, excess of heme can activate cell-damaging enzymes such as caspases and cathepsins [32]. *In vitro*, heme can denature DNA by an oxidative mechanism. The mitochondria of cells are especially sensitive to heme toxicity. An excess of heme leads to a short-term increase in mitochondrial respiration followed by a decrease and then complete cessation of oxygen uptake [32]. At the same time, small amounts of peroxides, which are usually formed during mitochondrial respiration, exacerbate the prooxidant and other damaging effects of heme [32]. In addition to direct cytotoxic effects, heme can cause kidney damage due to its pro-inflammatory action. For example, heme in renal tubular epithelial cells and in the kidney *in vivo* induces the release of chemokines. Administration of heme enhances the expression of adhesion molecules *in vivo* in the intestine, liver, and pancreas; this is accompanied by leukocyte recruitment and increased vascular permeability [32].

The main enzyme involved in the degradation of excess heme in the body is heme oxygenase. In mammalian cells heme oxygenase is a 32 kDa protein that degrades hemin to biliverdin, carbon monoxide (CO), and free iron. Usually, heme oxygenase (HO-1) expression in mammalian cells is low or below detectable limits, except for tissues involved in erythrocyte metabolism (liver, spleen) [32]. Some substances, for example, hemin, some metals, xenobiotics, synthetic metalloporphyrins increase the expression and activity of HO-1 in many cells, for example, hematopoietic, hepatic, epithelial, and endothelial [32]. Products of the reaction catalyzed by HO-1 have various positive effects. CO exhibits vasodilating, anti-apoptotic, and anti-inflammatory actions [32]. Free iron ions induce formation of iron-binding and iron-transporting proteins [32]. A decrease in blood iron levels inhibits the growth of pathogens. Biliverdin (a bile pigment) is a powerful antioxidant, scavenging oxidants and reducing NADPH oxidase activity [32].

The main function of bacterial heme oxygenases is to obtain iron from the eukaryotic host [33]. For the vital activity of most bacteria *in vitro*, the presence of 0.4–4 μM iron in the environment is necessary. Since the concentration of free iron in the human body is below 0.4 μM, the main source of iron for bacteria is heme; heme oxygenases are responsible for iron release from it. Heme oxygenases have been isolated from both Gram-positive and Gram-negative bacteria [34]. In the last decade, several bacterial heme oxygenases required for iron release have been identified in such pathogenic bacteria as *Corynebacterium diphtheriae*, *Neisseria meningitidis*, and *P. aeruginosa*, as well as *Listeria monocytogenes*, *Bacillus anthracis*, and *S. epidermidis* [35]. Some bacterial heme oxygenases and mammalian heme oxygenases share a common structure and are characterized by a similar mechanism of heme degradation. Such bacterial heme oxygenases have been denominated as canonical oxygenases.

In contrast to the bacteria described above, the structure of heme oxygenases from *S. aureus* and *Mycobacterium tuberculosis* and the mechanism of heme degradation differ from the mammalian and other bacterial heme oxygenases and they are referred to as noncanonical ones [36]. For example, in *S. aureus*, two enzymes are responsible for heme degradation: bacterial heme oxygenases IsdG and IsdI. These enzymes are components of a system of 9 proteins that bind host hemoglobin, extract heme, transfer it from the environment to the bacterial cytosol, and decompose heme. Unlike human heme oxygenase, which degrades heme to CO, iron, and biliverdin, the IsdG and IsdI proteins of *S. aureus* and *M. tuberculosis* degrade heme to staphylobilin and formaldehyde, as well as small amounts of CO and formic acid (Fig. 8) [37, 38]. In addition, a nonplanar (ruffled) structure is very important for the heme associated with the IsdG protein [38]. This heme state significantly changes the chemistry of O_2 activation on the heme molecule, resulting in the production

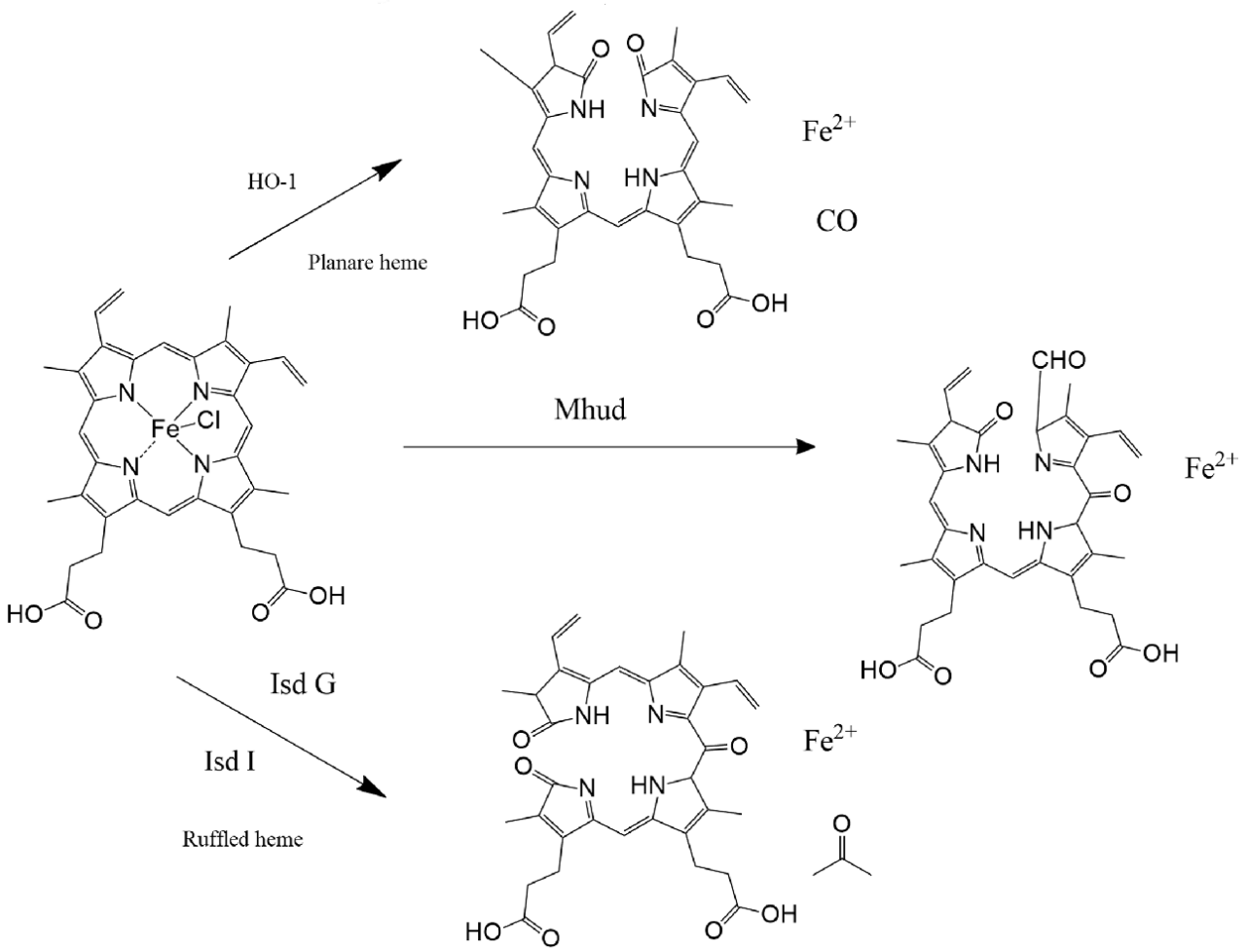


Figure 8. Differences between canonical mammalian heme oxygenase and non-canonical heme oxygenases IsdG and IsdI from *S. aureus* and non-canonical heme oxygenase MhuD from tuberculosis.

of a new tetrapyrol, staphylobilin, with an oxidized mesocarbon atom. Additionally, during the destruction of heme, an unusual product, formaldehyde, is formed. Although the biological role of these unusual products remains unknown, it has been found that mechanisms of formaldehyde deactivation exist both in the mammalian organisms and in bacterial cells [39].

In mammalian heme oxygenase, heme iron is bound to the His25 residue with the K_d values in the nanomolar range. The Asp140 residue organizes a network with water molecules, which directs the transient hydroxyl radical to the heme mesocarbon atom. *S. aureus* IsdG and IsdI bind heme via His76/77 with micromolar K_d values. Surrounding Asp6/7 and Trp66/67 residues are essential for enzymatic transformations [38].

M. tuberculosis heme oxygenase also catalyzes heme degradation without CO generation. This results in the formation of a new chromophore, mycobillin, containing an aldehyde group at the cleavage site of the heme moiety [38].

Despite the differences in the structures and mechanisms of heme degradation by canonical and noncanonical bacterial heme oxygenases, they have common conservative regions. For example, the canonical heme oxygenases from *L. monocytogenes* (Lmo2213) and *Bacillus subtilis* (HmoB) share 25.3% similarity in their structure. In the case of the noncanonical *S. aureus* heme oxygenase (IsdG), the similarity is 25.7%. At the same time, the structural similarity between the *B. subtilis* and *S. aureus* heme oxygenases is about 15% [40]. The structural similarity of canonical *P. aeruginosa* and *N. meningitidis* heme oxygenases is 33%, while the similarity with mammalian and human heme oxygenases is less than 15% [41]. This inspires certain optimism in terms of creating selective inhibitors of bacterial heme oxygenases that affect many bacterial pathogens, but not mammalian heme oxygenase [41].

6. THE SIGNIFICANCE OF THE INDUCTION AND INHIBITION OF MAMMALIAN HEME OXYGENASE FOR THE FIGHT AGAINST VIRAL AND BACTERIAL INFECTIONS

Despite the availability of different generations of antimicrobials, infectious diseases are still among the top 10 causes of death worldwide. The leading

infectious diseases are HIV/AIDS, tuberculosis, and malaria. According to the WHO, in 2016 there were 36.7 million cases of HIV, 10.4 million cases of tuberculosis, and 480,000 cases of multidrug-resistant tuberculosis. In 2016, 216 million cases of malaria were reported (including 445,000 fatal cases) [42].

Good evidence exists in the literature that some known pharmacological agents can lead to the expression of HO-1. These include such pharmacological agents as hemin, anesthetics (sevoflurane or isoflurane), statins, melatonin, as well as such substances as curcumin, resveratrol, caffeic acid phenethyl ester, ethyl ferulate, piceatannol, sulforaphane, carnosol, rosolic acid, α-lipoic acid [43]. The ability of these substances to induce the expression of heme oxygenase has been found *in vitro*, and for some substances *in vivo*.

Hemin is the most potent heme oxygenase inducer; it has been approved by FDA for the treatment of acute porphyria at a dose range of 1–4 mg/kg. In the human study activation of HO-1 expression by hemin was found in healthy volunteers. It was infused as a Pangematin solution (hemin for injections) diluted with 25% albumin at the dose 2.4 mg/kg (the infusion rate of 60 ml/h). This dose corresponds to the dose used for the treatment of acute porphyria. Dilution with 25% albumin is necessary to improve hemin stability, to reduce the incidence of phlebitis in patients, to prevent anticoagulant effect, and to improve efficacy. It was shown that 24 h after the administration of hemin, there was a 4-5-fold increase in the concentration of heme oxygenase in the blood plasma, and a 5-9-fold increase in its catalytic activity. The increase in heme oxygenase concentration persisted after 48 h [44]. In addition to the ability to induce mammalian heme oxygenase, hemin sodium salt has its own antibacterial activity [45, 46]. Besides that, hemin, covalently modified with amino acids and peptides, has its own both antibacterial and virucidal activity [47–49].

In the liver of rats with experimental toxic hepatitis induced by CCl_4, administration of heptral, mexidol, and methyluracil led to an increase in the relative expression of the *hmox1* gene encoding heme oxygenase as compared to the group of animals treated with CCl_4 only [50]. Thus, it can be assumed that in the case of toxic liver damage induced by CCl_4, administration of the above drugs led to an increase in protective and adaptive mechanisms, increasing the activity of the *hmox1* gene [50]. *In vitro* some statins at micromolar concentrations induced heme oxygenase expression in ECV304 endothelial cells and human epithelial cells [51]. *In vivo*, simvastatin induced HO-1 expression in vascular and cardiac smooth muscle cells. The authors believe, that HO-1 induction may explain some of the anti-inflammatory and cytoprotective pleiotropic (having several genetic effects) effects of statins [52].

Two inhalational anesthetics, isoflurane and sevoflurane, increased heme oxygenase levels and promoted cytoprotection during heart and liver surgery [53]. Long-term treatment of a man with isoflurane for 12 days promoted the recovery and reduction of bronchospasm in pneumonia caused by herpes simplex [54]. Sevoflurane improved pulmonary mechanics and gas exchange in a number of cases in infants with severe bronchiolitis and acute respiratory distress syndrome [55]. Recently, hyperbaric oxygen therapy has been proposed to improve oxygenation in patients with COVID-19. Its usefulness is associated with an increase in the level of HO-1 and a cytoprotective effect [56].

Heme oxygenase can play a dual role, both positive and negative, depending on the amount of heme metabolism products and the cellular environment. Overexpression of heme oxygenase can lead to cell damage, probably due to the temporary accumulation of catalytically active Fe^{2+} ions or toxic concentrations of CO or bilirubin, which can limit or suppress the body's defenses [53]. Expression of heme oxygenase can lead to normalization of blood pressure [57], exhibit a positive effect in acute pancreatitis [58], acute myocardial infarction [59], and anti-inflammatory and healing effects [60]. Expression of heme oxygenase induced by various factors/agents can lead to the manifestation of antibacterial and antiviral effects. Currently, the safety and efficacy of various HO-1 induction strategies, such as delivery of the HO-1 gene or the use of chemicals capable of inducing heme oxygenase, remain a major concern; in general, such studies are limited to the preclinical stage [53].

6.1. The Influence of Heme Oxygenase on the Course of Diseases Caused by Microbial Infections

Sepsis is one of the leading causes of death worldwide; it is characterized by a systemic inflammatory response and can induce a hyperinflammatory response leading to multiple organ failure. In sepsis, erythrocyte lysis results in hemoglobin release; its subsequent decomposition yields free heme, which reaches the circulatory system. Heme induces pro-inflammatory mediators that can lead to tissue damage and cell death [61].

Expression of HO-1 activated by heme and the enzyme can fight microbial infections by releasing CO and biliverdin. Biliverdin induces expression of the anti-inflammatory interleukin IL-10 and inhibits the action of pro-inflammatory cytokines (IL-6, MCP-1). CO also plays an important role in fighting bacterial infections. Induction of heme oxygenase contributes to the protection of mice against infection caused by *Salmonella typhimurium* [62, 63].

In addition to hemin, some metalloporphyrins, acting as inducers or inhibitors of mammalian heme oxygenase, have their own antibacterial activity. Sodium

salts of various metalloporphyrins showed antibacterial activity against the Gram-positive bacteria *S. aureus*, *Mycobacterium smegmatis*, *Yersinia enterocolitica*. For example, the MIC of the sodium salt of the cobalt complex of protoporphyrin IX was 20 μg/ml, 40 μg/ml, and 20 μg/ml, respectively, for the *Y. enterocolitica*, *S. aureus,* and *M. smegmatis* strains respectively, and for the sodium salt of the tin complex of protoporphyrin IX, these values were 20 μg/ml, >40 μg/ml, and 20 μg/ml, respectively [64].

The authors of [65] evaluated the effectiveness of the sodium salt of the cobalt complex of protoporphyrin IX (HO-1 activity inducer), and the sodium salt of the tin complex of protoporphyrin IX, (HO-1 activity inhibitor), in acute and persistent infection with *S. typhimurium* in mice *in vivo*. Mice were treated with i.p. injections of these compounds of protoporphyrin IX at a dose of 5 mg/kg. After 24 h, the animals were infected with *S. typhimurium* (CFU of 10^6 cells/ml). In the group of mice treated with cobalt protoporphyrinate 20% of animals survived, while all mice that did not receive its injections died. This effect is associated with the induction of HO-1 [65]. Cobalt protoporphyrinate significantly reduced the bacterial load in the spleen, liver, mesenteric lymph nodes, gallbladder, feces; at the same time, an increase in the number of bacteria was observed in the blood as compared to the control group. This may be due to the ability of hemoglobin to bind CO released during the decomposition of heme by heme oxygenase; this allows bacteria circulating in the blood to avoid the bactericidal action of CO. It should be noted that neither cobalt protoporphyrinate nor tin protoporphyrinate have their own (direct) activity against *S. typhimurium* [65].

Induction of heme oxygenase can have a negative effect, increasing chronic inflammation and the immunosuppressive state of a patient with sepsis [66]. The use of a heme oxygenase inhibitor in *K. pneumoniae*-induced sepsis increased neutrophil migration to sites of inflammation, attenuated lung injury, and reduced patient mortality [67]. In [68], the activity of the sodium salt of the zinc complex of protoporphyrin IX, an inhibitor of mammalian heme oxygenase, as well as the sodium salt of hemin, a heme oxygenase inducer, was evaluated at the late stage of a mouse model of sepsis induced by ligation and puncture of the caecum. During the experiment, hemin and the zinc complex of protoporphyrin were dissolved in 0.2 M NaOH and after neutralization (pH 7.4) were injected intravenously at a dose of 20 mg/kg and 25 mg/kg, respectively, 24 h and 36 h after infection. After 10 days of observation the survival rate of the control group, which did not receive such treatment, was 46.7%. The administration of the sodium salt of the zinc complex of protoporphyrin IX to mice increased their survival rate to 80%, while the administration of hemin, on the contrary, reduced it to 10%. In addition, the zinc complex of protoporphyrin IX decreased bacterial CFUs in the spleen and abdominal cavity of mice, while hemin, on the contrary, increased this parameter.

The antibacterial effect of the sodium salt of zinc protoporphyrin IX was associated with a decreased decline in the number of T-cells, B-cells, and dendritic cells [68]. The number of immune cells is an important characteristic of immunosuppression caused by sepsis. During experiments, it was demonstrated that in mice with sepsis, the level of T-cells ($CD4^+$ and $CD8^+$) decreased, while the zinc complex of protoporphyrin IX slowed down this decrease [68]. The authors did not illustrate the effect of hemin on the level of T-cells; however, they investigated the effect of CO. For this purpose, mice were intravenously injected with CORM-2 (Carbon-Monoxide-Releasing Molecule-2) at a dose of 8 mg/kg 24 h after ligation and cecal puncture, inducing sepsis. CORM-2 administration decreased the number of T-cells as compared to mice with untreated sepsis. The authors did not evaluate the intrinsic antibacterial activity of the sodium salt of the zinc complex of protoporphyrin IX and the sodium salt of hemin [68]. Nevertheless, certain evidence exists in the literature [64] that the sodium salt of the zinc complex of protoporphyrin IX has an antibacterial effect against *S. aureus* and *M. smegmatis* (MIC 3.2 μg/ml and 20 μg/ml, respectively), and 10^{-5} M sodium salt of hemin caused the death of 99% of *S. aureus* cells [46].

Thus, overexpression of heme oxygenase induced by hemin administration reduces bacterial clearance (the rate at which bacteria are cleared from the body) and increases mortality in mice with sepsis, and also reduces the number of immune T-cells. This leads to depletion of immune cells and immunosuppression. Therefore, the control of HO-1 expression is an important task in the clinical practice, especially in the treatment of diseases causing immunodeficiency [68].

6.2. The Influence of Heme Oxygenase on the Course of Tuberculosis

The current recommendation for initial treatment of tuberculosis includes the standard first line regimen of rifampicin, isoniazid, pyrazinamide, ethambutol, which are used for 6–9 months [69]. Such a long-term treatment causes negative side effects. If the long-term use of drugs is accompanied by impaired treatment regimen, then re-infection may occur, as well as the development of resistance to antibiotics. In this regard, there is a clear need in the development of effective drugs that can more quickly and effectively control *M. tuberculosis* in infected patients. The tin complex of protoporphyrin IX was studied as an agent against *M. tuberculosis* in mice [69]. Mice were injected intravenously daily at a dose of 5 mg/kg with tin protoporphyrinate dissolved in 0.1 M aqueous NaOH and diluted 10 times with

phosphate-buffered saline. The study of the activity of the tin complex of protoporphyrin IX was carried out in comparison with commonly used antibiotics for the treatment of tuberculosis, such as rifampicin (at a dose of 10 mg/kg), isoniazid (25 mg/kg), pyrazinamide (150 mg/kg). For this purpose mice were injected with a cocktail of three antibiotics, and after 60 days — a cocktail of two antibiotics — rifampicin and isoniazid. During the experiment, mice were infected with *M. tuberculosis* (CFU of 10^2 cells/ml), and treatment was started 4 weeks after infection. Three weeks after the start of the treatment, daily doses of the sodium salt of the tin complex of protoporphyrin IX decreased CFU by 1 lg. A similar result was obtained for a combination of drugs consisting of rifampicin, isoniazid, pyrazinamide. The simultaneous use of the sodium salt of the tin complex of protoporphyrin IX, rifampicin, isoniazid, pyrazinamide after 3 weeks decreased CFU by 2 lg as compared with the control group. Prolonged administration of the tin complex of protoporphyrin IX for 9 weeks did not lead to a further decrease in the CFU of *M. tuberculosis*. Combined injections of the reference drugs (rifampicin, isoniazid, pyrazinamide) 17 weeks after the start of administration reduced the CFU of *M. tuberculosis* to 10^2 cells/ml. The addition of the tin complex of protoporphyrin to this combination of drugs after 17 weeks reduced the CFU to an undetectable level (Fig. 9).

The antibacterial effect of the tin complex of protoporphyrin IX is not due to direct antibacterial action, since *in vitro* the tin complex of protoporphyrin IX did not demonstrate inhibitory antimicrobial activity against *M. tuberculosis* at a concentration of 125 μM. According to the authors, the antibacterial effect of the tin complex of protoporphyrin IX against *M. tuberculosis* is associated with the inhibition of mammalian heme oxygenase [69].

6.3. The Effect of a Heme Oxygenase Inducer on Pneumonic Plague Caused by Yersinia Pestis

Plague is a multi-organ disease accompanied by sepsis and necrosis of immune tissues and liver, as well as bronchopneumonia and uncontrolled bacterial growth [70]. A heme oxygenase inducer, the cobalt complex of protoporphyrin IX, has been used to treat pneumonic plague caused by *Y. pestis* [70]. It was dissolved in 0.1 M NaOH and after neutralization with HCl to pH 7, was administered intravenously to mice twice before infection with the plague bacillus and every 48 h for 10 days after infection at a dose of 5 mg/kg. This treatment decreased animal mortality and by the 9th day of the experiment, the surviving mice showed no signs of the disease. The decrease in mortality was accompanied by an increase in bacterial clearance due to activation of the innate immune response, an increase in the level of mammalian heme oxygenase, and a decrease in blood serum and lung levels of cytokines [70].

The combined use of the cobalt complex of protoporphyrin IX at a dose of 5 mg/kg and doxycycline at a dose of 20 mg/kg increased the survival rate of infected animals and in the best therapeutic regimens it reached 95%.

Thus, the cobalt complex of protoporphyrin IX provides a high survival rate of mice infected with *Y. pestis*, and the combined use of cobalt protoporphyrinate and doxycycline in animals results in a synergistic effect [70].

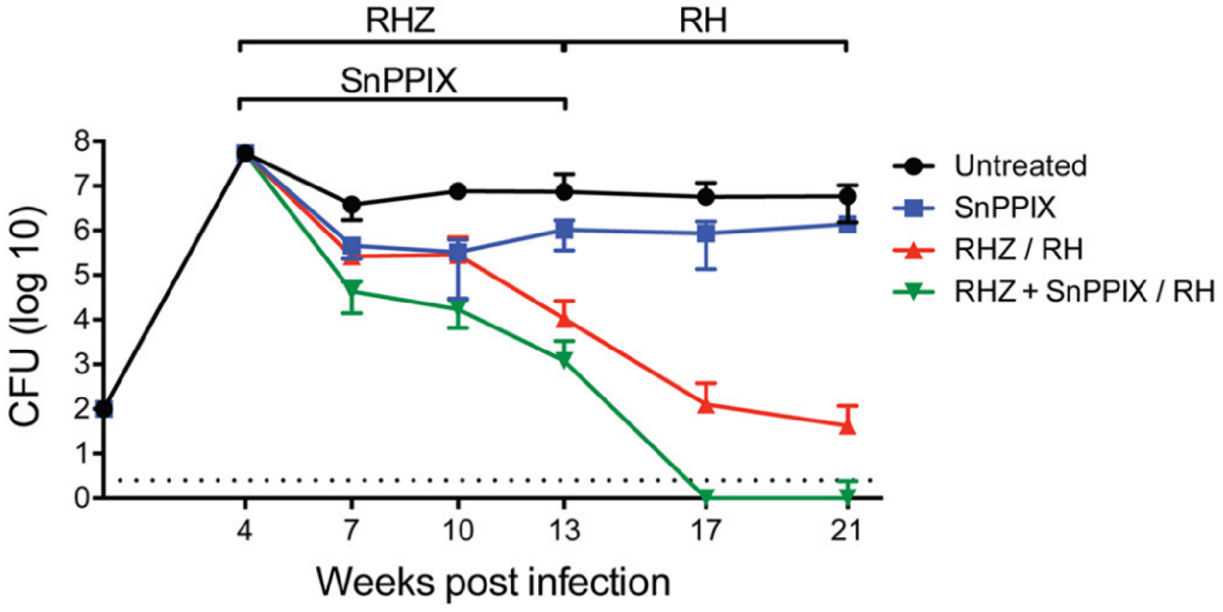


Figure 9. Decreased CFU of *M. tuberculosis* in *in vivo* experiments in mice after exposure to protoporphyrin tin complex sodium salt (SnPPIX) and a combination of rifampicin (R), isoniazid (H), and pyrazinamide (Z) (adapted from [69]).

6.4 Involvement of Heme Oxygenase in Inhibition of Immunodeficiency Virus (HIV)

Acquired immunodeficiency syndrome (AIDS) has claimed more than 35 million lives worldwide, and 36.7 million people are currently living with HIV [42]. In 2016 alone, about 1 million people died from AIDS-related complications [42]. In the 1980s, Tsutsui and Mueller showed in an *in vitro* model that a 100 μM aqueous solution of hemin sodium salt completely inhibited the activity of reverse transcriptase of the Rauscher mouse leukemia virus [71].

Hemin sodium salt at a concentration of 10^{-4} M inhibited HIV replication *in vitro* [71]. Hemin was dissolved in 0.2 M NaOH and then HCl was added to pH 7.4. The resulting solution was used to treat monocytes 24 h before infection with the immunodeficiency virus, then either simultaneously or 4 h later, and 24 h after infection. A significant suppression of HIV virus replication in monocytes was noted when hemin was added not only before but also after infection and no cytotoxic activity of the hemin sodium salt was observed. In a further experiment, the efficacy of hemin sodium salt was evaluated *in vivo* in diabetic mice. For this purpose, five days after infection, the animals were injected intravenously with hemin sodium at a dose of 4 mg/kg for 2 weeks. After 14 days of the treatment, the viral load of HIV in the blood serum decreased by six times as compared with the control group [72]. This antiviral effect is associated with the ability of hemin to induce heme oxygenase, since the addition of the sodium salt of the tin complex of protoporphyrin IX (an inhibitor of mammalian heme oxygenase activity) reduced the ability of hemin to inhibit HIV replication [72].

6.5. The Effect of a Heme Oxygenase Inducer on Hepatitis B Virus

According to data release 2022, about 296 million people worldwide are infected with hepatitis B virus (HBV). Approximately 5–10% of the infected people develop a chronic infection. Some of these patients develop active hepatitis, which can progress to cirrhosis and liver cancer [73].

The authors of the study [74] described the activity of a heme oxygenase inducer, sodium salt of cobalt protoporphyrinate, against HBV replicated in hepatoma cells. The cobalt complex of protoporphyrin IX, dissolved in 0.2 M NaOH solution and then adjusted to neutral pH, was administered to mice i.p. at a dose of 10 mg/kg 24 h before infection. Induction of heme oxygenase with the sodium salt of the cobalt complex of protoporphyrin IX suppressed HBV replication directly in hepatocytes at the post-transcriptional stage by reducing the stability of the HBV nucleocapsid protein. In addition, there was a significant inhibition of transaminase release and reduction in hepatic necroinflammatory damage.

6.6. The Effect of Heme Oxygenase Inducer on Hepatitis C Virus

According to the WHO, 58 million people are currently living with chronic hepatitis C infection. In 2019, hepatitis C caused approximately 290,000 deaths [75]. It is known that HO-1 overexpression can inhibit hepatitis C virus replication in human hepatoma cells [76]. Biliverdin, a product of the HO-1 catalyzed heme degradation reaction, can inhibit hepatitis C virus replication [76]. The mechanism of the antiviral action of biliverdin is associated with the ability to inhibit the NS3/4A proteinase of the hepatitis C virus [76].

Another independent study showed that Fe^{2+} and Fe^{3+} iron ions, another product of the heme degradation reaction, inhibited HCV replication. They bind to a protein "pocket" needed for incorporation of Mg^{2+} to the hepatitis virus RNA polymerase (NS5B) and cause enzyme inhibition [77]. Thus, biliverdin or its derivatives, as well as iron ions, may be useful in the future development of drug(s) for the treatment for hepatitis C virus [76, 77].

6.7. The Effect of a Heme Oxygenase Inducer on the Course of Dengue Fever

The dengue virus (DENV) is an infection transmitted by arthropods. Approximately 390 million cases of this viral infection occur each year, with 96 million people developing clinically significant symptoms [78]. Treatment of Dengue virus-infected human liver cells (Huh-7) with mammalian heme oxygenase inducers, solutions of hemin (2.5–10 μM) and cobalt complex of protoporphyrin IX (7.5–30 μM), led to a dose-dependent decrease in the level of Dengue virus RNA [79]. For example, 10 μM hemin reduced the level of Dengue virus RNA by approximately 80%, and 30 μM cobalt protoporphyrinate by 90%. Addition of the tin complex of protoporphyrin IX (a specific inhibitor of heme oxygenase induction) decreased the antiviral activity of both hemin and cobalt protoporphyrinate [79]. This may indicate that the antiviral effect of metalloporphyrins is mainly due to the functioning of mammalian heme oxygenase, rather than the direct antiviral action of the substances.

In vitro, the products of hemin decomposition by heme oxygenase, CO and iron ions (Fe^{3+}), did not possess antiviral activity. Biliverdin at a concentration of 100 μM reduced the level of Dengue virus RNA by 90%. At the same time, bilirubin, a product of biliverdin reduction, also did not exhibit the antiviral activity. HO-1 induction resulted in inhibition of all 4 Dengue virus serotypes (DENV 1–4) [79].

In experiments performed on Dengue virus-infected mice, administration of cobalt protoporphyrinate (50 mg/kg) provided survival of 80% of the animals [79].

6.8. The Effect of a Heme Oxygenase Inducer on Zika Virus

The Zika virus is transmitted primarily through mosquito bites, but sexual transmission and blood transfusion from an infected person is also possible. Currently, there is no specific drug for the treatment of this viral infection, but a corresponding vaccine is being developed [80].

Since immune responses are of key importance for protection against viral infections, the authors [81] suggested that activation of a safe and effective innate immune response of the host would be a logical direction towards the development of an antiviral drug. For this purpose, the drug Pangematin, which includes hemin sodium salt as an active component, has been used. The study was performed using monocytic macrophages. The data obtained *in vitro* revealed an increased expression of heme oxygenase HO-1 by hemin (as a component of Pangematin). After 48 h hemin at a concentration of 100 μM completely inhibited Zika virus replication in the monocytic macrophages. The authors investigated the effectiveness of adding hemin 1 h before infection, during or 1 h after infection. The incubation was carried out for 48 h. The addition of hemin equally effectively suppressed virus replication, regardless of its administration before or after infection [81].

6.9. Activity Against COVID-19 Virus

From 2020 to 2022, more than 573 million cases of COVID-19 infection have been recorded with a mortality rate of 1.11% [82]. Currently, various antiviral drugs against HIV, influenza viruses, Ebola, hepatitis, cytomegalovirus, and herpes are being used to treat COVID-19 [83]. However, none of them is approved for the treatment of COVID-19. In the context of the rapid spread of COVID-19 around the world, the search for substances capable of suppressing the replication of this virus, becomes relevant and hemin attracts certain attention in this context [83]. There is currently no specific treatment for COVID and most strategies are mainly symptomatic [84]. Patients with metabolic syndrome, the elderly persons (particularly men) have increased morbidity, mortality, and low levels of stress proteins, especially extracellular HO-1 [85]. In addition, decreased levels of heme in COVID-19 may further reduce the amount of HO-1 in the body. These risk factors trigger a cytokine storm that leads to multiple organ failure and eventually death [85]. The cytoprotective and anti-inflammatory properties of HO-1 may reduce the risk of such complications.

The use of HO-1 inducers may prevent or cure COVID-19 diseases. Animal models have shown that suppression of animal HO-1 genes increases the risk of lung damage in sepsis [86]. In addition, the COVID-19 virus blocks production of heme, the main inducer of heme oxygenase. In this regard, it should be noted that the Han population of Taiwan is protected from COVID-19 due to the high level of heme oxygenase expression [87].

An *in vitro* study of the effect of hemin on SARS-CoV-2 virus replication in African green monkey kidney Vero 76 cells has shown that treatment of cells 1 h before infection with SARS-CoV-2 with 6.25 μM hemin increased heme oxygenase levels, and 12.5 μM hemin markedly suppressed viral replication. The ability of hemin to suppress SARS-CoV-2 replication both before and after infection suggests that hemin can inhibit the virus at the stage of intracellular replication [83].

The study of hemin toxicity performed using Vero 76 cells has shown that at its concentration of 169.9 μM, 50% of the cells died, while exposure of the Vero 76 cells, infected with SARS-CoV-2, to 0.68 μM hemin caused a 50% decrease in the virus titer. Thus, the activity of hemin against COVID-19 is approximately 250 times higher than its toxicity against Vero 76 cells [83].

In order to detect the antiviral activity of heme oxygenase itself, the authors infected Vero 76 cells, overexpressing monkey HO-1, with SARS-CoV-2. The replication of SARS-CoV-2 in the cells overexpressing heme oxygenase was suppressed. The study of antiviral activity against the SARS-CoV-2 virus of hemin decomposition products showed that 10 μM and 30 μM $FeCl_3$ suppressed the expression of SARS-CoV-2 RNA by 50% and 60%, respectively, and 10 μM and 50 μM biliverdin — by 67% and 75% respectively. Model substances capable of releasing CO did not demonstrate any activy against COVID-19 [83].

In order to elucidate the nature of the antiviral effect of hemin by heme oxygenase induction in cells, an additional study of the antiviral activity of a mixture consisting of hemin (25 μM) and the zinc complex of protoporphyrin IX (10 μM and 30 μM) was carried out against Vero 76 cells infected with SARS-CoV-2 (Fig. 10). The zinc complex of protoporphyrin IX (an inhibitor of HO-1 enzymatic activity) demonstrated little activity against the SARS-CoV-2 virus. A mixture consisting of hemin and the zinc complex of protoporphyrin IX significantly reduced the level of SARS-CoV-2 virus RNA as compared to hemin (Fig. 10), thus suggesting a synergistic effect of the substances added in the mixture. It should be noted that the zinc complex of protoporphyrin inhibits only the enzymatic activity of heme oxygenase, but at the same time induces its expression. At the same time treatment of SARS-CoV-2-infected Vero 76 cells with a mixture of small interfering RNA (blocking the expression of heme oxygenase) and hemin did not suppress viral replication.

The results obtained indicate that inhibition of the enzymatic activity of heme oxygenase does not affect its antiviral activity, and the expression of HO-1 may be accompanied by production of interferons with antiviral activity [83].

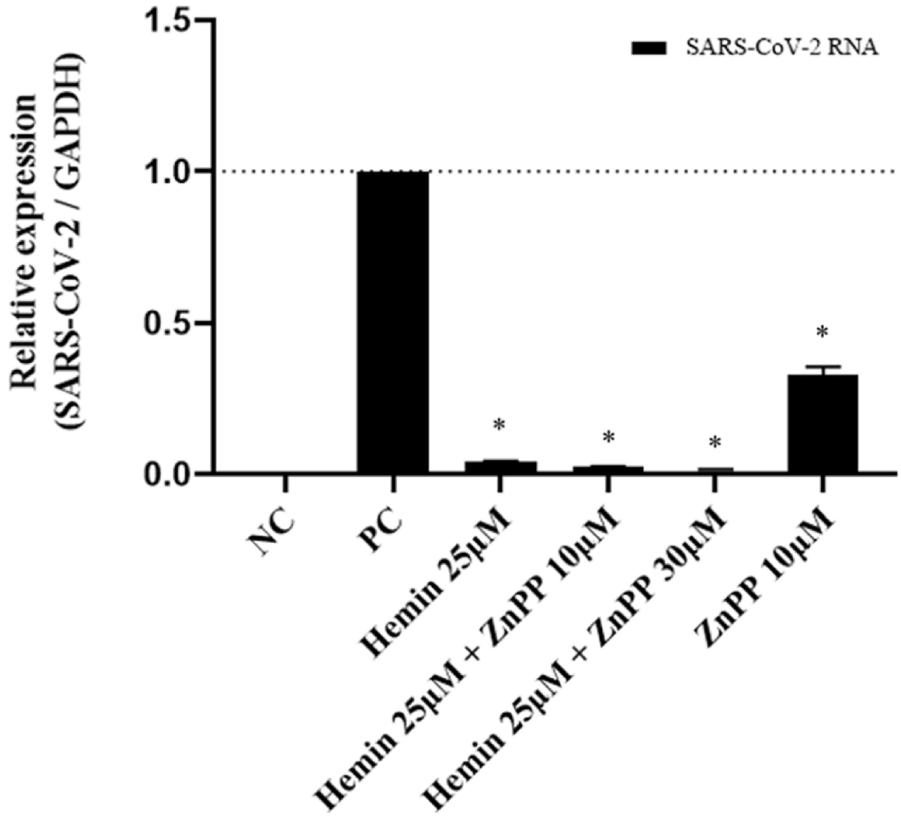


Figure 10. Antiviral activity of hemin, a mixture of hemin with a zinc complex of protoporphyrin against the COVID-19 virus. An asterisk indicates statistically significant differences *p<0.001 (adapted from [83]).

The data discussed above differ from the data on the effectiveness of hemin arginate (as a part of Normosang) against COVID-19 obtained on Vero-E6 renal epithelial cells and Calu-3 lung cancer cell line. Vero-E6 and Calu-3 cells were exposed to 64.4 μM hemin 24 h before SARS-CoV-2 infection or simultaneously with cell infection. At 48 h and 72 h after infection of Vero-E6 and Calu-3 cells, respectively, no significant decrease in the virus titer was noted after exposure to hemin arginate [88].

Differences in the results obtained by different authors may be due to different experimental conditions, for example, using different types of cells and forms of hemin, as well as differences in initial virus titers. Results of another study of the antiviral activity of hemin arginate have been reported in [89]. Hemin arginate as a part of the Normosang preparation at concentrations of 1.25 μM and 2.5 μM was added to Vero cells (CFU 1.5×10^5 cells/ml) immediately after infection with the SARS-CoV-2 virus (initial virus titer 10^6). Three days after exposure to 2.5 μM hemin the virus titer remained unchanged, thus indicating inhibition of the viral replication rate.

Hemin arginate prevented death in 80% of SARS-CoV-2 virus-infected Vero cells in mice *in vivo*. Hemin arginate was administered intravenously at a dose of 3 mg/kg on days 4, 5, and 6 after infection. On day 8, the mice were sacrificed and the virus titer in the lungs was determined. In animals treated with heminate arginate, the virus titer in the lungs of mice decreased to 10% of the initial value [89].

Positive results of hemin arginate (Normosang) administration were obtained in humans, on 6 volunteers (3 women and 3 men) with COVID-19. Patients suffered from concomitant diseases (bronchial asthma, arterial hypertension, dyslipidemia, type 2 diabetes mellitus, overweight). Patients received infusions of 3 mg/kg hemin arginate. During hemin arginate administration or shortly thereafter, there was a decrease in the need for supplemental oxygen, an improvement in their health status, and a reduction in the length of their hospitalization [89].

The mechanism of hemin arginate action on the SARS-CoV-2 virus is associated with its induction of heme oxygenase in mammalian cells. One of the main advantages of hemin is the availability of its approved dosage forms (Normosang, Pangematin) suitable for intravenous administration. It is also known that hemin suppresses the action of pro-inflammatory cytokines and exhibits other beneficial properties [62]. However, neither the papers [87, 88] nor the application [89] clearly formulated and quantified the beneficial effects of hemin. In addition, there are no *in vivo* studies demonstrating a reduction in mortality from SARS-CoV-2 infection of animals treated with hemin. Currently, hemin can be classified as a drug helper.

7. POSSIBILITIES OF INHIBITION OF BACTERIAL HEME OXYGENASE TO FIGHT BACTERIAL INFECTIONS

We found the first mention of the usefulness of bacterial heme oxygenase inhibition in [64]. The authors of that report suggested that the antibacterial effect of the gallium complex of protoporphyrin IX (Ga-PPIX) could be due to its ability to enter the cell through the heme uptake system. At the same time, Ga-PPIX cannot be utilized by bacterial heme oxygenase and this leads to their death. Later it was found that the cobalt, gallium, manganese, and zinc complexes of protoporphyrin IX (Co-PPIX, Ga-PPIX, Mn-PPIX, and Zn-PPIX) could bind to IsdG and IsdI proteins, but were not cleaved [90]. This can lead to the accumulation of these compounds in the bacterial cell. The accumulated metalloporphyrins can block cellular processes that require the participation of iron and thus cause cell death [90]. It was shown in that Ga-PPIX exhibited inhibitory activity against a Gram-negative clinical strain of *P. aeruginosa* [91]. This effect occurs under conditions of iron deficiency in the nutrient medium and is abolished by additional amounts of iron (hemin). The scenario of the antibacterial effect includes penetration of Ga-PPIX through the heme uptake system into the cell. Intracellular Ga-PPIX is then integrated into the respiratory cytochromes Cco-1, Cco-2 and Cio, located in mitochondria, instead of heme, thus disrupting cellular respiration. These cytochromes are found only in bacteria. It should be noted that we did not find any references in the literature about possibility of inhibition of mammalian heme oxygenase by Ga-PPIX.

In addition to the compounds described above, the research performed using computer simulation in combination with experimental studies, revealed low molecular weight compounds that could bind and inhibit the activity of heme oxygenases from *N. meningitidis* (nm-HO) and *P. aeruginosa* (pa-HO) [41]. The *N. meningitidis* (nm-HO) and *P. aeruginosa* (pa-HO) heme oxygenases were isolated, and the K_d values for the complexes of these heme oxygenases with the tested compounds were determined by fluorescence titration. The most effective among the studied compounds was the substance shown in Figure 11.

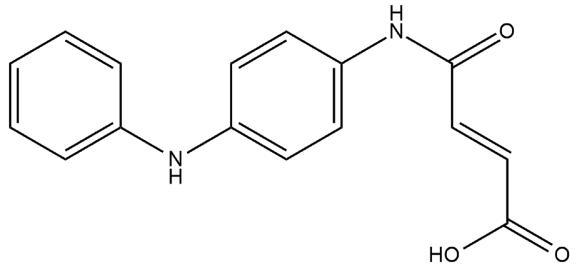


Figure 11. Structure of (E)-3-(4-(phenylamino) phenylcarbamoyl)acrylic acid, the most effective inhibitor of *N. meningitidis* (nm-HO) and *P. aeruginosa* (pa-HO) bacterial heme oxygenases.

In the course of further work, their ability to inhibit the activity of bacterial heme oxygenase inside the cell was evaluated. For this purpose, *E. coli* cells expressing *N. meningitidis* heme oxygenase were treated with potential inhibitors. It was found that at a concentration of 1500 μM the compound shown in Figure 11 almost completely inhibited *in vitro* the activity of *N. meningitidis* heme oxygenase expressed in *E. coli.* Next, the authors evaluated the antibacterial activity of this compound against the Gram-negative bacterium *P. aeruginosa MPA01*. At a concentration of 250 μM, the compound partially inhibited growth of *P. aeruginosa MPA01* cells. The achieved antibacterial effect could be abolished only by adding iron-containing pyoverdin to the medium, which was an alternative source of iron for bacteria, and its absorption was not associated with heme oxygenase. The addition of hemoglobin to the medium did not abolish the antibacterial action of the compound. At the same time, the level of heme oxygenase expression inside the bacterial cell remained unchanged under the influence of this compound.

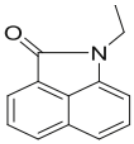
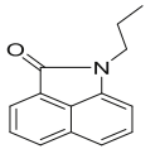
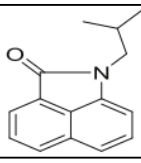
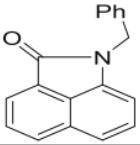
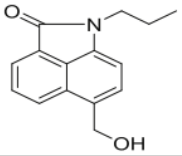
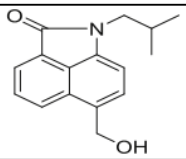
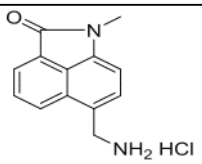
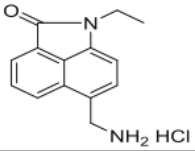
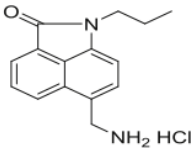
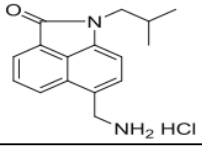
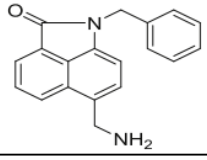
Thus, the antibacterial effect of the compound shown in Figure 11 is obviously associated with the ability of this substance to penetrate into the bacterial cell, bind to bacterial heme oxygenase, and inhibit its activity without affecting its level [41].

The active site of *P. aeruginosa* heme oxygenase (HemO) has a unique structure. For example, it is smaller in size (7.3 $Å^3$) than the active site of mammalian heme oxygenase (43–59 $Å^3$), and also differs in the mode of heme binding (heme binds to the active site of HemO in a significantly rotated (100°) orientation) [92]. The authors believe that using these data it is possible to develop a selective inhibitor of *P. aeruginosa* heme oxygenase (HemO) [92].

Taking into consideration the results of X-ray studies of the crystal structure of bacterial heme oxygenase HemO, a number of its potential inhibitors were synthesized (Table 1). Most of the synthesized compounds were characterized by the K_d values ranged from 1.5 μM to 180 μM. According to the authors, these substances bind to the active site of bacterial heme oxygenase HemO with moderate affinity and may be suitable inhibitors of bacterial heme oxygenase [92].

The study of the antibacterial activity of the synthesized compounds was carried out in the iron deficient medium (dialyzed tryptic soy broth; DTSB) and also in the medium with a higher iron content (Luria broth, LB). Most of the compounds tested had a satisfactory antimicrobial activity. For example, the MIC_{50} of synthesized compounds varied from 26 μg/ml to 260 μg/ml (Table 1) [92]. Some compounds, despite the low K_d value, had low antimicrobial activity (e.g., compounds 7b and 7c, see Table 1). According to the authors, this is due to the presence of certain substituents that prevent

Table 1. Structures and activity of potential inhibitors of bacterial heme oxygenase HemO

Substance code	Formula	K_d (μM)	MIC_{50} (DTSB*) (μg/ml)	MIC_{50} (LB*) (μg/ml)
2b		36	174	100
2c		46	240	80
2d		14	230	150
2e		8	42	26
4c		67	250	230
4d		65	260	120
7a		90	58	52
7b		1.9	180	89
7c		1.5	160	160
7d		180	230	120
7e		78	220	180

*DTSB – iron deficient medium, LB – medium enriched with nutrients (contains peptides, peptones, vitamins, iron, and microelements), K_d – dissociation constant (adapted from [92]).

the inhibitory effect of the compounds *in vitro*. At the same time, compound 7d exhibited antibacterial activity, despite the higher K_d value. This may be explained by employment of a different mechanism of the antibacterial action.

In [93], three possible strategies for inhibition of the noncanonical bacterial *S. aureus* heme oxygenase have been proposed. The first is to create a competitive inhibitor acting at the active site of bacterial heme oxygenase. However, the creation of such inhibitor is difficult due to the tight binding of heme to the bacterial heme oxygenases IsdG and IsdI. For example, the K_d values of such complexes are 1.4 nM and 12.9 nM, respectively [93]. The second strategy is to create a non-competitive inhibitor. Such inhibitors are, for example, cyanides and azides. They bind to the IsdG-heme iron, block the access of molecular oxygen and interfere with enzymatic activity. The third way involves the creation of an allosteric inhibitor of bacterial heme oxygenase. At present, inhibitors of the canonical heme oxygenase of another bacterium, *P. aeruginosa*, are being developed on the basis of the third strategy. However, there are no significant achievements in this field so far.

It should be noted that to date there are no commercially available antibiotic drugs with the convincingly elucidated action of bacterial heme oxygenase.

In connection with the development of resistance to antibacterial agents, including modern ones, the creation of new effective drugs with unique mechanisms of the antibacterial action is an important task. Its solution, may include creation of drugs that act on bacterial heme oxygenase. Some similarity of the active sites of heme oxygenases of various bacteria can make it possible to create universal agents that act on the heme oxygenases of many bacteria. However, to date, there are only a small number of works devoted to this proble. At the same time, the known inhibitors of bacterial heme oxygenase have insufficiently high antibacterial activity.

CONCLUSIONS

Based on the literature data discussed above, some conclusions can be drawn about the further perspectives and directions in the development of new antimicrobial agents. It seems promising to use siderophores to create antibiotics capable of overcoming bacterial resistance. This was demonstrated by the implementation of the Trojan horse strategy for a modified antibiotic, Fetroja (cefiderocol), approved by the US FDA [5]. The essence of this approach is the covalent attachment of the siderophore residue to the antibiotic molecule. Currently, such strategy is widely used in research on the synthesis of new antibiotics. Another way to create antibacterial agents may be the practical use of the mammalian lipocalin, which has so far been proposed to be used only for the preservation of platelets.

A new developing direction in the creation of innovative antimicrobial materials is also interesting [94]. In this context, a fibrous polymeric material containing hemin and possessing some antibacterial activity may serve as an example [95].

Amino acid and peptide derivatives of hemin have already demonstrated pronounced antimicrobial and antiviral activity; they are candidates for their further development as antimicrobial agents. It seems useful to clarify the mechanisms of their antibacterial action. An interesting direction in this area may be the further study of the antiviral effect of hemin and its derivatives, which is associated with its effect on heme oxygenase.

Contradictory data regarding the effectiveness of hemin against the SARS-CoV-2 (COVID-19) virus indicates the clear need for further research of both hemin and its derivatives in this direction.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ОБМЕН ЖЕЛЕЗА В КЛЕТКЕ КАК МИШЕНЬ ПРИ СОЗДАНИИ ПОТЕНЦИАЛЬНЫХ АНТИМИКРОБНЫХ И ПРОТИВОВИРУСНЫХ СРЕДСТВ

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Поиск и создание инновационных антимикробных препаратов, действующих в отношении резистентных и полирезистентных штаммов бактерий и грибов, является одной из важнейших задач современной биоорганической химии и фармацевтики. Поскольку железо необходимо для жизнедеятельности практически всех организмов, включая млекопитающих и бактерий, белки, участвующие в его обмене, могут служить в качестве потенциальных мишеней при создании новых перспективных антимикробных агентов. К таким мишеням относятся эндогенные биомолекулы млекопитающих — гемоксигеназы, сидерофоры, белок 24p3, — а также гемоксигеназы и сидерофоры бактерий. Определённый интерес представляют и другие белки, которые ответственны за доставку железа в клетки и его баланс между бактериями и организмом хозяина. В обзоре обобщены данные по созданию ингибиторов и индукторов (активаторов) гемоксигеназ, селективных для млекопитающих и бактерий, обсуждаются особенности их механизмов действия и строения. На основании рассмотренных литературных данных сделан вывод о перспективности использования гемина — самого мощного индуктора гемоксигеназы — и его производных в качестве потенциальных антимикробных и противовирусных агентов, в частности против COVID-19 и других опасных инфекций. При этом важная роль отводится продуктам деградации гемина под действием гемоксигеназ, в том числе *in vivo*. Определённое внимание уделено данным по антимикробному действию не содержащих железа протопорфиринатов, а именно комплексов с Co, Ga, Zn, Mn, их преимуществам и недостаткам по сравнению с гемином. Модификация известного антибиотика цефтазидима молекулой сидерофора повысила эффективность его действия против резистентных бактерий.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: железо; гемоксигеназа; сидерофоры; антибактериальная и противовирусная активность; гемин; белок 24p3

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