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REVIEWS

The promise of endogenous and exogenous riboflavin in anti-infection

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ABSTRACT

To resolve the growing problem of drug resistance in the treatment of bacterial and fungal pathogens, specific cellular targets and pathways can be used as targets for new antimicrobial agents. Endogenous riboflavin biosynthesis is a conserved pathway that exists in most bacteria and fungi. In this review, the roles of endogenous and exogenous riboflavin in infectious disease as well as several antibacterial agents, which act as analogues of the riboflavin biosynthesis pathway, are summarized. In addition, the effects of exogenous riboflavin on immune cells, cytokines, and heat shock proteins are described. Moreover, the immune response of endogenous riboflavin metabolites in infectious diseases, recognized by MHC-related protein-1, and then presented to mucosal associated invariant T cells, is highlighted. This information will provide a strategy to identify novel drug targets as well as highlight the possible clinical use of riboflavin.

Introduction

Drug resistance of bacteria, fungi and even viruses is increasingly becoming a serious problem worldwide. Antimicrobial-resistant bacteria (such as *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Streptococcus pneumoniae* and *Salmonella typhimurium*) have been found in hospitals and community settings [1,2]. Excessive use of antifungal drugs to treat humans and in other applications has led to increasing resistance [3]. The resistance of *Candida* and *Aspergillus* species notably presents a major problem in clinical treatment [4]. In addition, the issue of virus resistance cannot be ignored; the resistance of viruses (such as hepatitis B virus [5] and influenza virus [6]) has become a major challenge to human public health. Meanwhile, the emergence of many new resistance mechanisms also increases the difficulty in treating resistant microorganisms and viruses [7]. Our current, limited antimicrobial drugs can no longer solve the growing problem of drug resistance [8–10]. Therefore, it is urgent to develop additional antimicrobial drugs. Sulfonamides, the first generation of antibiotics [11], have recently been shown to target *de novo* folate synthesis in pathogens [12,13]. Therefore, microbial cellular targets and pathways, such as endogenous riboflavin biosynthesis, may provide targets for novel drug discovery [14,15].

Riboflavin (vitamin B$_2$) was isolated from milk whey in the late 1870s as a water-soluble compound. It is indispensable to adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which participate in electron transport, and metabolism of lipids, drugs, and xenobiotics. Because of the absence of a system to produce riboflavin, riboflavin in humans is mainly obtained from the diet. However, bacteria and fungi, such as *Aspergillus fumigatus*, *Candia albicans*, *Escherichia coli*, *Mycobacterium tuberculosis*, and *S. typhimurium*, have the ability to produce riboflavin [16–18]. Silencing or downregulation of genes of the endogenous riboflavin biosynthetic pathway may be beneficial tin treating fungal skin infections [19]. Furthermore, exogenous riboflavin has also been shown to exhibit anti-infectious effects in infectious diseases [20,21].

In this review, the role of the endogenous riboflavin synthesis pathway, which may be a target for the development of antimicrobial agents, and exogenous riboflavin, against infectious diseases is provided. The effects of exogenous riboflavin on immune cell and cytokine gene and heat shock protein gene expression are also presented. Moreover, the relationship between endogenous riboflavin and mucosal associated invariant T (MAIT) cell is highlighted. These data provide a comprehensive review of the use of endogenous and exogenous riboflavin in anti-infection.

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Endogenous riboflavin synthesis pathways and the FMN riboswitch target in pathogens

Biosynthesis and transport of riboflavin

The riboflavin biosynthesis pathway is summarized in Figure 1. Riboflavin is formed by consuming guanosine triphosphate (GTP) and ribulose 5-phosphate (Ru5P) [9]. GTP cyclohydrolase II catalyzes GTP to form 2,5-diamino-6-ribosylamino-4(3 H)-pyrimidinedione phosphate (1). The first step is common to both bacteria and fungi, whereas the second steps are different. In bacteria, (1) is converted into 5-amino-6-ribosylamino-2,4(1 H,3 H)-pyrimidinedione 5′-phosphate (2) to form 5-amino-6-ribitylamino-2,4(1 H,3 H)-pyrimidinedione 5′-phosphate (4). However, in fungi, (1) is converted into 2,5-diamino-6-ribosylamino-4 (3 H)-pyrimidinedione 5′-phosphate (3) [22,23]. Then, (4) is dephosphorylated to form 5-amino-6-ribitylanimo-2,4(1 H,3 H)-pyrimidinedione (5). Another compound involved in riboflavin synthesis is
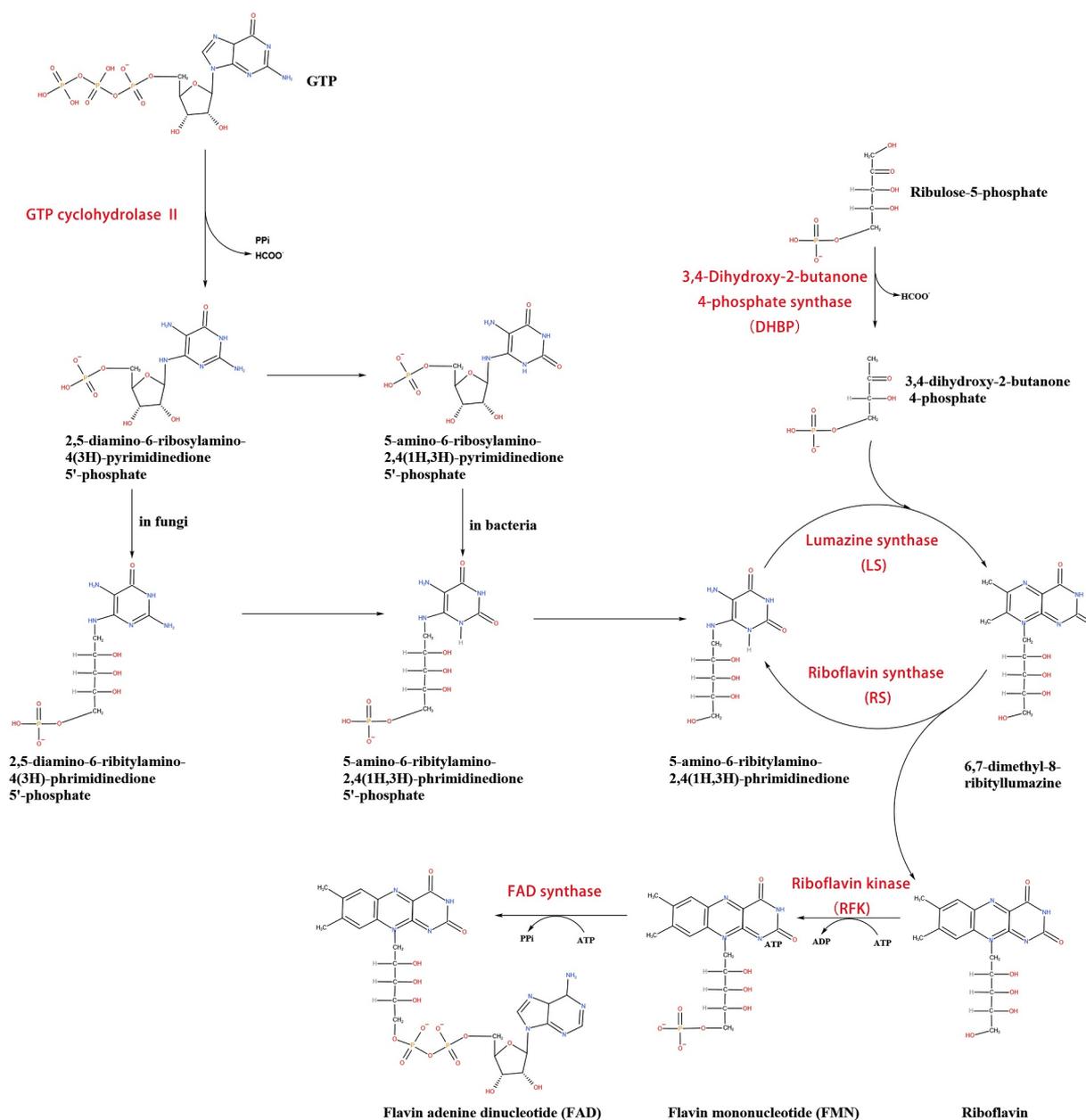
Figure 1. Biosynthesis of riboflavin, FMN and FAD.

Riboflavin is formed by GTP and Ru5P, and key enzymes are in red (reproduced from reference [37]). 5-OP-RU: 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil, 5-OE-RU: 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil

3,4-dihydroxy-2-butanone 4-phosphate (6), which is converted from Ru5P catalyzed by 3,4-dihydroxy-2-butanone 4-phosphate synthase. (5) and (6) are catalyzed by Lumazine synthase (LS) to form 6,7-dimethyl-8-ribityllumazine (7). Then, riboflavin synthase (RS) catalyzes formation of the LS substrate (5) and riboflavin. Riboflavin is usually converted to FMN and FAD. Riboflavin kinase catalyzes riboflavin to form FMN, which is then converted into FAD by FAD synthase [24,25]. Until now, the endogenous riboflavin synthesis pathway in viruses has not been found.

In addition, some microorganisms are able to take up exogenous riboflavin. In early 1979, components of the cell membrane were found to be involved in riboflavin transport [26]. Riboflavin transporters introduce riboflavin into cells; these transporters include RibU of *Lactococcus lactis*, RibM of *Actinobacteria and Streptomyces davaonensis* [27], ImpX of *Fusobacterium nucleatum* [28], RibZ of *Clostridium difficile*, RibV of *Mesoplasma florum*, and RibXY of *Chloroflexus aurantiacus* [29]. However, exogenous riboflavin may suppress the biosynthesis of riboflavin in some cases [30]. The relationship between the uptake system and the *de novo* biosynthesis pathway remains unclear [29]. Most fungi are able to biosynthesize riboflavin, but the uptake of riboflavin has been relatively less studied. Wild type *Saccharomyces cerevisiae* has a riboflavin efflux system but cannot take up exogenous riboflavin [31]. The expression of *MCH5* appears to be necessary for the uptake of riboflavin in auxotrophic *S. cerevisiae* mutants [32]. Unlike bacteria, fungi transport riboflavin through a passive process that dose not consume any energy [26,32]. The riboflavin transport systems in pathogenic fungi and viruses require further study. Human riboflavin transporters (*hRFVT1, hRFVT2,* and *hRFVT3*) belong to the SLC52 family of solute carriers, which show low homology with bacterial or fungal riboflavin transporters [33]. Therefore, the riboflavin transport in bacteria or fungi may provide ideas for developing antimicrobial agents.

Targeting the FMN riboswitch

FMN riboswitches are broadly conserved and consist of non-coding RNA structural elements. In the presence of FMN, they regulate the biosynthesis and transport of riboflavin in bacteria [34,35]. Importantly, they are specific to bacteria and absent in humans [36]. Here, several antibacterial agents, which include analogues of the riboflavin biosynthesis pathway, such as ribocil, roseoflavin (RoF), 8-demethyl-8-aminoriboflavin (AF),

and 5-(3-(4-fluorophenyl)butyl)-7,8-dimethylpyrido [3,4-b]quinoxaline-1,3(2 H,5 H)-dione (5FDQD), protect against the pathogens by targeting the FMN riboswitch.

Ribocil directly binds the FMN riboswitch and inhibits *ribB* expression, inducing bacterial death by blocking the riboflavin biosynthesis pathway in *S. aureus* and *E. coli* [37]. Further resistance mutation and whole-genome sequencing identified the ribocil target at the FMN riboswitch in *E. coli* [38,39]. However, exogenous riboflavin reduced the antibacterial activity of ribocil *in vitro* [37]. Interestingly and contrastingly, ribocil and RoF not only block riboflavin biosynthesis but also inhibit uptake from the environment in *S. aureus* and MRSA [34,40].

RoF is produced by *S. davaonensis* and *S. cinnabarinus* [41], and its main antibacterial spectrum is Gram-positive bacteria. The FMN riboswitch is a target for RoF in *Bacillus subtilis* [42] *E. coli* [15], *F. nucleatum* [43], and *Listeria monocytogenes* [44,45], which may be primarily responsible for its antibiotic activity. On the one hand, promiscuous riboflavin kinases catalyze RoF to form toxic RoF mononucleotide (RoFMN), which negatively regulates the *ribB* FMN riboswitch in *E. coli* [15,46]. *L. monocytogenes* does not contain genes that encode riboflavin biosynthetic enzymes. RoF was also shown to target the FMN riboswitch and inhibit the growth of *L. monocytogenes*. Surprisingly, RoF can increase the pathogenicity of *L. monocytogenes* in the absence of the FMN riboswitch [44,45]. However, the mechanisms require further study. On the other hand, the toxic RoFMN and RoF adenine dinucleotide (RoFAD) restricted growth of *B. subtilis* and *S. davaonensis* [45,47–49]. RoFMN and RoFAD can also be isolated from *E. coli* flavoproteins. Thus, flavoproteins may be another target for RoF in addition to FMN riboswitches [50]. However, human FAD synthase can accept RoFMN and disrupt the activity of flavoenzymes, which may be disadvantageous to human metabolism [40,51].

AF, also produced by *S. davaonensis*, is less toxic to host. Similar to RoF, AF negatively regulates *ribB* expression, which is controlled by the *ribB* FMN riboswitch. Human flavokinase converts AF to 8-demethyl-8-amino-riboflavin mononucleotide (AFMN). In contrast to RoF, human FAD synthase cannot accept AFMN. Therefore, it may provide a better structure to develop antibacterial compounds [51]. Furthermore, 5FDQD defends against *Clostridium difficile* infection through binding to FMN and triggering the function of the FMN riboswitch. However, many aerobes and

Gram-negative anaerobes are not sensitive to 5FDQD [52]. This is likely because the FMN riboswitch is not highly conserved across different bacteria. Thus, antibiotics targeting the FMN riboswitch are a narrow spectrum. Overall, FMN riboswitches are the main target of several existing inhibitors of the riboflavin synthesis pathway. However, the function and application of FMN riboswitches in fungi have been rarely studied. The question is whether the FMN riboswitch exists in fungi? If it does exist, the function of the FMN riboswitch in regulation of riboflavin biosynthesis will require further investigation in fungi.

Inhibiting enzymes in the riboflavin synthesis pathway

LS and RS were identified in fungi and bacteria [30] and can be inhibited by antimetabolites [53]. Mutations of LS genes have emphasized the essential role of riboflavin biosynthesis in pathogen survival. For example, there are two LS isoenzyme-related genes (*ribH1* and *ribH2*) in *Brucella abortus*. The double mutant of *ribH1* and *ribH2* does not survive without exogenous riboflavin [54]. Thus, inhibitors of these enzymes are logical candidates for development as antibiotics [55]. Crystal structure analysis of icosahedral LS from *S. typhimurium* also supports the above-mentioned conclusion [56]. Several potent LS inhibitors were discovered by a high throughput screening approach. However, cell membrane permeability remains an issue for the therapeutic use of these compounds [57]. Further investigations are needed to enhance cell membrane permeability *in vivo* and *in vitro*. RS is highly conserved across pathogenic microorganisms, and may be a suitable target for alternative antibiotics [58]. High-throughput screening technology was used to discover and develop covalent hydrates of trifluoromethylated pyrazoles, which can inhibit RS in *M. tuberculosis* [59]. Using the same technology, several inhibitors of *B. abortus* RS were discovered [60]. One compound could inhibit LS in *B. subtilis* and *M. tuberculosis* and RS in *E. coli* [61]. Mutation of both LS and RS is a rare event, but can lead to a low rate of resistance in strains [16]. Therefore, this compound can be used as a lead structure for designing dual inhibitors.

3,4-Dihydroxy-2-butanone-4-phosphate synthase (DHBP) catalyzes Ru5P to form (**6**) and formate (Figure 1). A competitive inhibitor of DHBP in *Vibrio cholerae*, 4-phospho-d-erythronohydroxamic acid, has been described [62,63]. DHBP is essential to *Helicobacter, Mycobacterium, Salmonella* species, and

S. pneumoniae [[64-]]. Moreover, in eukaryotes and prokaryotes, FMN and FAD are catalyzed by FAD synthase and RFK, respectively (Figure 1). The two are involved in a plethora of vital processes. Therefore, the most important thing is to identify the different characteristics of FAD synthase/RFK in pathogens and hosts. However, the characteristics of FAD synthase in *S. pneumoniae* are different from those in other bacteria [65]. In addition, several FAD synthase inhibitors were shown to inhibit the growth of *M. tuberculosis* and *S. pneumoniae* [66].

To date, studies of endogenous riboflavin synthesis pathway inhibitors have been primarily performed in bacteria, and have resulted in the design of several inhibitors. Riboflavin biosynthesis and uptake are essential not only for invasion but also during dissemination. *RIBB* and *RIB1* encode the GTP cyclohydrolase II enzyme, which converts GTP into (**1**) (Figure 1). Studies have shown that the *RIBB* mutant attenuates the virulence of *A. fumigatus* and inhibits its survival in hosts [67]. *CaRIB1Δ/Δ* deletion strains show no toxicity in HeLa cells and are completely avirulent in a mouse model [68–70]. *RIB2* encodes the 2,5-diamino-6-ribitylamino-4(3 H)-pyrimidinone 5′-phosphate deaminase of *Histoplasma capsulatum*, which catalyzes (**1**) into (**3**) (Figure 1). Disruption of the *RIB2* gene prevents growth and proliferation of *H. capsulatum* in macrophages and severely attenuates its virulence [71]. FlcA, FlcB, and FlcC are important for FAD accumulation and *A. fumigatus* virulence, and FlcA-C belong to the flavin transporter family. The virulence of the Δ*flcA*, Δ*flcB*, and Δ*flcC* mutant strain is lower, than that of the wild type, thus resulting in a higher survival rate of infected mice [71]. In conclusion, GTP cyclohydrolase II enzyme, DHBP, LS, RFK, RS, and FAD synthase, which are involved in the riboflavin synthesis pathway, can be used as antibacterial and antifungal drug targets (Table 1). However, RFK and FAD synthase are also present in humans [30]. Thus, the application of RFK and FAD synthase inhibitor against bacterial and fungal

Table 1. Inhibitors of enzymes in the riboflavin synthesis pathway.

Inhibitors	Target species	Reference
DHBP inhibitors	*Vibrio cholerae*	[62]
LS inhibitors	*Schizosaccharomyces pombe, C. albicans, B. abortus;* and *M. tuberculosis*	[55,57,61]
RS inhibitors	*M. tuberculosis, B. abortus* and *E. coli*	[59–61]
RFK inhibitors	*S. pneumoniae* and *Corynebacterium ammoniagenes*	[65]
FAD inhibitors	*C. ammoniagenes, M. tuberculosis* and *S. pneumoniae*	[66]

infections in the human body should be considered carefully.

Immune response of endogenous riboflavin

It is necessary to understand how the endogenous riboflavin biosynthesis pathway of pathogens affects the human immune response. 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil are intermediates in the microbial riboflavin biosynthesis pathway, and are considered to be the biological signature of microbial infection in mammals, which are formed by (5) combined with methylglyoxal or glyoxal [72] (Figure 2a). The major histocompatibility class I-like antigen-presenting molecule, MHC-related protein-1 (MR1), captures these pyrimidine intermediates and forms a complex. MAIT cell antigen receptors accept MR1-antigen complexes and then trigger MAIT cell immune responses, which lead to protection of the host from pathogens at mucosal surfaces [73–75]. In the early life of humans, the development of MAIT cells requires exposure to microorganisms with the ability to synthesize riboflavin. Subsequently, MAIT respond to cutaneous microbes, which are beneficial to tissue repair. Furthermore, MR1, which presents riboflavin metabolites, is necessary for MAIT cells to recognize pathogens [76]. Besides the activation and enrichment of MAIT cells, the production of cytokines is also a response to microbial antigens [77]. For example, *Legionella longbeachae* induces MAIT cell activation and rapid pulmonary accumulation in an MR1-dependent manner in pulmonary *L. longbeachae*-infected mice. Interferon (IFN)-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), and Interleukin (IL)-17 produced by activated MAIT cells enhance host immune protection [78]. Granzyme B and perforin, the secretions of MAIT cells, can also kill target cells to fight infection [79,80]. In addition, they promote the accumulation of CCR1+ and CCR5+ immune cells to the lung, preventing *mycobacterial* infection [80]. It is worth noting that MAIT cells are polarized to the Th1 phenotype and migrate to the infectious site during *M. tuberculosis* infection [81,82]. In addition, fungi (including *Aspergillus ssp.*, *C. albicans*, and *Mucorales* species) can be recognized by MAIT cells in an MR1-dependent manner [83–85].

However, immune responses of MAIT cells are different in different kinds of infections. For example, *A. fumigatus* infection induces MAIT cells to produce a large amount of TNF and less IFN-γ, in contrast to *C. albicans* infection, which induces MAIT cells to mainly produce IFN-γ. Differences in the MAIT cell response to *C. albicans* and *E. coli* can be seen in the sensitivity of recognition, release of cytokines, and immune response [84,86]. Viruses cannot produce riboflavin metabolites to form MR1-antigen complexes. MAIT cells depend on cytokines, but not MR1, which play a protective role in influenza virus, dengue virus, and hepatitis C virus infection [87–89] (Figure 2b). Activated MAIT cells can limit hepatitis C replication, and the mechanism should be further explored [88]. However, MAIT cells are activated and exhausted in most viral infections [90,91]. The antivirus activity of MAIT cells was induced in human immunodeficiency virus infection [92,93]. Thus, MAIT cells can be activated by different types of microbial stimuli and against bacteria, fungi, and viruses in different response patterns [86]. Thus, MAIT cells also promote cytokine and chemokine release, as well as promote migration of immune cells to sites of infection, which enhances host immunity and exerts an anti-infectious effect (Figure 2b). However, MAIT cells cannot be activated by *A. fumigatus* strains lacking *ribB* [84]. Hence, it is worth considering that whether inhibitors of the riboflavin biosynthesis pathway affect the formation of MR1-antigen compounds, resulting in pathogen evasion from MAIT cells. Further investigations are needed.

Anti-infection effect of exogenous riboflavin

Direct anti-infective effect of riboflavin

Riboflavin can be used as an antibiotic in the treatment of various infectious diseases. Intravenous injection of highly purified riboflavin (80 mg/kg) greatly reduced the virulence of *E. coli* and *S. aureus* as well as the production of proinflammatory cytokines and nitric oxide (NO) induced by lipopolysaccharide (LPS) [20]. Exogenous riboflavin (10 mg/kg) could also protect mice against LPS-induced shock by increasing the level of heat shock protein 25 (Hsp25) to decrease mortality [94]. In addition, exogenous riboflavin (10 mg/kg) and riboflavin combined with aminolevane increased survival of mice with LPS-induced shock [95]. Riboflavin (20 mg/kg) also decreased IL-6 and macrophage inflammatory protein-2 concentrations and mRNA transcription levels in mice injected with LPS. It also reduced plasma elevated NO levels by reducing expression of inducible NO synthase (*iNOS*) gene [96]. Supplementation with 300 mM riboflavin reduced the mortality of LPS-stimulated macrophages and induced expression of Hsp72 in macrophages [97]. These data illustrate that intravenous injection of riboflavin has antibacterial effects.
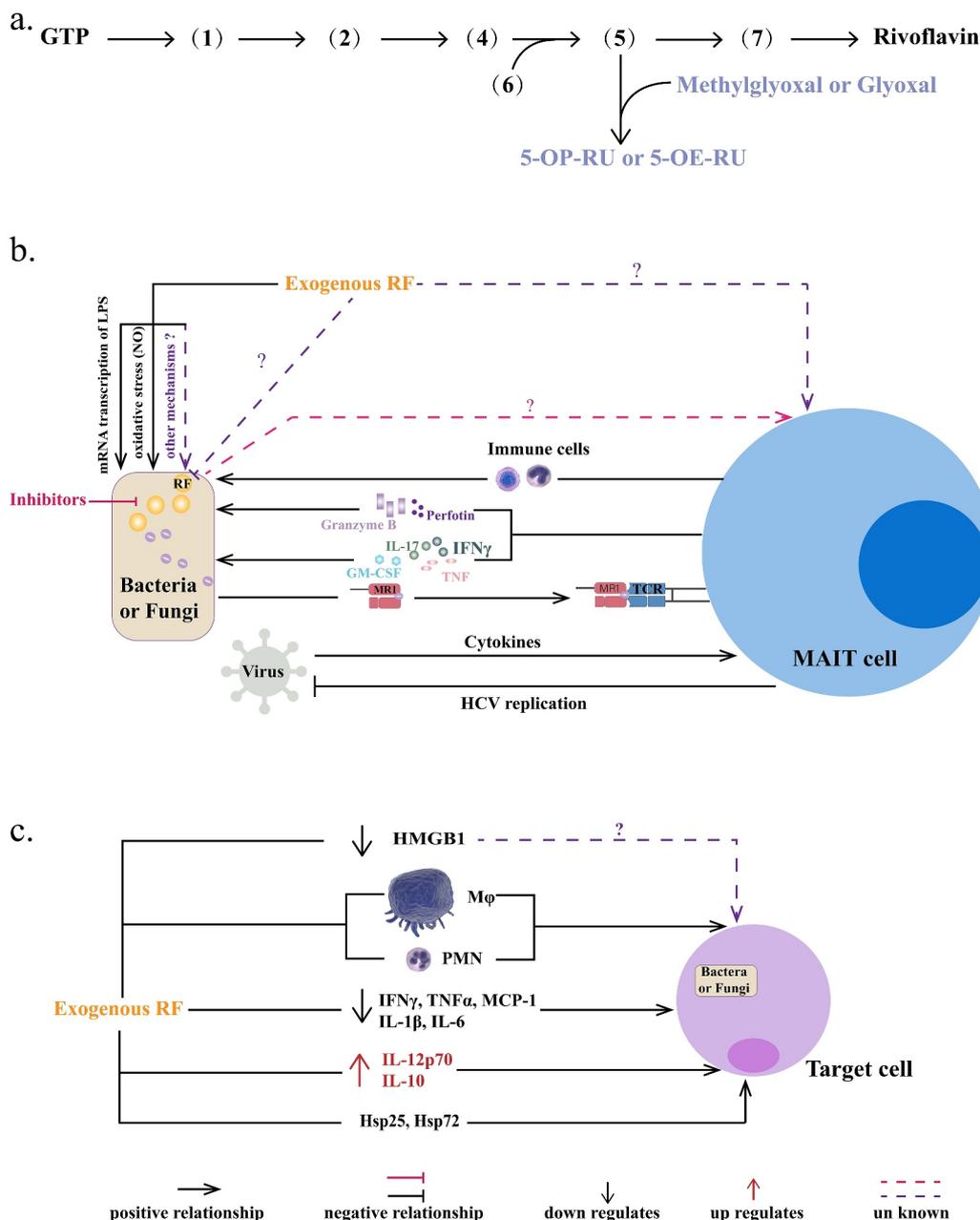
Figure 2. Immune responses of endogenous/exogenous riboflavin and exogenous riboflavin against infection. a. The formation of pyrimidine adducts. 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) are formed by **(5)** combining with glyoxal or methylglyoxal in bacteria. RF: riboflavin. **b. Interaction between riboflavin and MAIT cells**. MR1 recognizes and presents the signals of the riboflavin synthesis pathway to MAIT cells triggering different types of immune responses in bacteria and fungi. 1) MIAT cells produce granzyme B and perforin to kill the target cell directly. 2) MAIT cells release cytokines, including IFN-γ, GM-CSF, IL-17, and TNF. 3) MAIT cells facilitate other immune cells to protect the host from infection. However, fungi are recognized in an MR1-dependent manner and, therefore, MAIT cells response to fungi are not exactly same as to bacteria. Viruses cannot biosynthesize riboflavin, and depend on cytokines to activate MAIT cells. In most virus infections, MAIT cells levels were reduced and their functions were impaired, such as antimicrobial activity. In addition, activated MAIT cells can limit HCV replication, and the mechanism should be further explored. **c. The immune responses of exogenous riboflavin against infection**. Exogenous riboflavin affects infectious disease by regulating the function of immune cells and the release of cytokines/inflammatory factors. HMGB1: high mobility group box 1 protein, Mø: macrophage, PMN: polymorphonuclear cell. INF-γ: interferon-γ, TNF-α: tumor necrosis factor-α, MCP-1: monocyte chemoattractant protein-1, IL: interleukin, Hsp: heat shock protein.

In vivo, the dominant immune responses of C57BL/6 J, BALB/c, and CBA mice are different, which results in the different effects of riboflavin supplementation on fungal peritonitis. In general, riboflavin affects matrix metalloproteinase-9 activity, *iNOS* gene expression, and the migration of polymorphonuclear cells (PMNs) and macrophages [98]. The effect of riboflavin on zymosan-induced peritonitis in Swiss mice has also been studied. The results suggested that the effects of riboflavin on proinflammatory and anti-inflammatory cytokines were most significant. Different from LPS-induced infection, 20 mg/kg riboflavin was usually ineffective in zymosan-induced infection in Swiss mice [21], while 300 nM riboflavin supplementation of macrophages stimulated by zymosan significantly decreased Toll-like receptor 6, NO, iNOS, IL-1β, monocyte chemoattractant protein-1 (MCP-1), and keratinocyte chemoattractant levels [97]. Riboflavin supplementation reduced the release and expression of high mobility group box 1 protein (HMGB1) in zymosan-induced peritonitis mice and *in vitro* macrophage model. HMGB1 is responsible for activation of neutrophils or macrophages in recent studies [99]. In-depth studies of the mechanisms of action will be necessary in the future.

Exogenous riboflavin directly controls infection in three ways: 1) it inhibits transcription of bacterial LPS (Figure 2b); 2) it reduces the level of *NO* or expression *of iNOS*; and 3) it regulates the functions of innate immune cells (such as macrophages and neutrophils) and the levels of immunoreactive materials, including TNF, ILs, and IFN. The key factors affecting the antibacterial or anti-fungal effects of riboflavin are the time of administration and the dose [20,21,94,95]. Exogenous riboflavin regulation of immune responses is highlighted in Figure 2c. These immune responses also have a vital role in virus infection [100]. Thus, riboflavin may affect the occurrence and development of viral infection. In addition, the function and activation of MAIT cells participate in host defense during microbial or viral infection [88–91]. Therefore, exogenous riboflavin may directly affect the function of MAIT cells or influence MAIT cells' recognition of pathogens. Further experiments are needed to confirm these conclusions.

Indirect anti-infective effects of riboflavin

Riboflavin can also be used as a synergist to enhance the activity of antimicrobial or antiviral drugs. In 1996, Adelavin, a compound that contains liver extract and FAD, was used in patients with chronic hepatic-C and potentially enhanced the anti-viral effect of IFN [100].

Riboflavin showed significant synergistic activity with linezolid against MRSA infection [101]. Riboflavin in combination with azithromycin treatment eliminated *S. aureus* from blood, spleen, and synovial tissue of infected mice, reduced serum levels of TNF-α, INF-γ, and IL-6, and increased serum anti-inflammatory cytokines IL-12p70 and IL-10 [102].

Riboflavin, a natural photosensitizer, is widely used in photodynamic inactivation of microorganisms. Photo-illuminated riboflavin inhibits the biofilm formation of *S. aureus* and *E. coli* by inducing reactive oxygen species accumulation and oxidative stress, and destroying the respiratory system [103,104]. In combination with ultraviolet (UV) light A, riboflavin damages nucleic acids of pathogens (such as *S. aureus*, MRSA, and *P. aeruginosa* [105]) through the same mechanism [106,107]. Low concentrations of riboflavin (0.03%–0.07%) can improve the bactericidal effects of UV light A [108]. Interestingly, riboflavin/UVA combined with amphotericin B can enhance inhibitory effects on *A. fumigatus, C. albicans*, and *Fusarium*. Riboflavin/UV-A enhanced surface diffusion of amphotericin B and reduced vertical diffusion into the agar. This treatment strategy can be used to treat fungal infection keratitis [109]. Several studies have investigated the use of riboflavin to treat infectious keratitis. Clinical trials have shown that collagen cross-linking (CXL) with UV light-activated riboflavin has a positive role in the treatment of bacterial or fungal keratitis [110–113]. Conversely, no benefits of the CXL to treat infectious keratitis have been reported [114–117]. Based on current evidence, further investigations are needed to clarify the efficacy of CXL-UVA riboflavin for treatment of infectious keratitis. Moreover, riboflavin can also be used for virus inactivation [18]. Riboflavin and UV light reduced the infectious titer of severe acute respiratory distress syndrome coronavirus-2 [118,119], middle east respiratory syndrome coronavirus [120], dengue viruses [121], and ebolavirus [122] below the limit of detection. In summary, exogenous riboflavin plays a positive role in infectious diseases through combination with other drugs or therapies. Riboflavin's use as a synergist and photosensitizer are summarized in Table 2.

Exogenous riboflavin and immune responses

As an antioxidant, exogenous riboflavin has a vital effect on the treatment and prevention of infectious diseases directly or indirectly by regulating the immune response and redox state. The role of exogenous riboflavin in immune responses is summarized in Figure 2c. On the one hand, exogenous riboflavin facilitates the ability of

Table 2. Riboflavin's use as a synergist and photosensitizer.

Usage	Wavelength of light	Target organisms	Reference
Synergist	-	MRSA	[101,102]
	-	hepatitis C virus	[100]
Photosensitizer	450 nm	S. aureus	[103]
	355 nm	E. coli and P. aeruginosa	[104]
	365 nm	S. aureus; P. aeruginosa; MRAS; Staphylococcus epidermidis; A. fumigatus; C. albicans and Fusarium	[104,105,108,109]
	<400 nm	SARS-CoV-2; MERS-CoV; dengue viruses; ebolavirus	[118–122]

immune cells to protect the host from infection. For instance, riboflavin regulates the accumulation, infiltration, migration, and development of macrophages and PMNs [98,123,124]. Under some conditions, pathogens are disseminated through immune cells (such as macrophages and PMNs) [125]. It reduced the level of HMGB1 released by macrophages [126], although the underlying mechanism is unknown [99]. On the other hand, exogenous riboflavin decreases cytokine/chemokine levels (including those of TNF-α, IL-1β, MCP-1, and IL-6) and increases the levels of IL-12p70 and IL-10 [127–131]. Riboflavin also regulates the level of heat shock proteins, including Hsp25 and Hsp72 [97] (Figure 2c). Heat shock proteins can protect against cellular injury and death under harmful environmental conditions, such as infection [97,132133]. TNF-α is a well-known pro-inflammatory cytokine [133] that participates in inflammatory cell activation and recruitment [128]. TNF-α and INF-γ are partially responsible for LPS-induced iNOS mRNA expression [128]. Furthermore, TNF-α and INF-γ induced inflammatory cell death during SARS-CoV-2 infection, which may contribute to cytokine storm activation [127]. TNF-α is one of the reasons for the destruction of the immune defense during infection [129]. IL-10 primarily limits excessive inflammatory responses by inhibiting pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, and GM-CSF [130]. It also maintains tissue homeostasis and innate immunity to control infection [133]. Thus, TNF, ILs, and IFN play vital roles in the occurrence and development of infection. However, further studies are needed to determine the effects of exogenous riboflavin on infectious disease; its detailed effects on the immune system have yet to be elucidated.

Conclusion

In this review, anti-infectious roles of the endogenous riboflavin biosynthesis pathway, FMN riboswitch, and exogenous riboflavin are summarized. The data suggest that the riboflavin biosynthesis pathway may be a promising target for the development of novel classes of antibiotics and antifungals. In bacteria, the FMN riboswitch is the main target of existing riboflavin biosynthesis inhibitors, such as RoF, AF, Ribocil and 5FDQD. In addition, enzymes, such as LS, RS, riboflavin kinase, and FAD synthase, can also be targets for antibiotics [30,34]. However, few studies have developed inhibitors of the riboflavin biosynthesis pathway in fungi. Further studies are also needed to clarify whether enzyme inhibitors of riboflavin biosynthesis will be useful as a novel class of antifungal agents. In addition, MAIT cells recognize bacteria and fungi in an MR1-dependent manner, which requires the riboflavin synthesis pathway, while viruses depend on cytokines (Figure 2b). How MAIT cells respond to bacteria/fungi treated with inhibitors of the riboflavin biosynthesis pathway will require further investigation. Notably, if the inhibitors inhibit the production of pyrimidine adducts, MR1 cannot present antigen to MAIT cells and trigger an immune response in vivo, which may cause the effect of these inhibitors in vivo to be worse than that in vitro.

Furthermore, during infection, exogenous riboflavin inhibits the mRNA expression of LPS and reduces the overproduction of NO (Figure 2b), as well as regulates innate immune cells and cytokines (Figure 2c). However, whether exogenous riboflavin influences the de novo synthesis of riboflavin needs to be further explored. It also remains to be determined whether MAIT cells can respond to exogenous riboflavin or whether exogenous riboflavin can be presented by MR1 and compete with endogenous riboflavin/riboflavin precursors. Further understanding of these mechanisms will shed light on the role of endogenous and exogenous riboflavin in infection. In summary, further investigation of the endogenous riboflavin biosynthesis pathway and exogenous riboflavin will help lay the foundation for the development of new antimicrobials.

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Data available statement

Data sharing is not applicable to this article as no new date were created or analyzed in this study.

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